



National Institutes of Health  
National Institute of Mental Health  
6001 Executive Boulevard  
Bethesda, Maryland 20892

April 7, 2020

Ms. Allison Lucas  
Siri Glimstad LLP  
200 Park Avenue, Seventeenth Floor  
New York, NY 10166

Re: FOI Case No. 53816

Dear Ms. Lucas:

This is our final response to your February 14, 2020 Freedom of Information Act (FOIA) request addressed to Gorka Garcia-Malene at the National Institutes of Health (NIH). Your request was forwarded to me on February 14, 2020 because of my responsibilities under the FOIA at the National Institute of Mental Health (NIMH). You requested a search of Josh Gordon's email from May 20, 2017 to June 1, 2018 with "24814559" in the subject line.

Enclosed are 328 pages responsive to your request. This includes emails as well as all records that were attached to the emails received by Dr. Gordon. No information has been removed from the enclosed material.

If you feel that materials have been omitted that should have been made available to you, please write to me and I will consult with the NIH Freedom of Information Officer.

Please contact me on [301-443-6130](tel:301-443-6130) or at [LALBERTS@NIH.GOV](mailto:LALBERTS@NIH.GOV) if you have questions about your request. If you are not satisfied with the processing and handling of this request, you may contact the NIMH FOIA Public Liaison:

6001 Executive Blvd, Suite 6200  
Bethesda, MD, 20892-9667  
301-443-4335 (phone)  
[NIMHFOIA@mail.nih.gov](mailto:NIMHFOIA@mail.nih.gov) (email)

In certain circumstances provisions of the FOIA and Department of Health and Human Services FOIA Regulations allow us to recover part of the cost of responding to your request. Because the cost is below the \$25 minimum, there is no charge for the enclosed materials.

Sincerely,

Lisa D. Alberts  
FOIA Coordinator  
National Institute of Mental Health

Enclosures: 328 pages

**From:** [Gordon, Joshua \(NIH/NIMH\) \[E\]](#)  
**To:** [NIMH Executive Secretariat](#)  
**Subject:** FW: 1 selected item: 24814559 - PubMed  
**Date:** Monday, August 14, 2017 10:10:54 PM  
**Attachments:** [image001.png](#)  
**Importance:** High

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On request from Dr. Tabak, please route this through NIH OD Exec Sec

Josh

-----  
Joshua A Gordon, MD, PhD  
Director  
National Institute of Mental Health

---

**From:** Aaron Siri <aaron@sirillp.com>  
**Date:** Monday, August 14, 2017 at 4:48 PM  
**To:** "M. Joshua Gordon" <joshua.gordon@nih.gov>  
**Subject:** RE: 1 selected item: 24814559 - PubMed

Dr. Gordon,

I hope all is well.

I have not received a response to the emails below of July 10 and July 24.

The July 10 email was in response to a review you provided indicating it compared vaccinated and unvaccinated children (but which actually compares vaccinated children with vaccinated children who, at most, were missing MMR). As discussed at our meeting, I would like to see a study which supports the claim that the nearly two dozen doses of vaccines given in the first year of life (which would not include MMR and thimerosal) do not cause autism. I still await receipt of a study which supports same. Are you aware of any such study?

The July 24 email elaborated on my prior email and also sought to facilitate a meeting between with various experts in the field of aluminum adjuvant that do believe there is a connection between aluminum adjuvant in vaccines and autism. Are you willing to have this meeting?

Best regards,  
Aaron

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**From:** Aaron Siri  
**Sent:** Monday, July 24, 2017 6:18 PM  
**To:** 'Gordon, Joshua (NIH/NIMH) [E]' <joshua.gordon@nih.gov>

**Subject:** RE: 1 selected item: 24814559 - PubMed

Good evening Joshua,

As promised in my email below, I am following up regarding the research that has been conducted regarding aluminum adjuvants and neuro/psychiatric disorders, and to facilitate a meeting with you and the scientists conducting this research.

In recent years researchers have discovered that injected aluminum adjuvant travels into the brain, where it causes long term chronic inflammation, damage to neurons and behavioral abnormalities. These adverse effects occur at dosages (mcg/Kg body weight) even lower than dosages received by infants according to the CDC vaccine schedule.

Additionally, it is now well established that autism and other neuro/psychiatric disorders are caused by early life inflammation (i.e. elevated cytokines) in the brain. I have seen your published papers on immune activation and brain development so I presume you are aware of the immune activation findings. Aluminum adjuvant can cause chronic brain inflammation, and this establishes a biologically-plausible and empirically-supported mechanism for how vaccines may cause autism and other neurological disorders. None of the vaccine-autism studies to date tell us anything about the safety of aluminum adjuvants. There are no epidemiological studies showing that aluminum adjuvants do not produce these effects in humans.

Attached is a detailed explanation of the proposed mechanism for how aluminum adjuvants may cause autism. The mechanism suggests that aluminum adjuvant may cause other brain and neurodevelopmental disorders as well. Attached are also supporting letters from experts in the fields of aluminum toxicity. (Finally, I have also attached a more detailed analysis of Taylor 2014.)

I invite you to consider the arguments in the attached document and respond with your observations. I also invite you to share the document with colleagues, particularly if they may have insightful comments or rebuttals.

I also hope to facilitate a meeting with you and a number of the experts studying aluminum adjuvant toxicity, letters from a number of which are attached to this email. Assuming you are open to having this discussion, kindly have your office provide suggested dates/times for such a meeting.

Best regards,  
Aaron

---

**From:** Aaron Siri

**Sent:** Monday, July 10, 2017 4:16 PM

**To:** 'Gordon, Joshua (NIH/NIMH) [E]' <[joshua.gordon@nih.gov](mailto:joshua.gordon@nih.gov)>

**Subject:** RE: 1 selected item: 24814559 - PubMed

Good afternoon Joshua,

Thank you for sending me the below abstract/review article and it was great meeting at NIH. Really appreciate the opportunity to dialogue on the issue of vaccines and autism.

The abstract/review article you sent me below highlights the concern raised that there has never been a study assessing the relative risk of autism between vaccinated and unvaccinated child. To be sure, this review (and its abstract) leave the impression that the studies it relies upon compare “unvaccinated” children (no vaccines) with vaccinated children. Unfortunately, this is misleading since all 10 of the underlying studies relied upon for this review compared highly vaccinated children with highly vaccinated children. The only difference typically between the study and control groups was a single MMR vaccine or thimerosal vs. non-thimerosal vaccines. (I would be happy to provide you with a breakdown of each of the 10 studies reflecting same.) Meaning, what this review considers “unvaccinated” are vaccinated children typically only missing the MMR vaccine. Assuming the control children in these studies followed the current CDC recommended vaccination schedule, they would each have received 21 vaccine injections during the first 12 months of life excluding the MMR vaccine. Hence, these studies tell us virtually nothing about the relationship of vaccines to autism because they are not comparing vaccinated and unvaccinated children.

For example, the IOM stated in 2011 that there isn't a single study that supports the assertion that DTaP (injected at 2 months, 4 months, 6 months, etc.) does not cause autism, concluding that “The evidence is inadequate to accept or reject a causal relationship between diphtheria toxoid-, tetanus toxoid-, or acellular pertussis-containing vaccine and autism.” Attached is an excerpt of the discussion regarding autism and DTaP from the 2011 IOM report. (I am not aware of a single study regarding DTaP and autism that has been done since 2011.) As another example, the only study regarding Hepatitis B vaccine and autism I have located found a three-fold increase in the odds of an autism diagnosis for neonates that received the hepatitis B vaccine at birth compared that those that did not. (Gallagher CM, Goodman MS. 2010. Hepatitis B vaccination of male neonates and autism diagnosis, NHIS 1997-2002. J Toxicol Environ Health A. 73(24):1665-77.) There is simply no studies for the numerous other vaccines given to children during the first year of life with regard to their relationship with autism (except for the Mawson study which showed vaccination had an over 4 fold increase in autism risk but that study has some serious limitations).

As we discussed at the meeting, I really am open to seeing the evidence that the vaccination schedule, and in particular the cumulative impact of the 31 vaccine doses the CDC recommends a child receive in the first year of life, are not casually related to autism. I would gladly share that support with the community concerned with this issue with my personal endorsement. On the other hand, if that proof doesn't exist, that does not mean that vaccines cause autism. It just means that we need to really do the science necessary to rule out that possibility. (Seeking to assess the health outcomes of those receiving vaccines and those not receiving vaccines really is asking for nothing more than how all drugs are safety tested prior to licensure.)

I respected what appeared to be your thoughtful rather than reflexive reaction to the spirited discussion at NIH. Conducting a true study of the health outcomes between actually unvaccinated and vaccinated children (at least an initial quick and easy retrospective study) that shows no connection with autism should be something that everyone should want. If it shows no connection, it will likely provide the greatest relief to the portion of the autism community that thinks there may be a connection. Parents who think that it was their actions, in vaccinating their children, that lead to their child's condition would feel freed from that



guilt by knowing it wasn't the vaccines.

I look forward to your response and being persuaded that the science on the question of whether vaccines cause autism really is settled.

Thanks again in advance for your time and thoughtful consideration of this issue.

Best regards,  
Aaron

p.s. I have had a number of discussions with various aluminum adjuvant experts around the globe who believe there is a connection between the aluminum adjuvants in vaccines given in large quantities during the first six months of life and autism; I hope to soon send you a write-up regarding same for your consideration.

---

**From:** Gordon, Joshua (NIH/NIMH) [E] [<mailto:joshua.gordon@nih.gov>]

**Sent:** Wednesday, May 31, 2017 4:03 PM

**To:** Aaron Siri <[aaron@sirillp.com](mailto:aaron@sirillp.com)>

**Subject:** Fwd: 1 selected item: 24814559 - PubMed

-----  
Joshua A Gordon, MD, PhD  
Director  
National Institute of Mental Health

Begin forwarded message:

**From:** Sent by NCBI <[nobody@ncbi.nlm.nih.gov](mailto:nobody@ncbi.nlm.nih.gov)>

**Date:** May 31, 2017 at 4:00:01 PM EDT

**To:** <[Joshua.gordon@nih.gov](mailto:Joshua.gordon@nih.gov)>

**Subject: 1 selected item: 24814559 - PubMed**

This message contains search results from the National Center for Biotechnology Information ([NCBI](#)) at the U.S. National Library of Medicine ([NLM](#)). Do not reply directly to this message

Sent on: Wed May 31 15:58:39 2017

1 selected item: 24814559

PubMed Results

Item 1 of 1 ([Display the citation in PubMed](#))

1. Vaccine. 2014 Jun 17;32(29):3623-9. doi: 10.1016/j.vaccine.2014.04.085.  
Epub 2014 May 9.

# Vaccines are not associated with autism: an evidence-based meta-analysis of case-control and cohort studies.

[Taylor LE](#)<sup>1</sup>, [Swerdfeger AL](#)<sup>1</sup>, [Eslick GD](#)<sup>2</sup>.

Author information:

1

The Whiteley-Martin Research Centre, Discipline of Surgery, The University of Sydney, Nepean Hospital, Level 3, Clinical Building, PO Box 63, Penrith 2751, NSW, Australia.

2

The Whiteley-Martin Research Centre, Discipline of Surgery, The University of Sydney, Nepean Hospital, Level 3, Clinical Building, PO Box 63, Penrith 2751, NSW, Australia. Electronic address: [guy.eslick@sydney.edu.au](mailto:guy.eslick@sydney.edu.au).

## Comment in

- [Autism and vaccination: The value of the evidence base of a recent meta-analysis.](#) [Vaccine. 2015]
- [Answers regarding the link between vaccines and the development of autism: A question of appropriate study design, ethics, and bias.](#) [Vaccine. 2015]

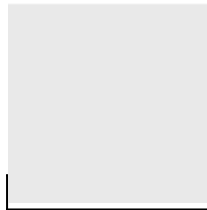
## Abstract

There has been enormous debate regarding the possibility of a link between childhood vaccinations and the subsequent development of autism. This has in recent times become a major public health issue with vaccine preventable diseases increasing in the community due to the fear of a 'link' between vaccinations and autism. We performed a meta-analysis to summarise available evidence from case-control and cohort studies on this topic (MEDLINE, PubMed, EMBASE, Google Scholar up to April, 2014). Eligible studies assessed the relationship between vaccine administration and the subsequent development of autism or autism spectrum disorders (ASD). Two reviewers extracted data on study characteristics, methods, and outcomes. Disagreement was resolved by consensus with another author. Five cohort studies involving 1,256,407 children, and five case-control

studies involving 9,920 children were included in this analysis. The cohort data revealed no relationship between vaccination and autism (OR: 0.99; 95% CI: 0.92 to 1.06) or ASD (OR: 0.91; 95% CI: 0.68 to 1.20), nor was there a relationship between autism and MMR (OR: 0.84; 95% CI: 0.70 to 1.01), or thimerosal (OR: 1.00; 95% CI: 0.77 to 1.31), or mercury (Hg) (OR: 1.00; 95% CI: 0.93 to 1.07). Similarly the case-control data found no evidence for increased risk of developing autism or ASD following MMR, Hg, or thimerosal exposure when grouped by condition (OR: 0.90, 95% CI: 0.83 to 0.98;  $p=0.02$ ) or grouped by exposure type (OR: 0.85, 95% CI: 0.76 to 0.95;  $p=0.01$ ). Findings of this meta-analysis suggest that vaccinations are not associated with the development of autism or autism spectrum disorder. Furthermore, the components of the vaccines (thimerosal or mercury) or multiple vaccines (MMR) are not associated with the development of autism or autism spectrum disorder.

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PMID: 24814559 [Indexed for MEDLINE]



**From:** [Gordon, Joshua \(NIH/NIMH\) \[E\]](#)  
**To:** [NIMH Executive Secretariat](#)  
**Subject:** FW: 1 selected item: 24814559 - PubMed  
**Date:** Sunday, August 20, 2017 11:48:50 AM  
**Attachments:** Alum-Autism.pdf  
Chris Shaw.PDF  
Roman Gherardi.pdf  
Chris Exley.pdf  
Analysis of Taylor 2014.pdf

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Attachments for NIH Exec Sec as requested, from 7/24 email.

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Joshua A Gordon, MD, PhD  
Director  
National Institute of Mental Health

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**From:** Aaron Siri <aaron@sirillp.com>  
**Date:** Monday, July 24, 2017 at 6:20 PM  
**To:** "M. Joshua Gordon" <joshua.gordon@nih.gov>  
**Subject:** RE: 1 selected item: 24814559 - PubMed

Good evening Joshua,

As promised in my email below, I am following up regarding the research that has been conducted regarding aluminum adjuvants and neuro/psychiatric disorders, and to facilitate a meeting with you and the scientists conducting this research.

In recent years researchers have discovered that injected aluminum adjuvant travels into the brain, where it causes long term chronic inflammation, damage to neurons and behavioral abnormalities. These adverse effects occur at dosages (mcg/Kg body weight) even lower than dosages received by infants according to the CDC vaccine schedule.

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Best regards,  
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For example, the IOM stated in 2011 that there isn't a single study that supports the assertion that DTaP (injected at 2 months, 4 months, 6 months, etc.) does not cause autism, concluding that “The evidence is inadequate to accept or reject a causal relationship between diphtheria toxoid-, tetanus toxoid-, or acellular pertussis-containing vaccine and autism.” Attached is an excerpt of the discussion regarding autism and DTaP from the 2011 IOM report. (I am not aware of a single study regarding DTaP and autism that has been done since 2011.) As another example, the only study regarding Hepatitis B vaccine and autism I have located found a three-fold increase in the odds of an autism diagnosis for neonates that received the hepatitis B vaccine at birth compared that those that did not. (Gallagher CM, Goodman MS. 2010. Hepatitis B vaccination of male neonates and autism diagnosis, NHIS 1997-2002. J Toxicol Environ Health A. 73(24):1665-77.) There is simply no studies for the numerous

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Aaron

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**Sent:** Wednesday, May 31, 2017 4:03 PM

**To:** Aaron Siri <[aaron@sirillp.com](mailto:aaron@sirillp.com)>

**Subject:** Fwd: 1 selected item: 24814559 - PubMed

---

Joshua A Gordon, MD, PhD  
Director  
National Institute of Mental Health

Begin forwarded message:

**From:** Sent by NCBI <[nobody@ncbi.nlm.nih.gov](mailto:nobody@ncbi.nlm.nih.gov)>

**Date:** May 31, 2017 at 4:00:01 PM EDT

**To:** <[Joshua.gordon@nih.gov](mailto:Joshua.gordon@nih.gov)>

**Subject:** 1 selected item: 24814559 - PubMed

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#### PubMed Results

Item 1 of 1 ([Display the citation in PubMed](#))

1. Vaccine. 2014 Jun 17;32(29):3623-9. doi: 10.1016/j.vaccine.2014.04.085. Epub 2014 May 9.

## Vaccines are not associated with autism: an evidence-based meta-analysis of case-control and cohort studies.

[Taylor LE](#)<sup>1</sup>, [Swerdfeger AL](#)<sup>1</sup>, [Eslick GD](#)<sup>2</sup>.

Author information:

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The Whiteley-Martin Research Centre, Discipline of Surgery, The University of Sydney, Nepean Hospital, Level 3, Clinical Building, PO Box 63, Penrith 2751, NSW, Australia.

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The Whiteley-Martin Research Centre, Discipline of Surgery, The University of Sydney, Nepean Hospital, Level 3, Clinical Building, PO Box 63, Penrith 2751, NSW, Australia. Electronic address: [guy.eslick@sydney.edu.au](mailto:guy.eslick@sydney.edu.au).

#### Comment in

- [Autism and vaccination: The value of the evidence base of a](#)

- [recent meta-analysis](#). [Vaccine. 2015]  
• [Answers regarding the link between vaccines and the development of autism: A question of appropriate study design, ethics, and bias](#). [Vaccine. 2015]

## Abstract

There has been enormous debate regarding the possibility of a link between childhood vaccinations and the subsequent development of autism. This has in recent times become a major public health issue with vaccine preventable diseases increasing in the community due to the fear of a 'link' between vaccinations and autism. We performed a meta-analysis to summarise available evidence from case-control and cohort studies on this topic (MEDLINE, PubMed, EMBASE, Google Scholar up to April, 2014). Eligible studies assessed the relationship between vaccine administration and the subsequent development of autism or autism spectrum disorders (ASD). Two reviewers extracted data on study characteristics, methods, and outcomes. Disagreement was resolved by consensus with another author. Five cohort studies involving 1,256,407 children, and five case-control studies involving 9,920 children were included in this analysis. The cohort data revealed no relationship between vaccination and autism (OR: 0.99; 95% CI: 0.92 to 1.06) or ASD (OR: 0.91; 95% CI: 0.68 to 1.20), nor was there a relationship between autism and MMR (OR: 0.84; 95% CI: 0.70 to 1.01), or thimerosal (OR: 1.00; 95% CI: 0.77 to 1.31), or mercury (Hg) (OR: 1.00; 95% CI: 0.93 to 1.07). Similarly the case-control data found no evidence for increased risk of developing autism or ASD following MMR, Hg, or thimerosal exposure when grouped by condition (OR: 0.90, 95% CI: 0.83 to 0.98;  $p=0.02$ ) or grouped by exposure type (OR: 0.85, 95% CI: 0.76 to 0.95;  $p=0.01$ ). Findings of this meta-analysis suggest that vaccinations are not associated with the development of autism or autism spectrum disorder. Furthermore, the components of the vaccines (thimerosal or mercury) or multiple vaccines (MMR) are not associated with the development of autism or autism spectrum disorder.

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PMID: 24814559 [Indexed for MEDLINE]





# SIRI & GLIMSTAD LLP

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## VIA EMAIL & FEDEX

July 24, 2017

U.S. Department of Health & Human Services  
National Institute of Mental Health  
Dr. Joshua A. Gordon, M.D., Ph.D.  
Director of the National Institute of Mental Health  
31 Center Drive, Suite 4A52; MSC 2116  
Bethesda, MD 20892

Re: *Aluminum Adjuvants*

Dear Director Gordon,

It was a pleasure meeting at the National Institutes of Health last month.

As a continuation of our discussion regarding vaccines and autism, and given your role as the Director of the Interagency Autism Coordinating Committee, I write to facilitate a meeting with you (and anyone else from CDC/FDA you deem appropriate) and a number of scientists involved in the research of aluminum adjuvants contained in many vaccines. Their work demonstrates that aluminum adjuvant injected intramuscularly travels into the brain and causes chronic neuroinflammation and behavioral abnormalities. These findings are relevant to autism because autism can be caused by neuroinflammation and elevated cytokines in the brain.

The appendix below summarizes some of this science connecting aluminum adjuvant to autism. Great care was taken to select high quality papers and we hope that the analysis below reflects same. Also attached are several endorsements of the conclusion reached below by many of the leading scientists studying aluminum adjuvants along with a curriculum vitae for each.

The agenda for the requested meeting would be to discuss the existing science reflecting on the safety of aluminum adjuvants -- both the science that supports safety and vice versa -- with an emphasis on its potential connection to autism. It is our hope that you accept this request. If so, kindly have your office provide proposed dates for this meeting.

Thank you for your time and attention to this issue and your work on behalf of the public.

Very truly yours,

A handwritten signature in black ink, appearing to read 'A. Siri', with a stylized flourish at the end.

Aaron Siri, Esq.

## **ALUMINUM ADJUVANTS IN VACCINES & AUTISM**

The Centers for Disease Control (CDC) asserts that vaccines and vaccine ingredients have been disproven as potential causes of autism. Statements by the CDC are generic, encompassing all vaccines and vaccine ingredients. For example, the CDC website states

*“There is no link between vaccines and autism.” “...no links have been found between any vaccine ingredients and autism spectrum disorder.” (CDC website, April 2017)*

The CDC’s statements are not supported by available science because the CDC’s evidence is limited to the MMR vaccine (Taylor 2014), thimerosal preservative (Taylor 2014) and vaccine antigen exposure (DeStefano 2013). Dr Frank DeStefano of the CDC’s Immunization Safety Office is co-author of a paper (Glanz 2015) which states:

*“To date, there have been no population-based studies specifically designed to evaluate associations between clinically meaningful outcomes and non-antigen ingredients, other than thimerosal.”*

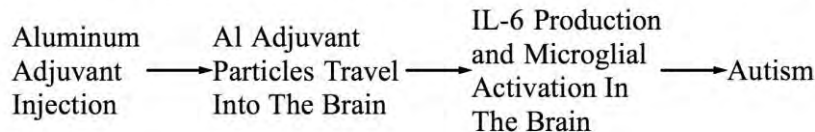
This statement applies to, among other vaccine ingredients, aluminum adjuvant, which is the primary focus of the Glanz 2015 paper. Studies of MMR vaccine cannot be used as evidence of safety for other vaccines, for example vaccines that contain aluminum adjuvant. The CDC’s overly-broad, generic assertions that no vaccines and no vaccine ingredients cause autism are therefore not supported by scientific evidence. In fact, the CDC statements are contradicted by a large, consistent and growing body of scientific evidence, including:

- 1) studies showing neurotoxic and neuroinflammatory effects (e.g. microglial activation) from dosages of aluminum adjuvants lower than or approximately equal to dosages received by infants according to the CDC vaccine schedule (Crepeaux 2017, Petrik 2007, Shaw 2013, Shaw 2009);
- 2) studies linking vaccines to immune activation brain injury (Zerbo 2016, Li 2015); and
- 3) studies showing that early-life immune activation is a causal factor in autism and other neurodevelopmental disorders and mental illnesses (e.g. schizophrenia) (Meyer 2009, Deverman 2009, Estes 2016, Kneusel 2014, Careaga 2017, Meyer 2014).

The accumulating evidence indicates that vaccine-induced immune activation, and aluminum adjuvants in particular, may cause mental illnesses and neurodevelopmental disorders, including autism.

Here we present evidence that aluminum adjuvants can cause autism and other brain injuries. Also, we explain why the studies allegedly demonstrating the safety of aluminum adjuvants do not show safety for adverse neurological outcomes.

# How Aluminum Adjuvants Cause Autism



**Fig 1: Proposed mechanism for how aluminum adjuvants cause autism. Each step is supported by replicated scientific studies.**

## Immune Activation: A Cause of Autism and Mental Illness

The developing brain can be injured by immune activation, with life-long consequences (Meyer 2009, Deverman 2009, Estes 2016, Kneusel 2014, Careaga 2017, Meyer 2014). Immune activation injury is linked to autism, schizophrenia, depression and other mental illnesses or neurodevelopmental disorders. Immune activation effects on the brain are mediated by immune system signaling molecules, especially cytokines (Estes 2016, Meyer 2014, Smith 2007, Choi 2016, Pineda 2013).

It is generally accepted that immune activation (e.g., from infection) during pregnancy is a risk factor for autism and schizophrenia in the offspring (Ciaranello 1995, Atladottir 2010, Brown 2012). The intensity of immune activation and cytokine expression appears to be an important factor for autism risk (Meyer 2014). Intense immune activation is associated with greater risk of autism (Careaga 2017, Atladottir 2010). Chronic inflammation is associated with greater risk of autism (Jones 2016, Zerbo 2014). However, there is no evidence that short-duration, low-intensity immune activation resulting from common childhood illnesses increase autism risk. Timing of immune activation in relation to stages of brain development is also an important factor (Meyer 2006, Meyer 2009).

Animal experiments have tested the effects of immune activation during pregnancy and postnatally on the development of the offspring (Meyer 2009, Deverman 2009, Estes 2016, Kneusel 2014, Careaga 2017, Meyer 2014). In these experiments, pregnant animals (mice, rats and monkeys have been used) or neonates are injected with a non-infectious immune activating substance such as “poly-IC” (which mimics a viral infection) or lipopolysaccharide (LPS, which mimics a bacterial infection). These substances cause immune system activation without infection. They induce fever and cytokine production and can have substantial effects on brain development if activation is sufficiently intense and if exposure occurs during vulnerable developmental stages.

Immune activation has been demonstrated in mice to cause the three core behavioral symptoms of autism (Malkova 2012). Immune activation has also been shown to cause behavioral abnormalities in monkeys that resemble behaviors in human schizophrenia and autism (Bauman 2014, Machado 2015). See Fig. 2. Immune activation also causes neuropathology in monkeys (Weir 2015).

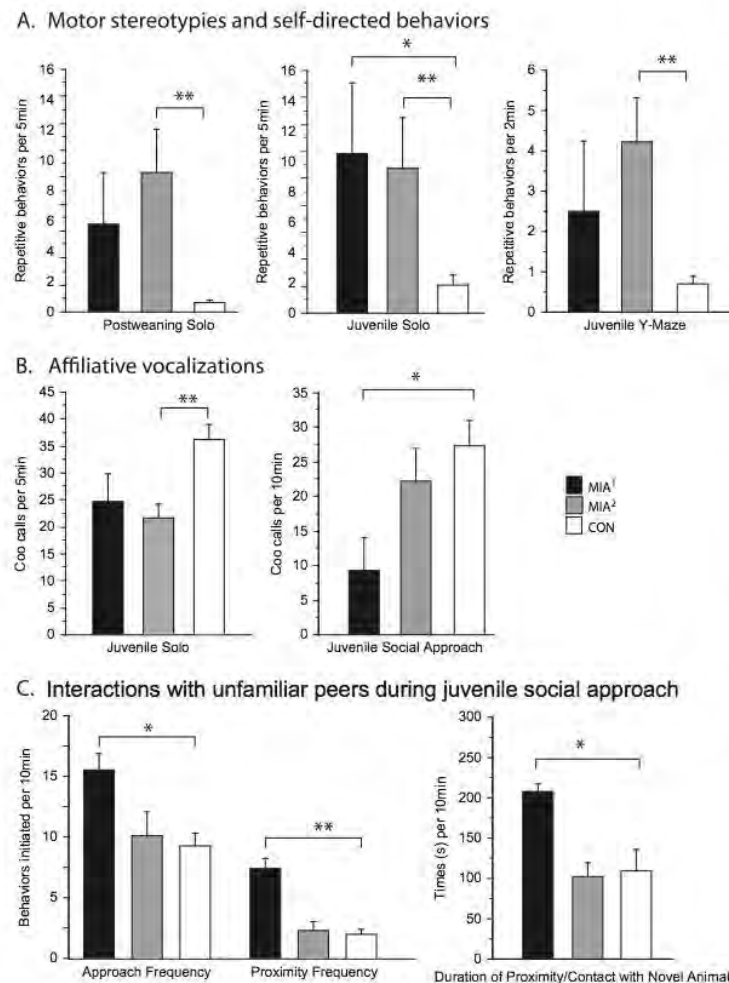
Immune activation also causes non-behavioral effects associated with human autism (citations here link immune activation with these effects):

- 1) reduction in Purkinje cells (Shi 2009);
- 2) mitochondrial dysfunction (Giulivi 2013);
- 3) increase in brain volume (from IL-6 exposure, Wei 2012(b)) and neuron density in the brain (Smith 2012);
- 4) long term chronic brain inflammation (Garay 2012); and
- 5) microbiome disruption (dysbiosis) (Hsiao 2013).

These non-behavioral similarities further support the relevance of the immune activation models to human autism. The non-behavioral (e.g., physiological) effects of immune activation have been reviewed (Labouesse 2015).

M.D. Bauman *et al.*

BIOL PSYCHIATRY 2014;75:332–341 335



**Figure 2.** (A) Maternal immune activation (MIA) offspring exhibit increased frequency of motor stereotypies and self-directed behaviors. Left panel: When observed alone in a large cage at 10 months of age, second trimester MIA (MIA<sup>2</sup>) animals produce significantly more repetitive behaviors than control animals (CON) (\*\* $p \leq .01$ ). The first trimester MIA (MIA<sup>1</sup>) offspring also produce more repetitive behaviors than control animals, but this difference does not reach statistical significance at 10 months ( $p = .06$ ). Middle panel: When observed alone at 22 months of age, MIA<sup>1</sup> offspring produce significantly more repetitive behaviors than control animals (\* $p \leq .05$ ). Second trimester MIA animals also produce significantly more repetitive behaviors than control animals at 22 months (\*\* $p \leq .01$ ). Right panel: When tested at 17 months of age in the Y-maze social preference assay, MIA<sup>2</sup> treatment animals produce significantly more repetitive behaviors than control animals (\*\* $p \leq .01$ ). (B) Maternal immune activation offspring display decreased affiliative vocalizations. Left panel: At 22 months, MIA<sup>2</sup> offspring produce significantly fewer coo calls than control animals (\*\* $p < .01$ ). Right panel: When observed with a novel conspecific at 24 months of age, MIA<sup>1</sup> offspring produce significantly fewer coo calls than control animals (\* $p \leq .05$ ). (C) Maternal immune activation offspring exhibit inappropriate interactions with unfamiliar conspecifics. Left panel: First trimester MIA offspring demonstrate inappropriate social interactions with an unfamiliar animal, as indexed by high frequency of approaching (\* $p < 0.05$ ) and more frequently moving within arm's reach of the unfamiliar animal (\*\* $p < .01$ ). Right panel: First trimester MIA offspring remained near the unfamiliar animal, as indexed by the duration of time spent in physical contact or within arm's reach of the unfamiliar animal (\* $p < .05$ ).

**Fig 2: Maternal immune activation in monkeys caused behavioral abnormalities in juvenile offspring resembling behaviors in both autism and schizophrenia. MIA1 (Black)= first trimester immune activation; MIA2 (grey) 2nd trimester immune activation; CON (white) saline control. From Bauman et al. 2014.**

The cytokines interleukin-6 (IL-6) and interleukin-17a (IL-17) have been identified as mediating the behavioral effects of immune activation (Smith 2007, Malkova 2012, Choi 2016,

Pineda 2013, Wei 2012(a), Wei 2013, Parker-Athill 2010, Wei 2016). The IL-6 findings have been replicated by different researchers using a variety of experimental methods. For example, in an experiment with poly-IC, abnormal behavior is almost completely prevented by simultaneous administration of IL-6-blocking antibody (Smith 2007, Pineda 2013). Injection of IL-6 by itself causes abnormal behavior that closely matches behavior resulting from poly-IC immune activation (Smith 2007). Inhibition of IL-6 signaling in a genetic autism model (BTBR mice) normalized social and repetitive behavior (Wei 2016). These results demonstrate that IL-6 is responsible for causing abnormal autism-like behavior.

The Patterson laboratory at CalTech was the first to report that IL-6 is responsible for causing the autism-like behavioral effects of immune activation (Smith 2007). Two papers from this research group state:

*“IL-6 is central to the process by which maternal immune activation causes long-term behavioral alterations in the offspring.” (Smith 2007)*

*“...blocking IL-6 prevents >90% of the changes seen in offspring of poly(I:C)-injected females, showing that gene expression changes, as well as behavioral changes, are normalized by eliminating IL-6 from the maternal immune response.” (Smith 2007)*

*“IL-6 is necessary and sufficient to mediate these effects since the effects...are prevented by injection of pregnant mice with poly-IC combined with an anti-IL-6 antibody, and are mimicked by a single maternal injection of IL-6.” (Garay 2013)*

Brain exposure to elevated IL-6 by engineered virus showed that IL-6 exposure, initiated after birth, caused autism-like behaviors (Wei 2012(a)). The Wei 2012(a) paper states:

*“We demonstrated that IL-6 is an important mediator of autism-like behaviors. Mice with an elevated IL-6 in brain developed autism-like behaviors, including impaired cognition ability, deficits in learning, abnormal anxiety-like trait and habituation, as well as a decreased social interaction initiated at later stages. These findings suggest that an IL-6 elevation in the brain could modulate certain pathological alterations and contribute to the development of autism.” (Wei 2012(a))*

More recent evidence shows that IL-17 acts downstream of IL-6 to cause autism-like behavioral abnormalities and atypical cortical development in mice (Choi 2016). Blocking either IL-6 or IL-17 prevents the autism-like behavior; an injection of IL-17 by itself causes the autism-like behavior (Choi 2016). IL-6 is known to induce IL-17 by promoting the development of Th17 cells which produce IL-17.

Immune activation animal models appear to be valid models for human neurological/psychiatric disorders, including autism (Estes 2016, Careaga 2017, Meyer 2014). The Estes 2016 review argues for the validity of the immune activation models to humans:

*“These MIA (maternal immune activation) animal models meet all of the criteria required for validity for a disease model: They mimic a known disease-related*

*risk factor (construct validity), they exhibit a wide range of disease-related symptoms (face validity), and they can be used to predict the efficacy of treatments (predictive validity).” (Estes 2016)*

Evidence suggests a mediating role for IL-6 and IL-17 in human autism. For example, IL-6 is significantly elevated in the cerebellum in human autism (Wei 2011) and is highly elevated in some brain regions of some autistic individuals (Vargas 2005). Treatment of human autistics with the anti-inflammatory flavonoid luteolin improves autistic behaviors in the individuals that also experience a decline in IL-6 blood levels (Tsiloni 2015). This result is consistent with a causal role for IL-6 in human autism. Also, IL-17 is elevated in human autism (Akintunde 2015, Al-Ayadhi 2012, Suzuki 2011). Vitamin D reduces IL-17 production (Bruce 2011, Wobke 2014, Drozdenko 2014) and improves autistic behaviors in humans (Saad 2016, Jia 2015). The vitamin D findings are consistent with a causal role for IL-17 in human autism.

IL-6 functioning appears to be similar or identical in mice and humans. No mouse-human differences in IL-6 functioning are described in a 2004 review (Mestas 2004). IL-6 functioning is quite conserved across species (Brown 2014). Central nervous system development in rodents and humans is governed by the same principles (Brown 2014). Hence, the fact that IL-6 causes autism-like behavioral abnormalities in animal models deserves a presumption of validity to humans.

Immune activation is a risk factor for autism, schizophrenia and other neurological/psychiatric disorders. The cytokines IL-6 and IL-17 are responsible for mediating the autism-like behavioral effects of immune activation in the animal models. The available evidence supports a causal role for IL-6 and IL-17 in human autism.

### **Maternal vs. Postnatal Immune Activation**

The timing of immune activation is an important factor influencing effects on the brain. The developing brain is vulnerable to immune activation injury; the mature, adult brain is apparently not nearly as vulnerable. Sensitivity to immune activation likely declines as the brain matures (Meyer 2014, Meyer 2007).

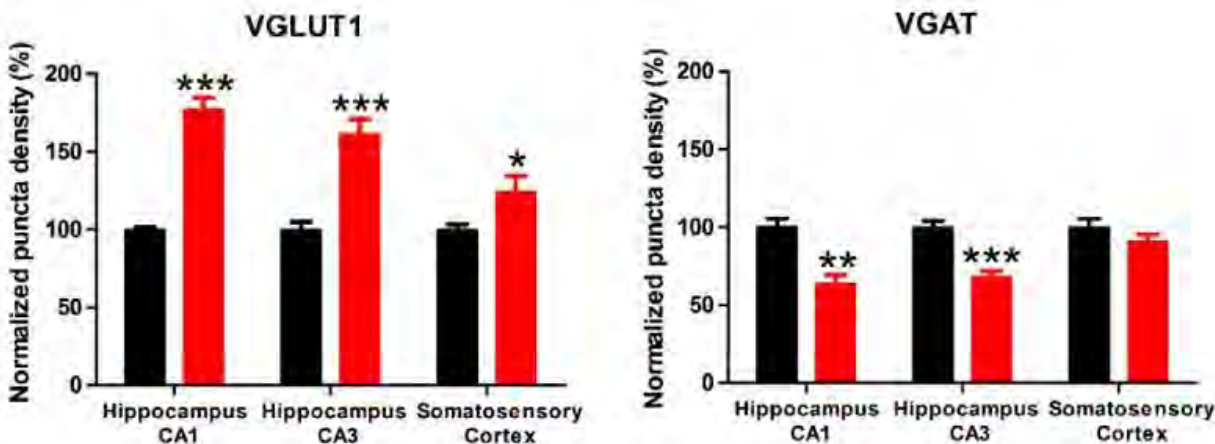
In most immune activation experiments, the offspring are exposed to immune activation during gestation (by stimulating the maternal immune system). In contrast, most vaccines are administered postnatally. This raises the question of whether postnatal immune activation can have similar effects on the brain as maternal immune activation. Diverse evidence indicates that the brain can be adversely affected by postnatal immune activation. Postnatal immune activation experiments, human case reports, and consideration of brain development timelines suggest that the human brain is vulnerable to immune activation injury for years after birth.

In the maternal immune activation experiments, inflammatory signaling and some cytokines (e.g. IL-6) traverse the placenta into the fetus. Consequently, immune activation in the mother causes immune activation and elevated cytokines in the fetus, and in the fetal brain (Oskvig 2012, Ghiani 2011).

Postnatal immune activation can have adverse neurological effects, including increased seizure susceptibility (Chen 2013, Galic 2008), learning and memory deficits (Harre 2008), and

an increase in excitatory synapse formation (Shen 2016). Seizure disorders, learning and memory dysfunction, and elevated excitatory signaling are associated with autism.

Elevated IL-6 in the brain in the postnatal period causes neuronal circuitry imbalance and mediates autism-like behaviors in mice (Wei 2012(a)). The circuitry imbalance observed in Wei 2012(a) was an excess of excitatory synapses and a deficit of inhibitory synapses. See Fig. 3. Excessive excitatory signaling is observed in human autism (Robertson 2016, Freyberg 2015). In fact, an imbalance between excitatory and inhibitory signaling (towards excess excitation) has been posited as a central characteristic of autism (Robertson 2016, Freyberg 2015).



**Fig 3: Elevation of IL-6 in the brains of mice (initiated shortly after birth) caused an increase in excitatory synapses (VGLUT1) and a decrease in inhibitory synapses (VGAT). Excessive excitatory signaling is observed in human autism. Red=Elevated IL-6; Black=Control. VGLUT1=excitatory synapses; VGAT=inhibitory synapses. \*P<0.05, \*\*P<0.01 and \*\*\*P <0.001. Adapted from Wei et al 2012(a).**

In a maternal immune activation experiment with mice (Coiro 2015), autism-relevant behavior and dendritic spine abnormalities (relevant to autism and schizophrenia) were ameliorated by administering an anti-inflammatory drug postnatally. The drug was started at birth and continued for 2 weeks, which roughly corresponds to age 2 in humans (Semple 2013). This result indicates that brain development is affected by postnatal inflammation, at times corresponding to when vaccines are given to humans.

Several case reports describe previously-healthy children that displayed sudden-onset autistic behavior during or subsequent to infection in the brain. All the cases had signs of intense brain inflammation. Here are brief descriptions:

*Delong 1981:* describes 3 children, ages 5, 7 and 11 with full-blown autistic behavior associated with brain inflammation. Brain inflammation was presumed in two cases and confirmed in one. The 5 and 7 year olds recovered completely, and the 11-year recovered partially.

*Marques 2014:* describes a previously healthy 32-month-old girl that suffered autistic regression from a viral central nervous system infection with associated brain inflammation.

*Ghaziuddin 2002*: describes a previously healthy 11-year-old boy that suffered permanent autistic regression after sudden onset herpes brain infection with associated brain inflammation.

*Gillberg 1986*: describes a previously healthy 14-year-old girl with permanent autistic regression from herpes brain infection with associated brain inflammation.

The most parsimonious explanation for these cases is that autistic behavior resulted from intense inflammation and cytokine production in the brain. Accordingly, these cases indicate that the human brain remains vulnerable to immune activation injury well into childhood, though the vulnerability almost certainly decreases with maturation. The susceptibility of older children to inflammation-induced autistic behavior strongly suggests that younger infants, of 0-2 years of age, are also vulnerable. It is not reasonable to claim, and there is no evidence to suggest, that the age range of 0-2 years (when most vaccines are given) is uniquely resistant to immune activation injury. All the available evidence indicates the opposite.

The immune activation experiments and case reports are consistent and indicate that immune activation and elevated cytokines in the postnatal period can cause brain injury.

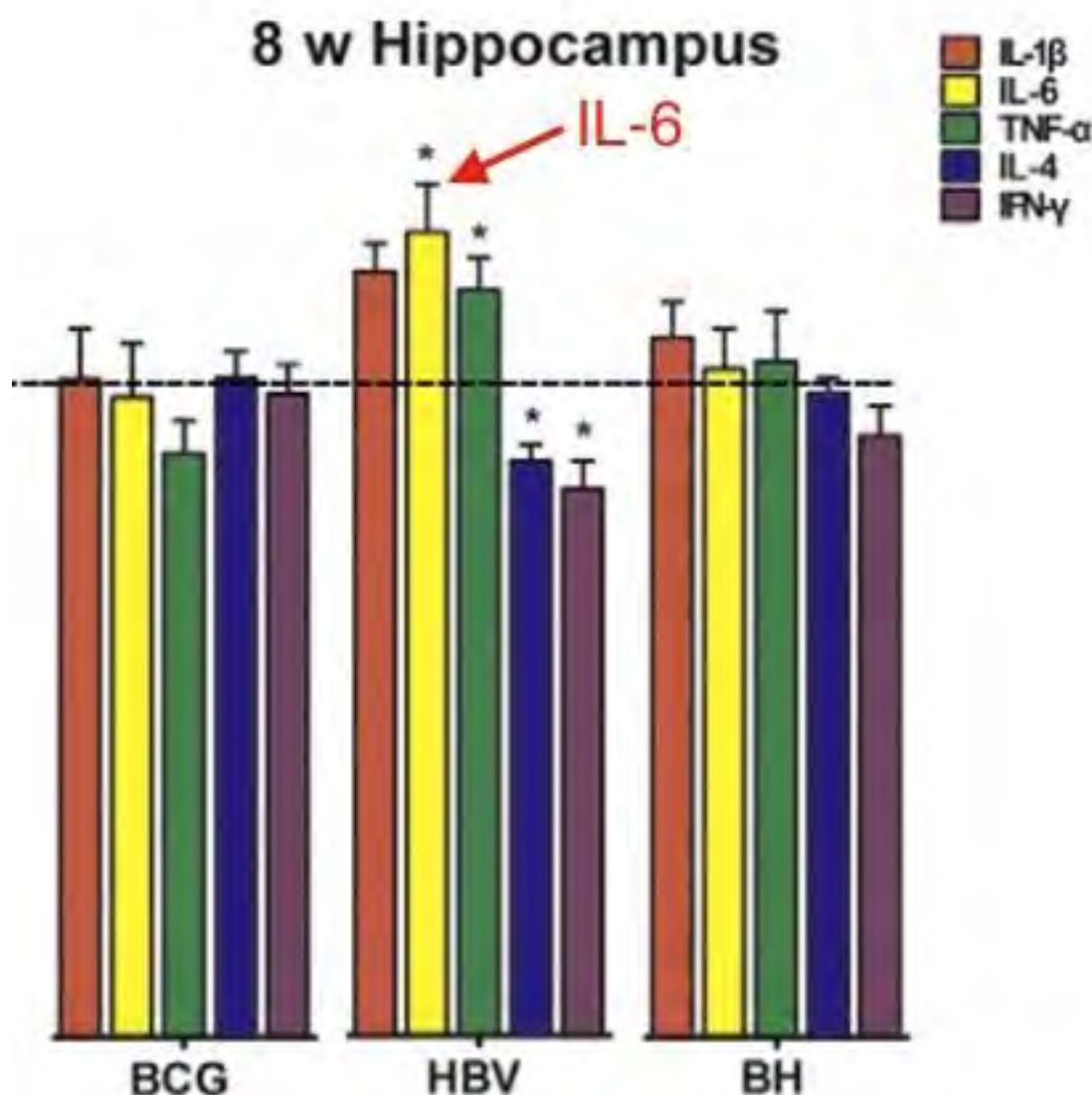
The next critical question to consider is whether vaccines can cause immune activation and elevated cytokines in the brain.

### **Postnatal Vaccination Affects Brain Development in Animal Model**

The first study to test the effect of postnatal vaccination on brain development was published in 2015 (Li 2015). In this experiment, neonatal rats were administered bacillus calmette-guerin (BCG) vaccine, hepatitis B (HBV) vaccine or a combination (BCG+HBV) timed to imitate human infant vaccination schedules. BCG and HBV vaccines produced opposite effects on the brain. Specifically, BCG enhanced synaptic plasticity and long-term potentiation (LTP, the basis for learning and memory); HBV inhibited synaptic plasticity and LTP. BCG and HBV vaccines also caused opposite changes in some synapse protein levels.

HBV vaccine (but not BCG vaccine) increased IL-6 gene expression in the brain; increased gene expression likely indicates an elevation in brain IL-6. The HBV vaccine contains aluminum adjuvant, and the BCG does not contain aluminum adjuvant. Hence, the aluminum adjuvant may be the ingredient responsible for the elevated IL-6 gene expression. See Fig. 4.





**Fig. 4: Hepatitis B vaccine, but not BCG vaccine, increased IL-6 gene expression in the brain at 8 weeks after neonatal vaccination. Hepatitis B vaccine contains aluminum adjuvant; BCG vaccine does not. Elevated IL-6 causes autism-like behaviors in animal models. \*P<0.05 Adapted from Li et al 2015.**

The Li et al study showed that the vaccines caused other changes in the brain, including 1) changes in long-term potentiation (LTP) (Hep B decreased LTP), 2) changes in dendritic spines, and 3) changes in synapse protein expression. Changes in synapse proteins and dendritic spines have been observed in human brain disorders.

Li et al. attribute the brain effects to changes in cytokine levels and immune polarization (Th1/Th2 polarization) induced by the vaccines. Aluminum adjuvants cause Th2 polarization. Li et al. state that the results suggest vaccines can interact by way of immune activation effects:

*“...our data suggested that combinations of different vaccines can mutually interact (enhance or counteract). The mechanism of synaptic plasticity*

*modulation through neonatal BCG/HBV vaccination may be via systemic Th1/Th2 bias accompanied by a specific profile of cytokines and neurotrophins in the brain.” (Li 2015)*

Li 2015 demonstrates that vaccines affect brain development by an immune activation mechanism. Further, since aluminum adjuvants induce Th2 activation and long term Th2 polarization, the Li 2015 results suggest that all aluminum-adjuvanted vaccines may cause adverse effects similar to the HBV vaccine. Accordingly, the Li 2015 results suggest that studies showing that immune activation causes neurological/psychiatric disorders are relevant to vaccine adverse effects.

### **Vaccines Are Given During Synaptogenesis**

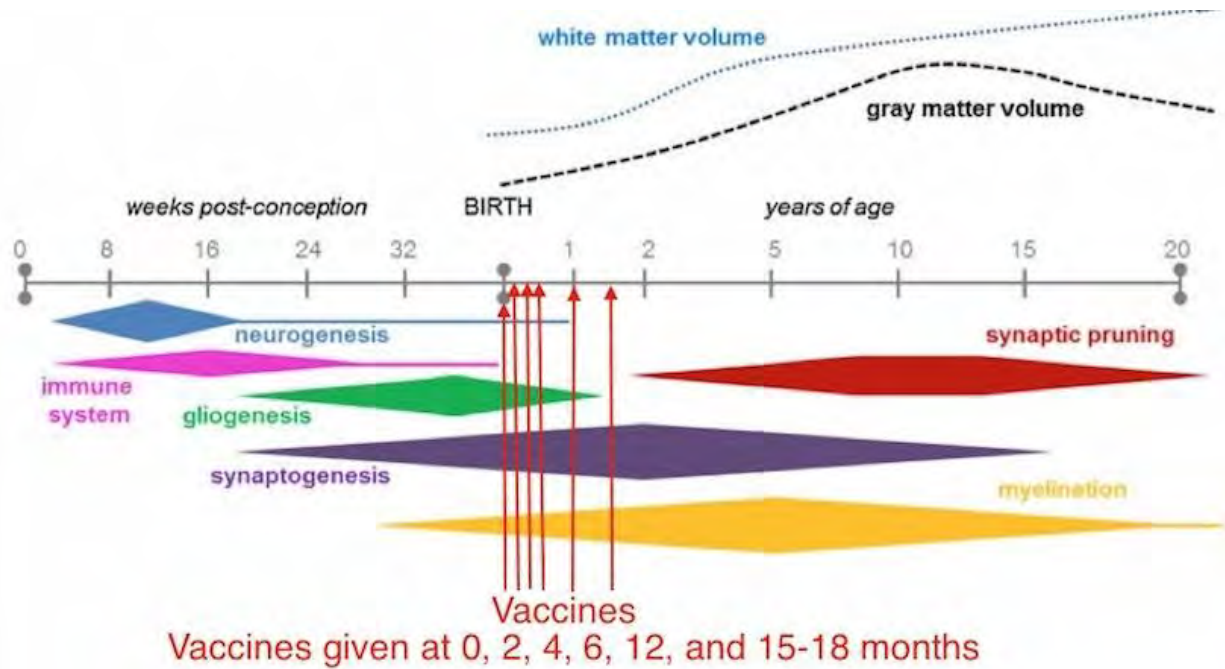
Another way to answer the question of brain vulnerability to immune activation is to consider the types of brain development processes occurring when vaccines are administered. Vaccines are given primarily in the first 18 months after birth. The human brain undergoes intense and rapid development during this period. Synaptogenesis (formation of synapse connections between neurons) is especially intense in this period.

The vulnerability of the developing brain to immune activation is apparently related to the specific types of brain development processes occurring (Tau 2010, Meyer 2006, Meyer 2007). Such processes include migration (movement of neurons to final locations in the brain), adhesion (formation of chemical-mechanical attachments between brain cells), and synaptogenesis (formation of synapse connections between neurons), among others (neurogenesis, gliogenesis, myelination etc).

Cytokines affect brain development processes. For example, elevated IL-6 affects migration, adhesion and synaptogenesis (Wei 2011). Elevated IL-6 in the postnatal period promotes an excess of excitatory synapses and a deficit of inhibitory synapses, and mediates autism-like behaviors (Wei 2012(a)).

In humans, a dramatic increase in synaptogenesis begins around the time of birth, and continues until about age 3 (Huttenlocher 1997, Tau 2010, Stiles 2010, Semple 2013). Vaccines are administered during this intense synaptogenesis. See Figs. 5-6. Elevated brain IL-6 induced by vaccination during synaptogenesis may cause an excitatory-inhibitory imbalance, towards excitation. An excitatory imbalance has been observed in human autism (Robertson 2016, Freyberg 2015).

Synaptogenesis tapers off through childhood and adolescence. This fact may explain why some older children and teens can suffer autistic regression after intense brain inflammation, but apparently become less vulnerable to immune activation brain injury with age.



**Fig. 5: Timeline of specific brain developmental processes in humans. Synaptogenesis is most intense during the first couple years of life, when vaccines are administered. Timing of vaccination according to the CDC vaccine schedule is shown. Elevated IL-6 during synaptogenesis may cause an excitatory-inhibitory synapse imbalance, towards excitation. Adapted from Semple 2013.**

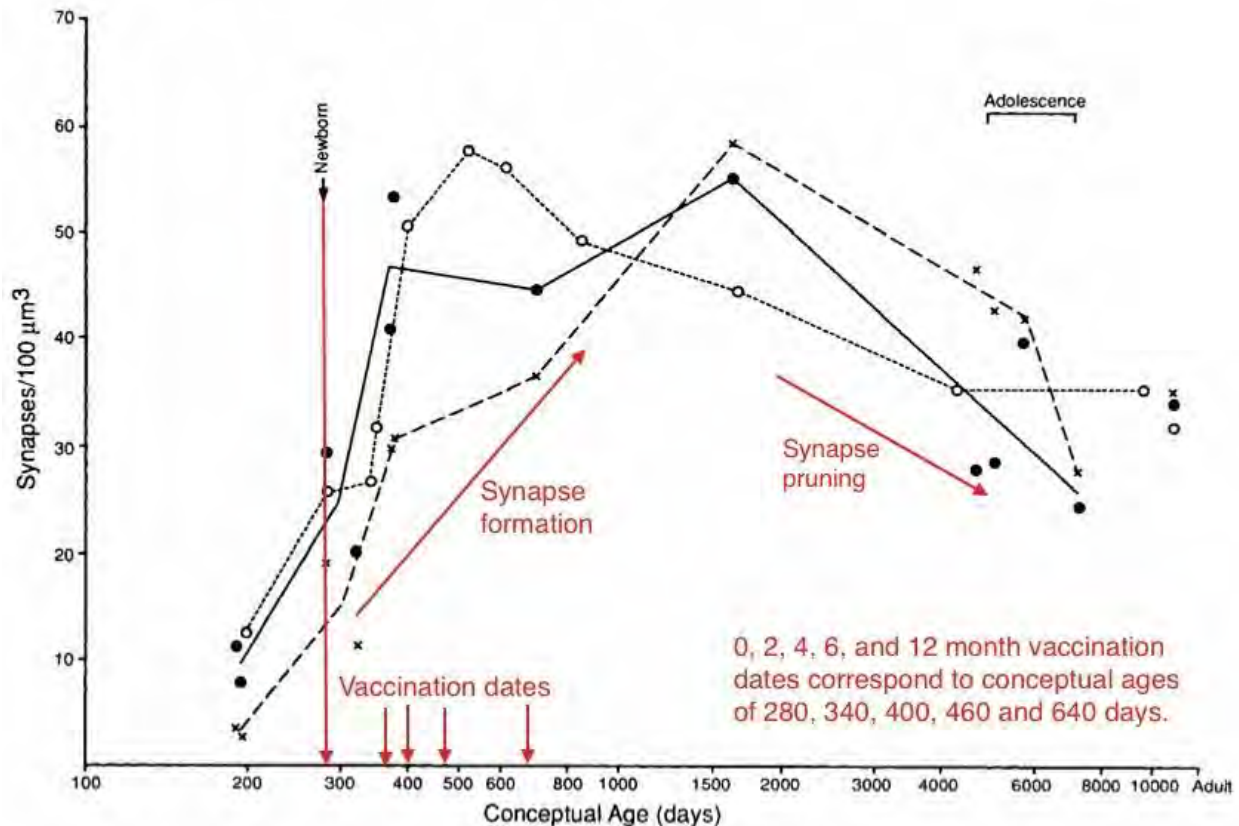


Fig. 2. Mean synaptic density in synapses/100  $\mu\text{m}^3$  in auditory, calcarine, and prefrontal cortex at various ages. Open circles, visual cortex (area 17); filled circles, auditory cortex; x, prefrontal cortex (middle frontal gyrus).

**Fig. 6: Measurements of synapse density in human cadavers of various ages indicate a dramatic increase in synapses in the first few years of life. Vaccines are administered during intense synapse formation. Elevated IL-6 during synaptogenesis may cause an excitatory-inhibitory synapse imbalance, towards excitation. Image adapted from Huttenlocher and Dabholkar 1997.**

Intense synaptogenesis occurs at ages 0-18 months, when many vaccines are administered. Consequently, vaccines may adversely impact synaptogenesis if they induce inflammation or IL-6 in the brain.

The timing of brain development processes in humans supports the idea that the human brain is vulnerable to immune activation and cytokines in the first few years after birth, when vaccines are administered. Disruption of synaptogenesis by vaccine-induced immune activation is a particular concern.

### **Aluminum Adjuvants: Neurotoxic At Vaccine Dosages**

Aluminum (Al) adjuvants have an essential role in many vaccines: to stimulate immune activation. Without Al adjuvants, these vaccines would have greatly reduced efficacy.

Aluminum adjuvants comprise sub-micron particles (primary particles) of aluminum compounds, typically  $\text{AlOH}$ ,  $\text{AlPO}_4$ ,  $\text{AlSO}_4$  or mixtures. The primary particles are typically

agglomerated into larger particles with sizes of about 2-20 microns (Harris 2012). The Al adjuvant materials have low solubility in water and body fluids. Al adjuvant particles are biopersistent and can remain in the body for months or years (Flarend 1997, Khan 2013, Gherardi 2001).

Aluminum ingested in the diet has low oral absorption (about 0.3%), is rapidly excreted by the kidneys, is (mostly) excluded from the brain by the blood-brain barrier, and is in a solubilized, Al<sup>3+</sup> ionic form (not particulate) These defenses are adequate for protecting the brain from natural levels of aluminum exposure. These protective mechanisms are unable to protect the brain from injected aluminum adjuvant particles. Al adjuvant particles are too large to be removed by the kidneys, and are carried across the blood-brain barrier by macrophages.

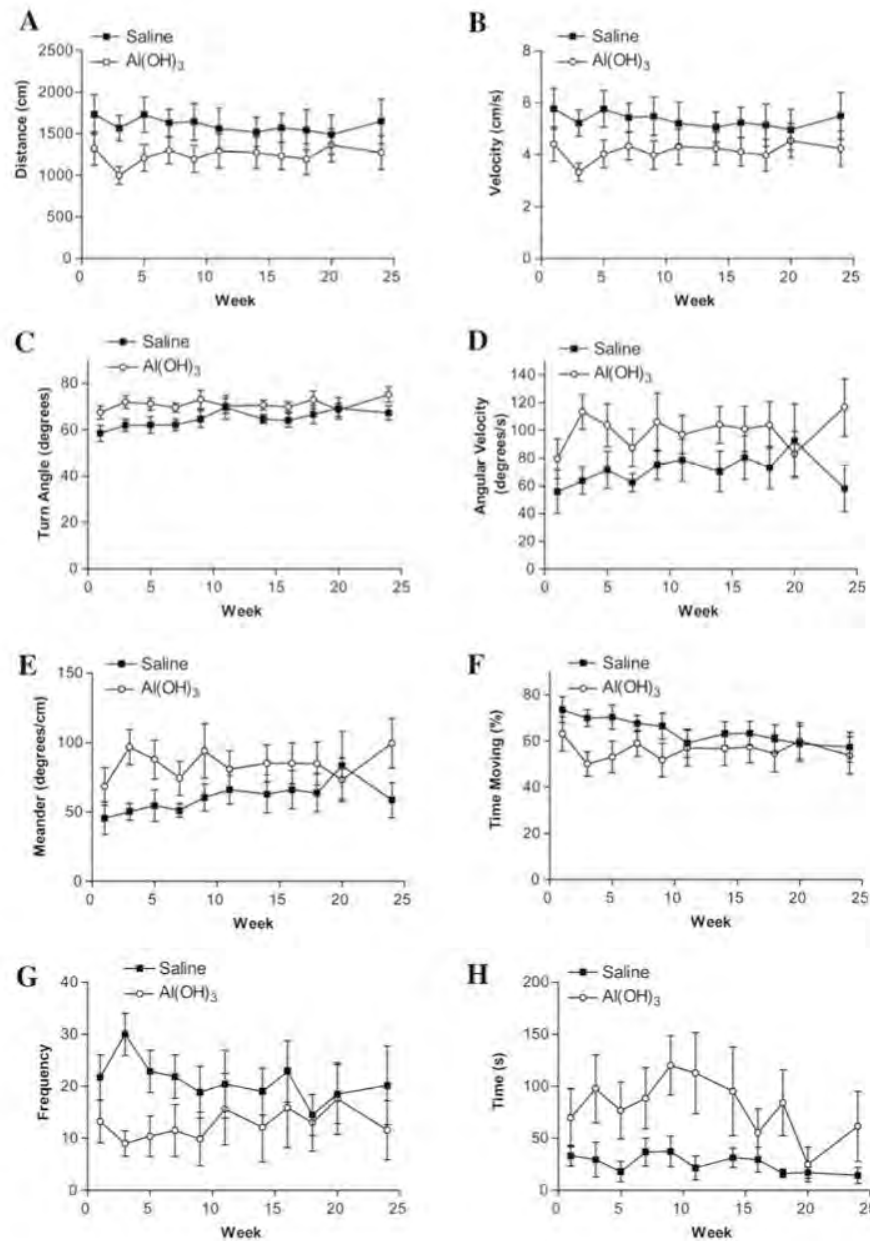
Dosages of aluminum adjuvants received by infants according to the CDC vaccination schedule are:

<b>Birth (Hep B):</b>	<b>74 mcg/kg (250 mcg for 3.4 kg infant)</b>
<b>2 month:</b>	<b>245 mcg/kg (1225 mcg for 5 kg infant)</b>
<b>4 month:</b>	<b>150 mcg/kg (975 mcg for 6.5 kg infant)</b>
<b>6 month:</b>	<b>153 mcg/kg (1225 mcg for 8 kg infant)</b>

These are maximum-possible dosages (because different vaccine products have different amounts) for average-weight infants.

Accumulating evidence shows that aluminum adjuvants have adverse neurological effects at dosages lower than or approximately equal to dosages infants receive from vaccines. These effects appear to depend on the particulate nature and biopersistence of the aluminum adjuvant. Injected Al adjuvant has adverse effects that are apparently mediated by the particles and independent of solubilized Al<sup>3+</sup> ions released by the slowly dissolving particles (Crepeaux 2017).

Al adjuvant injections in mice cause adverse effects at vaccine-relevant dosages of 100, 200, 300 and 550 mcg/Kg body weight (Crepeaux 2017, Shaw 2009, Petrik 2007, Shaw 2013). These include deficits in learning and memory (Shaw 2009), deficits in neuromuscular strength/function (Petrik 2007), and changes in locomotor activity and/or gait (Shaw 2009, Shaw 2013). Autism is associated with gait and movement abnormalities (Kindregan 2015) and memory dysfunction (Williams 2006).

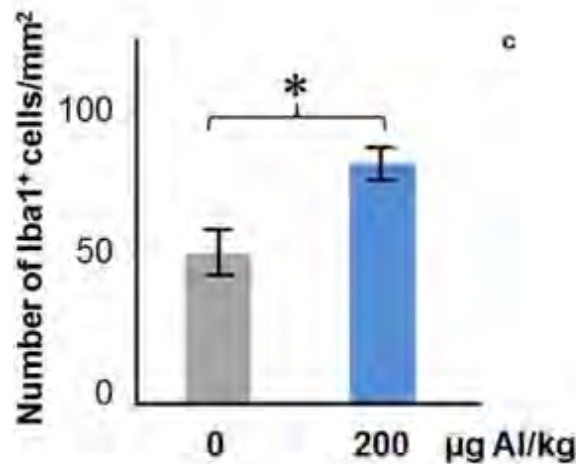


**Fig. 4.**

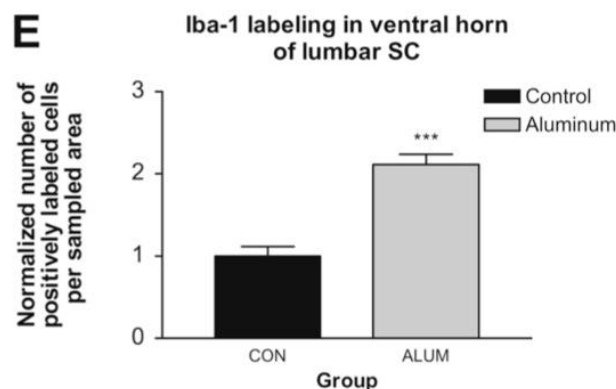
Open field movement analysis as an assessment of spontaneous activity and anxiety in control mice vs. mice injected six times with aluminum hydroxide. Aluminum hydroxide injected mice showed the following behavioural changes: (A) Shorter distances moved ( $***p < 0.0001$ ). (B) Slower movement ( $***p < 0.0001$ ). (C) Greater mean turn angle ( $***p < 0.0001$ ). (D) More rapid turning ( $***p < 0.0001$ ). (E) Greater meander ( $***p < 0.0001$ ). (F) Smaller percentage of time in overall movement ( $**p = 0.0030$ ). (G) Fewer entries into the centre of the open field ( $***p < 0.001$ ). Late entry into centre ( $***p < 0.0001$ ). (All measures, two-way ANOVA).

**Fig. 7: Dosage of 300mcg/Kg AlOH adjuvant caused large and persistent changes in exploratory behavior and movement in open field tests. This is an indicator of neurotoxicity. Human autistics also display abnormal movement and exploratory behavior. Adapted from Shaw and Petrik 2009.**

Al adjuvant dosages of 200mcg/Kg (as 3 x 66mcg/Kg) (Crepeaux 2017) and 300mcg/Kg (as 6 x 50mcg/Kg) (Shaw 2009) increased microglial activation in the ventral forebrain and lumbar spinal cord, respectively. The elevated microglial activation was measured about 6 months after Al adjuvant injection, which suggests that the microglial activation is chronic. Activated microglia indicate an ongoing inflammatory process and suggest the presence of elevated cytokines. Human autistics have activated microglia and elevated cytokines throughout the brain (Vargas 2005, Suzuki 2013, Li 2009).



**Fig. 8:** Al adjuvant (200mcg/Kg) caused an increase in microglial activation in the brain of mice. The protein iba1 indicates activated microglia. Measurements were performed 6 months after Al adjuvant injection, indicating that the microglial activation is a chronic condition. \*  $P < 0.05$ . From Crepeaux et al., 2017.



**Fig. 9:** Al adjuvant (300mcg/Kg) caused an increase in microglial activation in the lumbar spinal cord of mice. The protein iba1 indicates activated microglia. Measurements were performed 6 months after Al adjuvant injection, indicating that the microglial activation is a chronic condition. \*\*\* $p < 0.001$ , one-way ANOVA. From Shaw and Petrik 2009.

Activated microglia are implicated as a causal factor in autism, because microglia mediate inflammation in the brain. Microglia can produce IL-6 when in an activated state. A recent review on microglia and autism (Takano 2015) states:

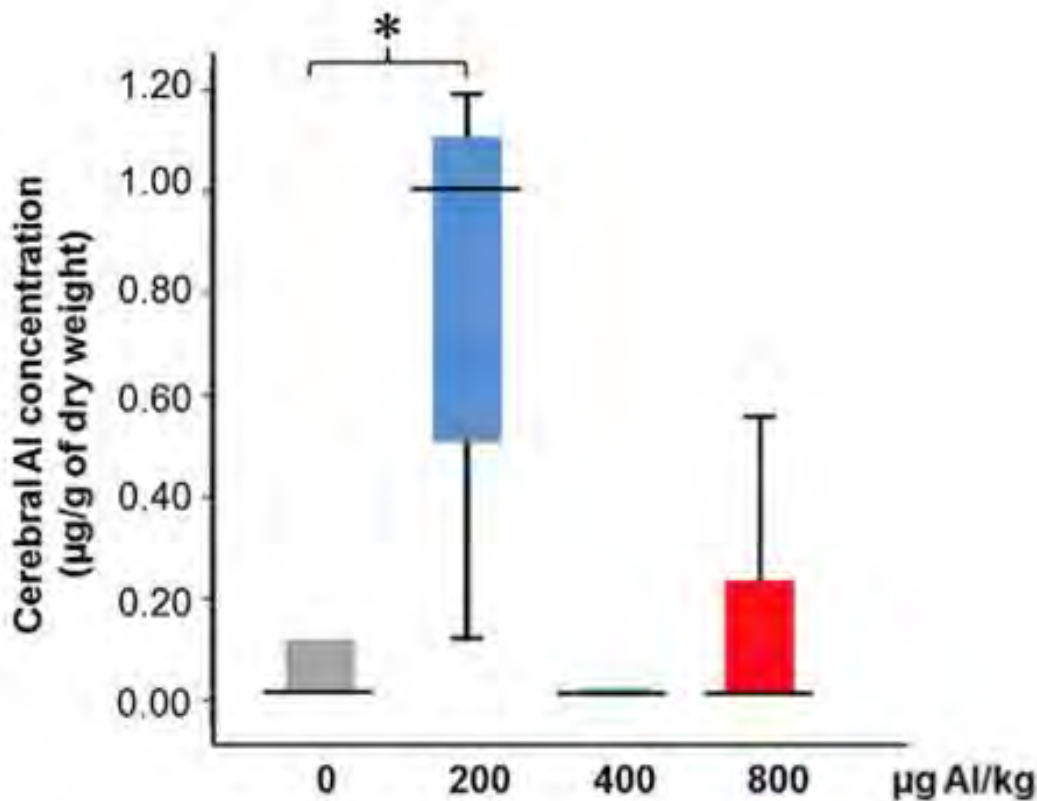
*“...any factors that alter the number or activation state of microglia either in utero or during the early postnatal period can profoundly affect neural development, thus resulting in neurodevelopmental disorders, including autism.”*  
(Takano 2015)

Microglia appear to play an important role in the causation of autism (Takano 2015, Kneusel 2014). Hence, the microglial activation caused by aluminum adjuvants suggests a role in autism.

Several studies show that Al adjuvants increase brain aluminum content (Crepeaux 2017, Flarend 1997, Shaw 2009, Khan 2013, Crepeaux 2015). A dosage of 200 mcg/Kg Al adjuvant caused a 50-fold increase in brain aluminum content in mice, from 0.02 ug/g to 1.0 ug/g dry weight of brain (Crepeaux 2017). These measurements were performed 6 months after the final injection, indicating that the Al persists in the brain long-term (Crepeaux 2017). See Fig. 10. Al adjuvants have been found to accumulate in the brain of mice up to one year after injection (Khan 2013). Crepeaux 2015 demonstrated persistence and increasing accumulation of Al adjuvant particles up to 270 days in spleen and lymph nodes of mice. Increasing accumulation of Al in distant organs over time suggests that toxic effects may increase with time, and may be delayed by months or years after exposure.

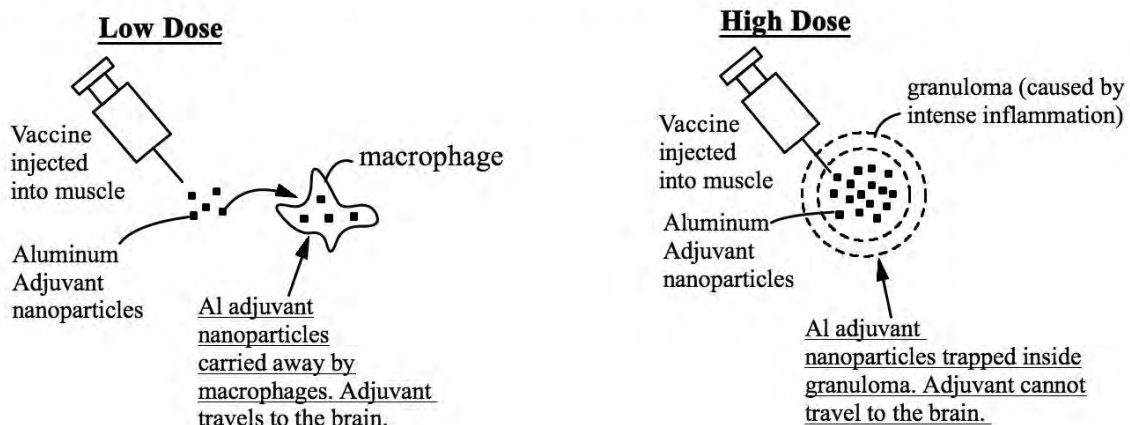
The 400 and 800 mcg/Kg doses used in the Crepeaux 2017 study did not cause adverse effects or elevated brain aluminum. The authors attribute this surprising inverted dose-response relationship to granulomas induced by the higher dosages. Granulomas trap the Al adjuvant at the injection site, thereby preventing its transport into the brain and other sensitive tissues. Granulomas occur after about 1% of vaccinations (Bergfors 2014). This is cause for concern because it indicates that, for 99% of vaccinations, the Al adjuvant can be transported around the body. It is not confined to a granuloma. See Fig. 11.





**Fig. 10: Dosage of 200 mcg/Kg Al adjuvant caused a 50-fold increase in brain aluminum content, from 0.02 to 1.00 ug/g dry weight, in mice. Higher dosages (400 and 800 mcg/Kg) did not increase brain Al content, presumably because the higher dosages caused a granuloma at the injection site. A granuloma traps the Al adjuvant at the injection site, thereby preventing systemic dispersal and transport into the brain. These measurements were performed 6 months after the final injection, indicating that the Al persists in the brain long-term. \*P<0.05. From Crepeaux et al., 2017.**

### **Proposed Mechanism For Inverse Dose-Toxicity Relationship:**



**Fig. 11: High dose Al adjuvant injection into the muscle causes a granuloma, which traps the Al adjuvant and prevents it from traveling into the brain. Low dose does not form a granuloma. Hence, the lower dose is free to travel to the brain. Consequently, the lower dose is more toxic than the higher dose. This mechanism explains the surprising inverted dose-toxicity results of Crepeaux et al. 2017.**

### **Particle Transport and Macrophage Chemotactic Protein (MCP-1)**

Aluminum adjuvants travel into the brain (Khan 2013, Crepeaux 2015, Crepeaux 2017, Shaw 2009, Flarend 1997). Al adjuvant particles are carried through the blood-brain barrier and into the brain by macrophages (Khan 2013). Transport is promoted by macrophage chemotactic protein-1 (MCP-1) (Khan 2013). MCP-1 causes macrophages to travel around the body and into the brain. Particle transport into the brain by macrophages is well-established and has been investigated for therapeutic applications (Choi 2012, Pang 2016).

MCP-1 is elevated in the brains of human autistics (Vargas 2005) and is elevated in the blood of neonates later diagnosed with autism (Zerbo 2014). This suggests that neonates with high MCP-1 will experience elevated Al adjuvant transport into the brain when injected with Al adjuvanted vaccines. This is consistent with Al adjuvants causing autism by inducing immune activation and elevated cytokines in the brain.

### **Aluminum Induces IL-6 Expression In The Brain**

Water-soluble aluminum salts (e.g.  $AlCl_3$ , Al lactate) induce elevated IL-6 in the brain and other tissues. In fact, aluminum appears to selectively induce IL-6 (Viezeleiene 2013). Studies of aluminum exposure and IL-6 expression in the brain include:

Cao 2016: Ingestion of 30 or 90 mg/kg/day aluminum (as  $AlCl_3$ ) for 90 days significantly increased gene expression of IL-6 and other cytokines in the brain (hippocampus).

Alawdi 2016: Ingestion of 3.4 mg/kg/day aluminum (as  $AlCl_3$ ) for 6 weeks caused a 4-fold increase in IL-6 in the brain (hippocampus). This dosage is far lower than the outdated “no observed adverse effects level” (NOAEL) oral dosages (26 and 62 mg/kg/day) used as benchmarks for toxicity threshold (Mitkus 2011, Offit 2003).

In fact, other experiments show that oral dosages of 3.4, 4, 5.6, 6, and 20.2 mg/Kg/day aluminum cause numerous adverse effects in mice or rats and hence the NOAEL for orally ingested Al is currently unknown (Alawdi 2016, Dera 2016, Sethi 2008, Sethi 2009, Bilkei-Gorzo 1993).

The induction of IL-6 may occur because aluminum strongly induces oxidative stress (Exley 2003). Oxidative stress induces IL-6 expression (Viezeleiene 2013).

### **CDC Website Cites Fatally Flawed Study Of Al Adjuvants (Mitkus 2011)**

Dosages of Al adjuvants received by infants increased dramatically as the vaccine schedule was expanded in the 1980s and 1990s. However, as the vaccine schedule expanded, the

increasing dosages of Al adjuvants were not tested for safety. Government agencies (HHS, NIH, CDC, FDA) have not pursued any new experimental work on Al adjuvant toxicity.

As evidence for the safety of Al adjuvants at today's higher dosages, the CDC cites a 2011 FDA study of aluminum exposure from vaccines (Mitkus 2011). The Mitkus study apparently represents the most up-to-date and best evidence for Al adjuvant safety in the present vaccine schedule. Mitkus 2011 is the only scientific evidence cited by the CDC and FDA websites in support of the safety of Al adjuvants.

The Mitkus 2011 study is a theoretical modeling study of Al adjuvant kinetics; it contains no new data concerning Al adjuvant toxicity (from animal models or epidemiology). Mitkus 2011 calculates a body burden of aluminum resulting from the slow dissolution of Al adjuvant particles, and compares the dissolved-aluminum body burden to a "minimal risk level" (MRL). The MRL is derived from a study of ingested Al toxicity in mice (Golub 2001). The Golub 2001 study provides the NOAEL (26 mg/kg/day ingested), which is converted into the MRL for human infants (based on 1mg/kg/day ingested) by using a safety factor of about 30.

The Mitkus study is fatally flawed for these reasons:

#### **(1) Mitkus assumes Al adjuvant particles are harmless**

Mitkus makes an unstated assumption that Al adjuvants have zero toxicity while in particulate form. Mitkus only considers the potential toxicity of aluminum ions (Al<sup>3+</sup>) released by the slowly-dissolving Al adjuvant particles.

Al adjuvants comprise low-solubility and biologically-persistent microscopic particles. The Mitkus analysis assumes that the particles are absolutely nontoxic and perfectly harmless, even when present in the brain and other organs. Mitkus provides no justification for this unstated assumption. Further, the assumption is contradicted by recent findings on Al adjuvant toxicity (Crepeaux 2017) and particulate toxicity generally. Particles can have toxic effects mediated by surface chemistry (e.g. surface charge and surface catalytic activity) and particle shape, among other characteristics of solid particles (Sharifi 2012, Podila 2013).

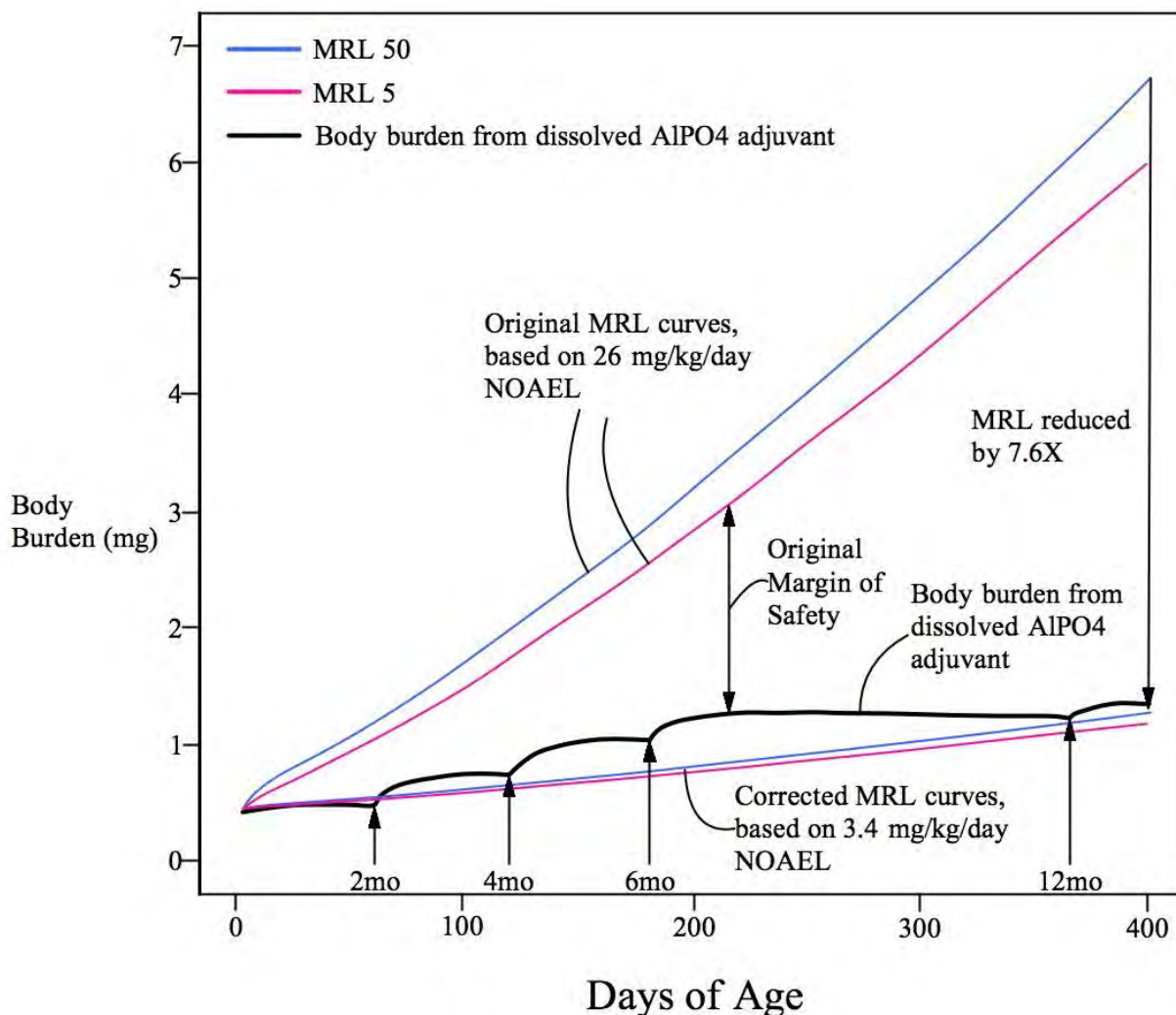
Several studies show injected Al adjuvants cause behavioral abnormalities, abnormal weight gain, learning and memory impairment, motor neuron death/apoptosis, neuromuscular strength deficits, chronic microglial activation/brain inflammation, and large (e.g. 50X) increases in brain and spinal cord aluminum content (Petrik 2007, Shaw 2009, Shaw 2013, Crepeaux 2017). These adverse effects occur at dosages less than or approximately equal to dosages received by infants according to the CDC vaccine schedule.

#### **(2) New research shows ingested Al harmful at dosages lower than 26 mg/Kg/day**

Mitkus assumes that Al adjuvant toxicity is mediated exclusively by solubilized Al (Al<sup>3+</sup> ions) released by the slowly-dissolving Al adjuvant particles. To establish a threshold toxicity level from the solubilized Al, Mitkus relies on a mouse feeding study (Golub 2001) reporting a "no-observed adverse effects level" (NOAEL) oral dosage of 26 mg/Kg/day ingested aluminum. Mitkus used a 30X safety factor for applying this dosage to humans, which is reasonable.

However, other experiments show that much lower oral dosages of 3.4, 4, 5.6, 6, and 20.2 mg/Kg/day aluminum cause adverse effects in mice or rats (Alawdi 2016, Dera 2016, Sethi 2008, Sethi 2009, Bilkei-Gorzo 1993). The adverse effects include chronic brain inflammation, learning and memory impairment, and kidney inflammation. So, the Mitkus analysis is wrong because 26 mg/kg/day is not a NOAEL. The “minimal risk level” (MRL) determined by Mitkus is too high by a factor of at least  $26/3.4 = 7.6$ . Using a corrected NOAEL of 3.4 mg/Kg/day (based on Alawdi 2016) results in vaccine aluminum exposure exceeding the MRL for AlPO<sub>4</sub> adjuvant, and approximately matching the MRL for Al(OH)<sub>3</sub> adjuvant. The new, corrected MRL lines indicate that Al phosphate adjuvant (Fig. 12) and Al hydroxide adjuvant (Fig. 13) from the CDC vaccine schedule may cause toxicity from the solubilized Al per se.

Since 3.4mg/Kg/day is not a NOAEL (adverse effects were observed at this dosage) the true NOAEL is less than 3.4/mg/Kg/day. See Figs. 12-13.



**Fig. 12: Body burden vs. MRL comparison chart for Al phosphate adjuvant (AlPO<sub>4</sub>) corrected in accordance with the new discovery (Alawdi 2016) that ingestion of 3.4 mg/kg/day Al causes adverse effects. The body burden exceeds the corrected MRL curve**

for almost the entire first year of life, indicating toxicity. The toxicity of Al adjuvant particles is a separate, additional issue. MRL 50 and MRL 5 refer to two different infant growth rates. Adapted from Mitkus et al., 2011.

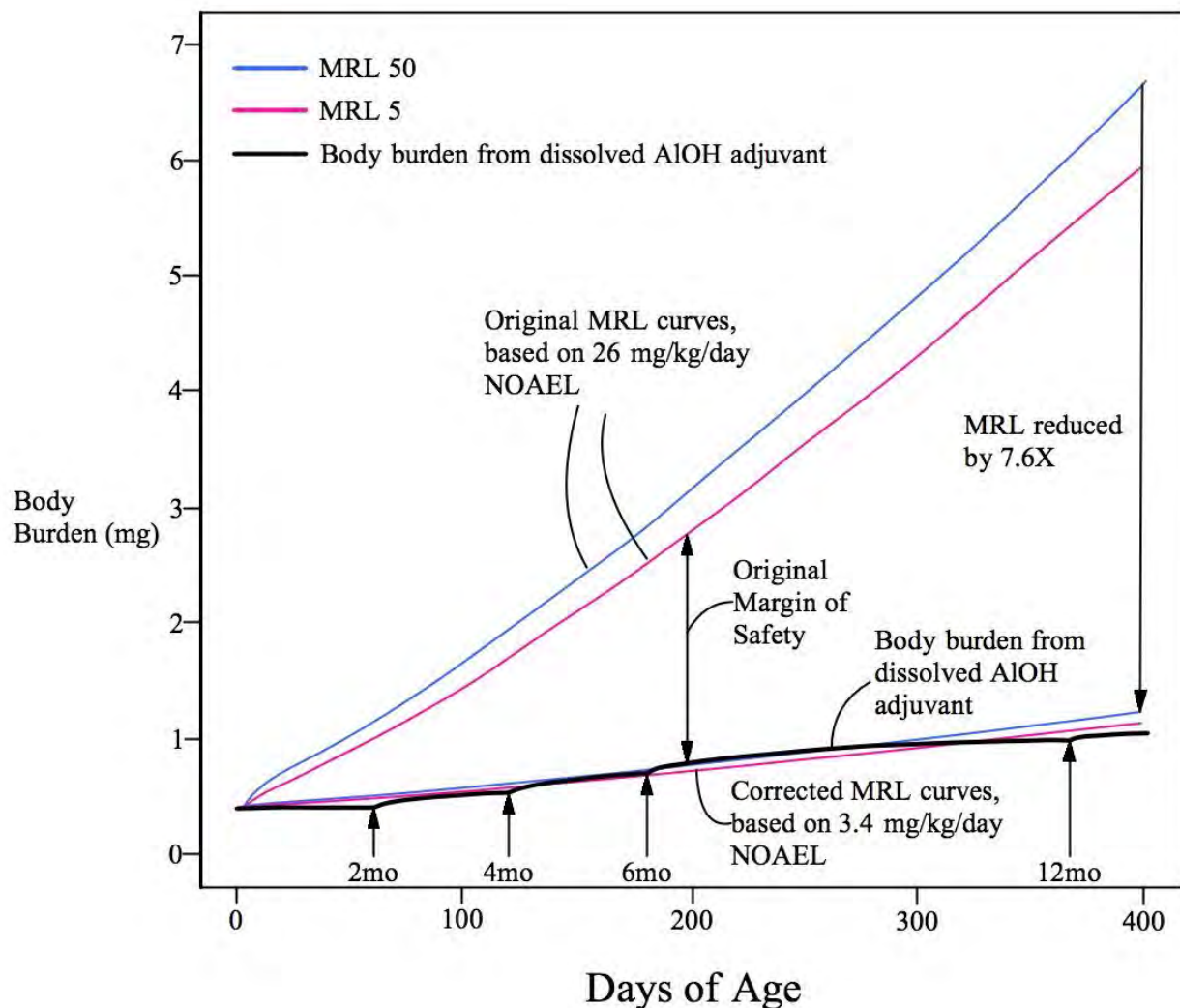


Fig. 13: Body burden vs. MRL comparison chart for Al hydroxide adjuvant (AlOH), corrected in accordance with the new discovery (Alawdi 2016) that ingestion of 3.4 mg/kg/day Al causes adverse effects. The body burden overlaps the new, corrected MRL, indicating borderline toxicity. The margin of safety is gone. MRL 50 and MRL 5 refer to two different infant growth rates. The toxicity of Al adjuvant particles is a separate, additional issue. Adapted from Mitkus et al., 2011.

### **(3) No Al adjuvant toxicity data cited, despite availability**

Mitkus does not cite any toxicity data for injected Al adjuvants. Mitkus instead uses toxicity data for ingested, non-particulate, water-soluble Al (Golub 2001, which used Al lactate) to derive the MRL. This data comes from a single study (Golub 2001).

So, remarkably, Mitkus claims a safe level of injected Al adjuvant exposure, without citing any Al adjuvant toxicity data. The error is unnecessary and neglectful because at least two animal studies of injected Al adjuvant toxicity were available prior to the Mitkus publication in 2011 (Petrik 2007, Shaw 2009). These papers were not cited or mentioned by Mitkus 2011.

Each of these three flaws is fatal for the validity of the Mitkus study in establishing the safety of aluminum adjuvants. Hence, the CDC is completely lacking valid evidence for the safety of Al adjuvants. This is especially true for safety regarding neurological and long-term outcomes, because other available studies of Al adjuvant safety (e.g., Jefferson 2004) do not consider (or are incapable to detecting) these outcomes.

### **CDC Fails To Investigate Toxicity of Al Adjuvants**

The CDC has conducted no epidemiological studies on long term safety (e.g. considering neurological outcomes) of Al adjuvants. There is one ecological study of country-level data, which reported an association between Al adjuvant exposure and autism (Tomljenovic 2011). However, being an ecological study, it is highly susceptible to confounding and biases.

Dr Frank DeStefano of the CDC's Immunization Safety Office is co-author of a feasibility study (Glanz 2015) on using the Vaccine Safety Datalink (VSD) to investigate the safety of individual vaccine ingredients. The paper focuses on Al adjuvants. It acknowledges that thimerosal is the only vaccine ingredient studied for autism or neurological safety, and that a possible association between Al adjuvants and autism has not been explored in epidemiological studies. Glanz 2015 states:

*“To date, there have been no population-based studies specifically designed to evaluate associations between clinically meaningful outcomes and non-antigen ingredients, other than thimerosal.”*

The CDC has not investigated Al adjuvant safety concerns, despite the accumulating scientific evidence of harm and evidence linking Al adjuvants to immune activation mechanisms of brain injury.<sup>1</sup>

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<sup>1</sup> However, the Glanz paper notes that studies of aluminum adjuvants are problematic because of expected small differences in exposures in the low and high exposure groups. Glanz 2015 concludes: “...children below the 10th percentile would be exposed to between 0 mg and 3.1mg, while children above the 90th percentile would be exposed to between 4.8 mg and 5.3 mg of aluminum from vaccines. It is unclear if such differences in aluminum exposure would be biologically meaningful.” (Glanz 2015). So, epidemiological studies may not provide reliable evidence for safety or harm. Controlled, prospective human trials of aluminum adjuvant exposure from vaccines will likely be prohibited for ethical reasons. Also, Al adjuvants are essential ingredients for Al adjuvanted vaccines. Consequently, it will be challenging to design studies of long term adverse effects of Al adjuvants in humans. Experiments in animal models can provide valuable information. Al adjuvants should be tested for effects on: 1) excitatory/inhibitory imbalance; 2) core symptoms of autism (social, communicative and repetitive/stereotyped behaviors); 3) IL-6, IL-17, and other cytokine levels in the brain; 4) other physiological abnormalities associated

## **Conclusion**

The science reviewed here tells a consistent and compelling story: that vaccines may cause autism by stimulating immune activation and elevated cytokines in the brain. Al adjuvants are implicated as a cause of autism because they can be transported into the brain, because they causes microglial activation at vaccine-relevant dosages, and because aluminum induces IL-6 in the brain.

In statements asserting no vaccine-autism link, the CDC cites scientific evidence that is not relevant to Al adjuvant safety or is incapable of disproving an Al adjuvant-autism link (Taylor 2014, DeStefano 2013, Mitkus 2011). In support of claims for Al adjuvant safety, the CDC relies on a profoundly flawed theoretical modelling study (Mitkus 2011). There is little scientific evidence supporting the safety of Al adjuvants, especially in relation to autism and other long term neurological outcomes.

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with autism (e.g. mitochondrial dysfunction, microbiome dysbiosis, Purkinje cell loss, cerebellum abnormalities etc); and 5) microglial activation and immune activity in the brain. Investigating these outcomes can provide valuable information concerning the safety of Al adjuvants.

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June 24, 2017

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Re: *Aluminum Adjuvants*

Dear Directors:

I am writing to you in regard to aluminum adjuvants in vaccines. This subject is one my laboratory works on intensively and therefore one where I feel that I have some expertise. In particular, we have studied the impact of aluminum adjuvants in animal models of neurological disease, including autism spectrum disorder (ASD). Our relevant studies on the general topic of aluminum neurotoxicity in general and specifically in regard to adjuvants are cited below.

These studies and the broader existing literature regarding aluminum toxicity, lead almost invariably to the conclusion that aluminum in any chemical form is always neurotoxic when administered to humans. Further, I am convinced that aluminum adjuvants in vaccines may contribute to neurological disorders across the lifespan. In adults, such adjuvant may induce macrophagic myofasciitis, a disease with neuropathological aspects. In children, there is growing evidence that aluminum adjuvants may disrupt developmental processes in the central nervous system and therefore contribute to ASD in susceptible children.

Despite the foregoing, the safety of aluminum adjuvants in vaccines has not been properly studied in humans even though, pursuant to the recommended vaccine schedule published by the Centers for Disease Control (CDC), a baby may be injected with up to 3,675 micrograms of aluminum adjuvant by six months of age.

In regard to the above, it is my belief that the CDC's claim on its website that "Vaccines Do Not Cause Autism" is wholly unsupported. Given this, I remain convinced that much more research on the role of aluminum adjuvant in vaccines and neurological disorders, including ASD, is warranted and should be a research priority for the NIH and other funding bodies.

Yours sincerely,

Christopher A. Shaw, Ph.D  
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#### Relevant Publications (Shaw Laboratory)

1. Crepeaux G, Eidi H, David MO, Baba-Amer Y, Tzavara E, giros B, authier FJ, Exley C, Shaw CA, Cadusseau J, Gherardi RK. Non-linear dose-response of aluminium hydroxide adjuvant particles: Selective dose neurotoxicity. *Toxicology*. 375:48-57. (2016).
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**THE UNIVERSITY OF BRITISH COLUMBIA**  
**Curriculum Vitae for Faculty Members**

**Date:** December 6, 2016

1. **SURNAME:** SHAW  
**SIGNATURE** \_\_\_\_\_
2. **DEPARTMENT/SCHOOL:** Ophthalmology and Visual Sciences/Faculty of Medicine
3. **FACULTY:** Medicine
4. **PRESENT RANK:** Professor **SINCE:** Dec. 2004
5. **POST-SECONDARY EDUCATION**

University or Institution	Degree	Subject Area	Dates
University of California Irvine	B.Sc.	Biology	1971
Hebrew University of Jerusalem	M.Sc.	Physiology	1974
Hebrew University of Jerusalem	Ph.D	Neurobiology	1979

**Title of Dissertation and Name of Supervisor**

Electrophysiological Studies of After potentials in Invertebrate Photoreceptors  
Supervisor: Prof. Peter Hillman

**Special Professional Qualifications**

Postdoctoral Fellow: 1979-1985  
Research Associate 1986-1988

6. **EMPLOYMENT RECORD**

(a) *Prior to coming to UBC*

University, Company or Organization	Rank or Title	Dates
Dalhousie University	PDF	1979-1985
Dalhousie University	Research Associate	1986-1988

(b) *At UBC*

Rank or Title	Dates
Assistant Professor	1988
Associate Professor	1993
Professor	2004

(c) *Date of granting of tenure at U.B.C.:*  
June/July, 1993

7. **LEAVES OF ABSENCE**

University, Company or Organization at which Leave was taken	Type of Leave	Dates
University of British Columbia	Sabbatical	Jan 1, 2005- 31 Dec, 2005
University of British Columbia	Sabbatical	September 1, 2013- August 31, 2014

8. **TEACHING (see attached teaching dossier)**

(a) *Areas of special interest and accomplishments*

Special interests in teaching include:

-Founder, 'Creativity' Study Group, Green College, UBC, 1994.

-Co-PI (with A. Kindler) on application to the Peter Wall Institute of Advanced Studies to organize a workshop on the subject of the neurobiology of creativity, 2000.

-Co-PI (with S. Goble and A. Kindler) for Thematic Lecture Series, "The Nature of Creativity: History, Biology, and Socio-Cultural Dimensions", Green College, 2001/02.

-Thematic Lecture Series: "The Olympic Games in Myth and Reality", Green College, Fall 2009.

(b) *Courses Taught at UBC*

Session	Course Number	Scheduled Hours	Class Size	Hours Taught			
				Lectures	Tutorials	Labs	Other
1990	Phys 510	30	30	30			
1991	Phys 510	30	30	30			
1991	Gross Anatomy	220	100			220	
1992	Phys 510	30	30	30			
1998	PBL	30	8		30		10
1999	PBL	30	8		30		10
2000	PBL	30	9		30		10
2001	PBL	30	8		30		10
2002	PBL	30	9		30		
2003	PBL	30	8		30		
2004	PBL	30	8		30		
2005	PBL	60	8		60		20
2006	PBL	60	8		60		20
2007	PBL	30	8		30		10
2008	PBL	30	8		30		10
2009	PBL	30	8		30		10
2010	PBL	30	8		30		10
2011	PBL	30	8		30		10
2012	PBL	30	8		30		
2013	PBL	30	8		30		



2014	PBL	20	8		20		
2015	Blood and Lymphatics; Endocrine	2 weeks; 4 weeks	8 students each				

(c) *Graduate Students Supervised and/or Co-Supervised*  
(PDFs also listed)

Student Name	Program Type	Year		Principal Supervisor	Co-Supervisor(s)
		Start	Finish		
Ningning Guo	M.Sc.	1990	1992	CA Shaw	
Steven Bowlsby	M.Sc.	1990	1992	CA Shaw	
Ruth Lanius	Ph.D	1992	1996	CA Shaw	
Allen J. Billy	PDF	1990	1992	CA Shaw	
Jaswinder S. Bains	PDF	1996	1999	CA Shaw	
Margaret Wong	M. Sc.	2001	2005	CA Shaw	
Jason Wilson	M.Sc, Ph.D	2001	2007	CA Shaw	
Jeff Schulz	M. Sc.	2002	2005	CA Shaw	
Erin Hawkes	M. Sc.	2003	2005	CA Shaw	
Swaraj Singh	M.Sc.	2003	2005	CA Shaw	
Reyniel Cruz-Aguado	PDF	2003	TBD	CA Shaw	
Michael Petrik	M.Sc.	2004	2006	CA Shaw	
Philip Ly	M.Sc.	2005	2007	CA Shaw	
Rena Tabata	M.Sc.	2006	2008	CA Shaw	
Grace Lee	Ph.D.	2005	2012	CA Shaw	
Yemi Banjo	M.Sc.	2007	2009	CA Shaw	
Trisha Kostaskey	M. Sc	2009	2011	CA Shaw	
Darryl Bannon	M.Sc.	2009	2015	CA Shaw	
Lucija Tomljenovic	PDF	2010		CAShaw	
Pierre Zwieggers	M. Sc.	2012	2015	CAShaw	
Sneha Sheth	M. Sc. Ph.D.	2012 2014		CAShaw CAShaw	Dr. Todd Woodward
Hongwu Liang	Visiting Scholar	2012	2013	CAShaw	
Alice Li	PDF	2013	2015	CAShaw	
Jess Morrice	M.Sc. Ph.D.	2014 2015		CAShaw	Dr Cheryl Gregory-Evans
Guillemette Crepeaux	PDF (in Universite Paris-Est)	2013-2014	2014	CAShaw	Dr Romain Gherardi, Universite Paris-Est)

Awards held by students and PDFs:

Ruth Lanius: MRC Studentship

Bryce Pasqualotto: McLeod Studentship (Alberta Heritage Foundation)

Jill McEachern: Epilepsy Canada Studentship

Pharmaceutical Manufacturers Council of Canada Studentship

MITACS Centre of Excellence Studentship

Erin Hawkes: NSERC Studentship

(also awarded Parkinson's Disease Foundation Scholarship, but unable to use it at specified time)

Jeff Schulz: Parkinson's Disease Foundation Scholarship

Jason Wilson: 1<sup>st</sup> prize, VGH Research Awareness week poster competition: graduate student division; Travel award to CIHR national poster competition, 2003.

Award of Excellence, Gold category, CIHR national poster competition, 2003.

Jason Wilson & Margaret Wong: Best Science Presentation at the 18<sup>th</sup> annual UBC Ophthalmology Research and Alumni Day May 2003

Jeff Schulz: Second Place Science Presentation at the 19<sup>th</sup> annual UBC Ophthalmology Research and Alumni Day, May 2003

Michael Petrik: Best Presentation at the 4<sup>th</sup> annual VGH Summer Student Symposium, 2003; Honor Mention in the 2005 CIHR National Student Research Poster Competition

Reyniel Cruz-Aguado: Best oral presentation, travel award, International Society for Neurochemistry, 2004

Daniella Winkler: NSERC Undergraduate Summer Research Award, 2005

Jason Wilson: Gold Prize in the 2005 CIHR National Student Research Poster Competition.

Philip Ly: Parkinson's Disease Foundation Summer Studentship, 2005

Benedict Wong: Parkinson's Disease Foundation Summer Studentship, 2006

Grace Lee: Student Award, Scottish Rite Charitable Foundation, 2007-2010.

Lucija Tomljenovic: Phenomenal presentation at the International Conference and Exhibition on Pharmavigilance & Clinical Trials. Chicago-North Shore, USA. October 1-3, 2012

Curtis May, FOM Summer Student Research Program Award, 2013

Sneha Sheth: Faculty of Medicine Graduate Award #6442, 2014

Sneha Sheth, 2014 Keele 11 Meeting Travel Bursary Award, 850 euros.

Additional graduate students supervised, but not graduated (see note below):

Marianne McCashin, 1988-1990 (Graduate Programme in Neuroscience).

Derrick March, 1990-1993 (Physiology).

Bryce Pasqualotto, 1993-1998 (Physiology)

Jill McEachern, 1993-2002 (Physiology)

Concerning the above students who did not graduate, please note the following: With the exception of M. McCashin who changed to another laboratory before withdrawing from the program, the other three students produced numerous papers between them. D. March was an author on 2 peer-reviewed publications and on a number of abstracts; B. Pasqualotto wrote and co-wrote a number of papers (13 full length) and additional abstracts, even sharing with me an invited editorship in a special issue of *Cellular and Molecular Life Science*; J. McEachern was a co-author on 6 major reviews, a number of abstracts, and co-edited *Toward a Theory of Neuroplasticity* (2000). She had additional publications during her tenure in the laboratory with various UBC collaborators. While it is regrettable that these students did not complete their degrees due to outside issues, it is nevertheless clear that each of these students was highly productive while in my laboratory, and at least 2 of these students (Pasqualotto and McEachern) achieved international recognition for their work.

Medical resident co-supervised:

Dr. Michele Mezei, 1994-1995 (C. Krieger, Principal supervisor).

The current status of the listed students/PDFs who have moved on is as follows:

Dr. Ningning Guo: Research Associate, Cornell University School of Medicine, NY.

Mr. Steven Bowsby: science writer

Dr. Ruth Lanius: Assoc. Professor, Psychiatry, Western Univ., London, ON.

Dr. Allen Billy: lecturer, Langara Community College.

Dr. Jaswinder Bains: practicing optometry, Vancouver.

Ms. Margaret Wong: Medical School, UBC

Mr. Jeff Schulz: Medical student

Dr. Swaraj Singh: Resident in Neurology, University of Arkansas

Ms. Erin Hawkes: Doctoral student

Dr. Jason Wilson: Resident in radiology

Dr. Reyniel Cruz-Aguado, lecturer, Douglas College

Michael Petrik, Optometry

The following undergraduate students have gone on to achieve professional degrees:

Dr. Brian Scarth: Psychiatrist, West Vancouver.

Dr. Lynn Huff: general practice, Vancouver.

Dr. John Bining, general practice, Vancouver.

Dr. Twyla Bergman, graduated UBC Medical School.

Mr. Michael Tjandrawijaja: Graduated, Calgary Medical School

Mr. Joseph Cheung: Genomics, University of Toronto, Ph.D.

Ms. Mandeep Mahay: graduated UBC Pharmacy.

Arash Seyedalikhani, graduate Univ. Calgary nursing school, Currently: RCMP officer

(d) *Continuing Education Activities*

(i) Green College, UBC: Associate Member, 1994-1996, 2000-present

(ii) Tutor observer, PBL program, 1998-present

(iii) UBC Faculty of Medicine Academic Advisor ('Mentor'), Class of 2004

(iv) Supervisor for students in the following categories:

1. Undergraduate honours thesis projects, 1988-present
2. Workstudy projects, 1988-present
3. 1<sup>st</sup> Job in Science and Technology (provincial program), 2000
4. Student Summer Works (provincial program), 1997-2000
5. Summer Career Placement Program, (Human Resources Canada), 2000

(v) Grand rounds, Dept. of Ophthalmology, 1989, 1994, 2009

(vi) Participant, Ophthalmology Research Day, 1988-present (either I and/or members of my laboratory have presented at each Ophthalmology Research Day since 1988).

(e) *Visiting Lecturer (indicate university/organization and dates) See attached*

(f) *Other*

**9. SCHOLARLY AND PROFESSIONAL ACTIVITIES**

(a) *Areas of special interest and accomplishments*

Edited/co-edited 3 books on topics in the neurosciences and related topics:

1. *Receptor Dynamics in Neural Development*, CRC Press, 1996. This book deals with the mechanisms and roles of receptor regulation in neural development and plasticity.
2. *Glutathione in the Nervous System*, Taylor and Francis, 1998. This book summarizes the various roles of GSH in the CNS and provides evidence supporting a major role of glutathione in various aspects of normal and abnormal synaptic activity.
3. *Toward a Theory of Neuroplasticity* (with J.C. McEachern), Taylor and Francis, 2000. This book represents the first synthesis of the vast and diverse realm of neuroplasticity studies and attempts to create a unified theory of the phenomenon.

(b) *Research or equivalent grants (indicate under COMP whether grants were obtained competitively (C) or non-competitively (NC))*

<b>Granting Agency</b>	<b>Subject (Title)</b>	<b>COM P</b>	<b>\$ Per Year</b>	<b>Year (fiscal)</b>	<b>Principal Investigator</b>	<b>Co-Investigator(s)</b>
BCHRF	Scholarship	C	37,000	1988-1992	Christopher Shaw	
BCHRF	Scholarship	C	10,000	1988-1989	Christopher Shaw	
MRC	Program Grant	C	112,500 total	1988-1990	Max Cynader	Researcher: Christopher Shaw
BCHRF	The Role of Steroids on Receptor Regulation	C	40,000 total	1988-1990	Christopher Shaw	
BCHRF	Equipment Grant	C	210,000 total	1989-1992	Christopher Shaw	
BCMSF	Molecular Basis of Synaptic Alterations in Amblyopia	C	10,250	1990-1992	Christopher Shaw	
NSERC	Receptors, Second Messengers and Ion Channels in Neuroglia of the Mammalian Neocortex	C	24,500	1990-1993	Christopher Shaw	
MRC	Role of Neurotransmitter Receptor Distribution and Function	C	10,875	1991-1992	Christopher Shaw	
MRC	Studentship	C	400	1991-1996	Christopher Shaw	Student: Ruth Lanus
BCHRF	Involvement of Protein Kinase C in ALS	C	83,554 total	1992-1994	Charles Krieger	Researcher: Christopher Shaw
BCHRF	Glutathione: The Geniculostriate Neurotransmitter?	C	15,000	1992-1993	Christopher Shaw	
Janssen-Ortho Inc.	Excitatory amino acid receptors in ALS	C	25,000 total	1993-1995	Christopher Shaw	
ALS (Canada)	Studies of Pathogenesis of ALS	C	46,250	1994-1996	Charles Krieger	Researcher: Christopher Shaw
NIH	GSH Receptor probes to study CNS development/dysfunction	C	31,770 total	1994-1998	Christopher Shaw	
AHFMR	Studentship	C	13,200	1995-1996	Christopher Shaw	
Calgary Foundation	Dynamic responses of cortical AMPA receptors to excitatory stimuli	C	3,300	1995-1996	Christopher Shaw	
ALSA (US)	Changes in the Glutathione Status of Cells in ALS	C	155,374 total	1995-1999	Christopher Shaw	
NSERC	Glutathione as an excitatory neurotransmitter in the CNS	C	94,600 total	1997-2001	Christopher Shaw	
Center for Neurologic Study	Stipend to supplement work on ALS grant	C	2,082	1997-1998	Christopher Shaw	
Scottish Rite Charitable Foundation	Examining the role of Novel Oxidative-/Excito-Toxins in the development of symptoms of neurological disease	C	35,000	1999-2002	Christopher Shaw	
MITACS	Studentship	C	17,500	1999-2002	Christopher Shaw	Student: Jill McEachern
ALSA (USA)	Mechanisms of action of a novel neurotoxin isolated from the seed of the cycad: Implications for ALS-PDC	C	72,109	2000-2001	Christopher Shaw	

ALSA (USA)	Cycad sterol glucoside toxicity and ALS-PDC: Implications for the etiology of ALS	C	135,875 total	2001-2003	Christopher Shaw	
NSERC	Genetic propensity for cycad neurotoxicity in a murine model of neurological disease	C	23,500	2001-2006	Christopher Shaw	
Green College	The nature of creativity: history, biology and socio-cultural dimensions		10,000	2001-2002	Christopher Shaw	With A. Kindler & S. Goble
Scottish Rite Charitable Foundation	Estrogen and apolipoprotein E as therapies for neurodegenerative disease	C	35,000	2002-2005	Christopher Shaw	
U.S. Department of Defense (Army Medical Research Acquisition Activity)	Implications of Cycad Neurotoxicity for ALS-PDC	C	1,128,253 total	2002-2007	Christopher Shaw	
Parkinson's Disease Foundation	Cycad and sterol glucoside neurotoxicity in vivo and in vitro models	C	\$3,950 total	2005-2007	Christopher Shaw	
ALSA (USA)	Roles of sterol glucoside neurotoxicity in ALS-PDC	C	286,014 total	2006-2009	Christopher Shaw	
NINDS (USA)	Time-lines of neural degeneration in ALS-PDC mouse model	C	1,352,555 total	2006-2011	Christopher Shaw	
Scottish Rite Charitable Foundation	Neurobiological basis of cycad toxicity in a mouse model of ALS-PDC	C	\$20,000 total	2007-2008	Christopher Shaw	
Scottish Rite Charitable Foundation	Neurobiological basis of cycad toxicity in a mouse model of ALS-PDC	C	\$20,000 total	2009-2010	Christopher Shaw	
NIH (USA) R03	Neurotoxicity of sterol glucosides: role in ALS-PDC	C	66,466 total	2007-2008	Christopher Shaw	
Pacific Alzheimer's Research Foundation	Role of progranulin in brain function and neuroprotection	C	344,846 total	2007-2009	Christopher Shaw	
ALS SC Bernice Ramsay Discovery Grant	Progranulin as a novel therapeutic for motor neuron rescue in an animal model of ALS	C	100,000 total	2010-2011	Christopher Shaw	
Lotus Foundation through the American foundation for UBC	The toxicity of polysorbate 80, sodium borate, and aluminum at clinically relevant concentrations in an in vivo animal model	NC	150,000	2011-2012	Christopher Shaw	
Dwoskin Foundation through the American Foundation for UBC	Aluminum vaccine adjuvants and neurodevelopment outcomes in an animal model of autism spectrum disorder	NC	250,000	2011	Christopher Shaw	
Katlyn Fox Foundation	Neurotoxic impacts of aluminum in CNS	NC	17,000	2010-2011	Christopher Shaw	

Katlyn Fox Foundation	Neurotoxic impacts of aluminum in CNS	NC	6,000	2012	Christopher Shaw	
Dwoskin Foundation through the American Foundation for UBC	Aluminum vaccine adjuvants and neurodevelopment outcomes in an animal model of autism spectrum disorder	NC	244,919	2012	Christopher Shaw	
Dwoskin Foundation through the American Foundation for UBC	Aluminum vaccine adjuvants and neurodevelopment outcomes in an animal model of autism spectrum disorder	NC	364,239	2013	Christopher Shaw	
Estate Grant (Luther Allyn Shourds Dean Bequest)	Impacts of environmental toxicity on children and across the lifespan	NC	862,280.14	2013 -	Christopher Shaw	
Katlyn Fox Foundation	Neurotoxic impacts of aluminum in CNS	NC	8,000	2014	Christopher Shaw	
Katlyn Fox Foundation	Neurotoxic impacts of aluminum in CNS	NC	5,000	2015	Christopher Shaw	

Granting Agency	Subject	COMP	\$ Per Year	Year	Principal Investigator	Co-Investigator(s)
Estate Grant (Luther Allyn Shourds Dean Bequest)	Impacts of environmental toxicity on children and across the lifespan	NC	109,600	2015	Christopher Shaw	
Katlyn Fox Foundation	Neurotoxic impacts of aluminum in CNS	NC	14,000	2016	Christopher Shaw	
CMSRI (was Dwoskin Foundation)	Impact of neonatal vaccination on neurodevelopment and immune –inflammatory responses in wild type mice	NC	260,251.78	2016-2017	Christopher Shaw	

**Total funds brought to UBC to date: \$6,731,424.80**

Please note that I am a former member of the BC 'node' of the national Mathematics Centre of Excellence (MITACS), which provided a student scholarship to one of my graduate students (Jill McEachern).

(c) *Invited Presentations (reverse chronological order) (note some of these are repeated in the next section)*

International (35):

Aluminium in the nervous system: A contributor to neurological diseases across the lifespan. *AutismOne*. Chicago, IL, USA, May 18-24, 2015.

CMSRI HPV Vaccine Safety. *AutismOne*. Chicago, IL, USA, May 18-24, 2015.

Toxicity of aluminum adjuvants in humans and animal models. In 3<sup>rd</sup> International Symposium on Vaccines (March 26, 2014), 9<sup>th</sup> *International Congress on Autoimmunity*. Nice, France. March 25-30, 2014.

Administration of aluminium in vaccine-related exposures in neonatal mice is associated with long term adverse neurological outcomes, Platform 19. *10<sup>th</sup> Keele Meeting*, Winchester, U.K., February 22-28, 2013.

The neurotoxicity of aluminum: implications for aluminum adjuvanted vaccines, *8<sup>th</sup> International Congress for Autoimmunity*. Granada, Spain, May 9-13, 2012.

Toxicity of aluminum in vitro and in vivo: relation to aluminum concentrations in humans, *9<sup>th</sup> Keele Meeting: "Aluminium and Life: Living in the Aluminium Age"*, Niagara-by-the-Lake, Hamilton, Ontario Canada, February 19-23, 2011.

Aluminum as a neurotoxin: the evidence from cell culture, in vivo, and human studies, *Vaccine Safety Conference*, Montego Bay, Jamaica, West Indies, January 3-8, 2011.

Neuropathology and neuroprotection of sterol glucosides: insights from ALS-PDC. *1<sup>st</sup> Neurodyn Corp. meeting on neuronal degeneration*, July 2010.

Aluminium hydroxide and Gulf War ALS: An *in vivo* model of motor neuron death. In Session 6. Animal Models of Aluminium Toxicity. *Eighth Keele Meeting on Aluminium*. Trest, Czech Republic. 21-25 February, 2009.

Sterols and sterol glucosides as causal factors in ALS and the interaction with genetic susceptibility. *4<sup>e</sup> Symposium sur la SLA de la Fondation André-Delambre*, Montreal, 25 September 2008.

Timelines of behavioural, anatomical, and biochemical changes in the CNS of an animal model of ALS-PDC of Guam. *18<sup>th</sup> International Symposium on ALS/MND*. Toronto, Canada, 2007.

An environmental model of neurological disease based on ALS-PDC of Guam, Department of Agricultural Sciences, Oregon State University, Oregon, USA, 2007.

ALS-PDC of the Western Pacific: A novel, predictive animal model, Department of Pathology, University of Washington, Seattle, Washington, USA, 2006.

Inflammation and neuronal cell death in an animal model of ALS-PDC of the Western Pacific, Quebec City, September 2006

Cycad toxicity studies. International Workshop on ALS-PDC, Guam, December 2005.

ALS-PDC: New insights from an old mystery. University of Maryland, Maryland, April 2005.

Cycad-induced neurodegeneration in a mouse model of ALS-PDC: Is the culprit really BMAA or is a novel toxin to blame? Xalapa, Veracruz, Mexico, Jan 2005.

Susceptibility and environmental factors in ALS, NIEHS Brainstorming Session, Research Triangle Park, Durham, North Carolina, May 2003.

Gene-environment interactions in neurological disease: prospects for prevention and early treatment, ALS Clinical Conference, ALSA regional meeting, Washington, DC, May 2003

Susceptibility and environmental factors in ALS, NIEHS Brainstorming Session II, Research Triangle Park, Durham, North Carolina, November 2002.

Reverse engineering neurological diseases, International Conference on Complex Systems, Nashua, NH, June 2002.

Excitotoxins/ALS-PDC, ALSA regional meeting on environmental factors and susceptibility genes in ALS, Keystone, Colorado, May 2002.

Glutathione and signal transduction in CNS, ISN conference, Buenos Aires, July, 2001

Mechanisms of cycad neurotoxicity: relation to ALS-PDC, Bodig-Lytico Research Group, University of Guam, 10<sup>th</sup> Pacific Science Inter-congress, Guam, June, 2001

Excitotoxicity and neurological disease, Department of Defense conference on Parkinson's disease, Bethesda, MD, 2001.

A murine model of ALS-PDC, University of San Diego, San Diego, March 2001

Combination of excitotoxicity and oxidative stress in the pathogenesis of neurological disease, Center for Neurologic Study, San Diego, 1998.

Glutathione in neurological disease: a new model, Center for Neurologic Study, San Diego, 1996

Receptor binding: theory and methods (2 lectures), Trinity College, Hartford, CN, 1990

Receptor regulation in cat visual cortex: development and plasticity, Neuroscience Group, University of Pittsburgh, Pittsburgh, 1989.

Receptor regulation in cat visual cortex: development and plasticity, Neuroscience Group, McGill University, Montreal, 1989.

Receptor regulation and cortical plasticity, Dept. of Psychobiology, University of California at Irvine, 1989

Mechanisms of acetylcholine receptor regulation in cortex, NATO conference on 'Receptors', Santorini, Greece, 1988.

Alterations in receptor distribution and characteristics in postnatal development, Dept. of Ophthalmology, University of Washington, Seattle, 1988.

Postnatal development of receptors in cat visual cortex: implications for neuroplasticity and the critical period, IBRO symposium, Budapest, 1987.

Mechanisms of receptor regulation, Neurobiology Program, Northeastern Ohio Universities College of Medicine, Ohio, 1987.

Receptor modifications during postnatal development of cat visual cortex, Neurobiology Program, Northeastern Ohio Universities College of Medicine, Ohio, 1986

#### Local (8):

Does a forgotten disease on Guam hold the key to understanding all neurodegenerative disorders? Green college Principal's Series January – April 2009, Thinking at the Edge of Reason: Interdisciplinarity in Action. Green College, University of British Columbia, BC, Canada. 10 February 2009.

Can your food give you Alzheimer's disease? Green College, University of British Columbia, Vancouver, Nov. 2001.

Behavioral assessment of toxicity of a novel neurotoxin, Dept. of Psychology, University of British Columbia, Vancouver, Oct. 2000.



ALS-PDC models, MITACS seminar, Dept. of Mathematics, University of British Columbia, Vancouver, September, 2000.

Cycad toxicity and ALS-PDC, Graduate Programme in Neuroscience discussion group, University of British Columbia, Vancouver, 2000.

Glutathione in the CNS, Dept. of Psychology, University of British Columbia, Vancouver, 1997.

Long-term potentiation: a new view (with J.C. McEachern), Dept. Psychology, University of British Columbia, Vancouver, 1997.

Alterations in receptor properties in postnatal development, Dept. Pharmacology, University of British Columbia, Vancouver, 1994.

(d) *Other Presentations*

**See list of abstracts for poster and slide presentations.**

Also, please note that I (or members of my laboratory) have presented research talks at every 'Ophthalmology In-House Research Day' from 1988-2010.

(e) *Other*

N/A

(f) *Conference Participation (Organizer, Keynote Speaker, etc.)*

Chair, Special Satellite Session: 3rd International Symposium on Vaccines and Autoimmunity, Ninth International Autoimmunity Congress. Nice, France. March 25-30, 2014.

Chair, Special Satellite Session: 2<sup>nd</sup> International Symposium on Vaccines and Autoimmunity, Eighth International Autoimmunity Congress. Granada, Spain. 2012. May 7-13, 2012.

Speaker, Toxicity of aluminum in vitro and in vivo: relation to aluminum concentrations in humans, 9<sup>th</sup> Keele Meeting: "Aluminium and Life: Living in the Aluminium Age", Niagara-by-the-Lake, Hamilton, Ontario Canada, February 19-23, 2011.

Organizer and Speaker, Aluminum as a neurotoxin: the evidence from cell culture, in vivo, and human studies, Vaccine Safety Conference. Montego Bay, Jamaica, West Indies. January 3-8, 2011.

Speaker, Understanding the Rules of ALS, 3<sup>rd</sup> Annual ALS BC dinner, speaker, November 2004.

ALS Society of Canada Research Forum, invited discussant, ALS Society of Canada, October 2004.

Biomarkers in Multiple Sclerosis, invited discussant, NINDS conference, Washington DC, April 2004.

Invited discussion panelist, Susceptibility and Environmental Factors in ALS, NIEHS Brainstorming Session 1, Durham NC, Nov, 2002.

Invited discussion panelist, Susceptibility and Environmental Factors in ALS, NIEHS Brainstorming Session 2, Durham NC, May 2003.

Keynote speaker, Glutathione and signal transduction in CNS, ISN meeting, Buenos Aires, 2001.

Keynote speaker, Mechanisms of cycad neurotoxicity: relation to ALS-PD. 10<sup>th</sup> Pacific Science Inter-Congress, Guam, 2001.

Organizer, Workshop on Neurobiology of Creativity in Art and Science, Peter Wall Institute of Advanced Studies, awarded 2002.

Co-Organizer, Green College Thematic Lecture series, The Nature of Creativity: History, Biology, and Socio-Cultural Dimensions, 2001/2002.

Keynote speaker, The effects of early diet on synaptic function and behaviour: pitfalls and potentials, Dobbing Conference, Phoenix, 1997.

Keynote speaker, Trinity College, Hartford, 1990.

Keynote speaker, IBRO, Budapest, 1987.

## 10. **SERVICE TO THE UNIVERSITY**

### (a) *Memberships on committees, including offices held and dates*

Recognized contribution to Experimental Medicine Program as supervisor to students (S Sheth and P Zwieggers), 2015 March 17

Appointed Associate Member, Department of Pathology and Laboratory Medicine, Term of appointment: July 1, 2014 – June 30, 2018. Letter of Appointment from Dr Gavin C.E. Stuart, Dean, UBC FOM, Jan 05, 2015.

Recognized contribution to Experimental Medicine Program as supervisor to students (D Bannon, S Sheth, P Zwieggers) as well as Committee Examiner for comprehensive exam (DBannon), 2014

Judge, UBC Medicine Undergraduate Research Forum, 2012

Tutor training for faculty development for the undergraduate Medical/Dental Curriculum, 2001-2002, & 2002-2003, 2010-11, 2011-12 school years

*Ad hoc* committee on Gender Equality

Chair, Thesis defense committees (listed below)

University Examiner, UBC

Admissions Committee, Graduate Programme in Neurosciences, 1990-1993

Judging panel committee, Ophthalmology Research Day 2002

### (b) *Other service, including dates*

Contributor to the core component of Faculty Development for the Undergraduate Medical/Dental curriculum by performing PBL tutorial observation, Fall Session 2012-13

Adjudicator, 2013 Killam Scholarship Award, Killam Program, UBC.

Adjudicator, 2012 Killam Graduate Teaching Assistants Awards, Faculty of Medicine, Dean's Office, Research

Contributor to the core component of Faculty Development for the Undergraduate Medical/Dental curriculum by performing PBL tutorial observation, Winter Session 2008-09, 2010-11

Participant, Workshop: Developing a Population-based Research Study of Neurological Conditions in Canada. Hosted by the Public Health Agency of Canada, Canadian Institutes of Health Research, Health Canada, and Neurological Health Charities Canada. Toronto. 2008 to present. Current Chair of the Risk Factors subcommittee and member of NHCC scientific advisory board.

Participant, Internal Review Process for the CIHR New Investigator Award Competition, conducted by the Office of the Vice president of Research and the Health Research Resource Office (HeRRO), September 2008

Member, Green College, 1994-1996, 2000-present

Tutor Observer, Faculty of Medicine PBL Program, 1998-present

Academic Advisor, Faculty of Medicine, Class of 2004, 2005

Promotion and tenure committees, Dept. of Ophthalmology, as required, 1988-present

Member of Judging panel, Ophthalmology Research and Alumni Day, May 2002

Participant, Dept. of Ophthalmology retreats

Green College: membership, lectures and Thematic Lecture series (2) (see above)

## **11. SERVICE TO THE COMMUNITY**

### **(a) *Memberships on scholarly societies, including offices held and dates***

Society for Neuroscience, 1979-present

### **(b) *Memberships on other societies, including offices held and dates***

### **(c) *Memberships on scholarly committees, including offices held and dates***

Member, Editorial Board, Journal of Controversies in Biomedical Research, 2016 – to date

Member, Research Committee, ALS Society of Canada

Member, Scientific Advisory Board for Neurological Health Charities Canada

Chair, Risk Factor Committee of the Scientific Advisory Board for Neurological Health Charities Canada

BC Health Research Foundation, Research Grants committee member, 1997-2000.

ALS Association, review panel, 2001, 2004, 2008.

AIBS review panel for Department of Defense grants on ALS, 2003 to present

NINDS Udall Centers (Parkinson's Disease) Reviews, Dec. 2003, March 2004

NINDS, special panel on oxidative stress and neurological disease, 2005

Chair, Risk Factor subgroup, Neurological Health Charities Canada, 2009 to present

Member, NHCC Scientific Advisory Board, 2011

(d) *Memberships on other committees, including offices held and dates*

UBC Faculty of Medicine Gender Issues Committee, committee member, 1993.

1. *Editorships (list journal and dates)*

1. Shaw, C.A. *Frontiers in Aluminum Toxicity and Human Disease*, EPFL Innovation Park, Lausanne, 2016.
2. Shaw, C.A. *Receptor Dynamics in Neural Development*, CRC Press, Boca Raton, 1996.
3. Shaw, C.A. *Glutathione in the Nervous System*, Taylor and Francis Publishers, Washington D.C., 1997.
3. Shaw, C.A. & McEachern, J.C. *Toward a Theory of Neuroplasticity*, Taylor and Francis Publishers, Psychology Press, 2000.
4. Shaw, C.A. & Pasqualotto, B.A. Introduction: tuning up the signal: regulation of postsynaptic receptor properties. *Cellular and Mol Life Sci.*, special edition, 57(11): 1495-1498 (2000)

See list of publications for books edited and multi-author reviews.

(f) *Reviewer (journal, agency, etc. including dates)*

Agencies:

ALS Association, 2005-2008  
 ALS Canada, Research Committee, 2008-present  
 ALSA Review Panel, Oct. 2001  
 BC Health Research Foundation, 1988-1997  
 CIHR (former Medical Research Council of Canada), 1988-present  
 Fond de Reserche Clinique du Quebec, 1995  
 Jewish General Hospital Foundation (Louisville, Kentucky), 1988  
 National Institutes of Health (USA), 1998-present  
 National Science Foundation (USA), 1988-present  
 Natural Science and Engineering Research Council (Canada), 1988-present  
 Neuroscience Charities Canada, 2008 to present (Chair, "Risk Factor" Subcommittee)  
 NINDS, panel on oxidative stress and neurological disease, 2005  
 NINDS-Udall Center Parkinson's Research Program Grants review panel, Dec 2003  
 Telthon Combatti La Distrofia Muscolare (Italy), 1998-2000  
 US Department of Defense, AIBS Review Panel, Nov 2003, 2004, 2008  
 Wellcome Trust, Nov 2003  
 Whitehall Foundation, 2000

Journals: (all 1988-present)

Annals of Medicine  
 Allergy, Asthma and Clinical Immunology  
 BMC Medicine  
 BMC Neuroscience  
 Brain Research  
 Case Reports in Rheumatology  
 Cell Biology and Toxicology  
 Chemosphere  
 Developmental Brain Research  
 Entropy  
 Environmental Research

Environmental Science and Pollution Research  
 European Journal of Neuroscience  
 Free Radical Biology and Medicine  
 Hippocampus  
 Journal of Comparative Neurology  
 Journal of Developmental Disabilities  
 Journal of Inorganic Biochemistry  
 Journal of Medical Case Reports  
 Journal of Neural Transmission  
 Journal of Neurochemistry  
 Journal of Neurophysiology  
 Journal of Neuroscience Research  
 Journal of Neuroscience and Behavioural Health  
 Molecular Brain Research  
 Neurobiology of Aging  
 Neuroscience  
 Pharmacology, Biochemistry and Behavior  
 PLOS1  
 Proceedings of the National Academy of Science (USA)  
 Trends in Pharmacological Sciences  
 Trends in Neuroscience  
 Vaccine; March 2015, Awarded Recognized Reviewer Status

(g) *External examiner (indicate universities and dates)*

University examiner: defense of Elissa Strome (supervisor, Dr. D. Doudet), UBC, 2006

Chair of the final doctoral oral examination of Robert Gerl in Experimental Medicine, UBC (2003).

University examiner: defense of Rachael Heisel (supervisor, Dr. S. Kim), Dept of Medicine, UBC (2002)

University examiner: defense of Magdalena Luca (supervisor Dr. L. Kesler), Dept. of Mathematics, UBC (2001)

University examiner: defense of Dr. Lisa Kalynchuk (supervisor, Dr.J.P.J. Pinel), Dept. of Psychology, UBC (1999)

(h) *Consultant (indicate organization and dates)*

Covalent Associates, 1993-1998

IGT Pharma, 1997-2000

MITACS (Mathematics Centre of Excellence), 1998-2003

Shaw Neural Dynamics (founder, president, and CSO): 2001-2005

Thomas Paine Institute (director) 2004-2008

Neurodyn Corp., 2005 - present

(i) *Other service to the community*

Member of the Army Reserve (officer), 1991-2010

*(Please note that in this role I have been involved in public education projects concerning the Ottawa Accord)*

Supervisor for Science summer program, University Hill Secondary School, 2000

Candidate for Parliament, Vancouver Quadra, Nov 2000;

Candidate for Vancouver City Council, 2008, 2011

Lecture to community organizations (Scottish Rite Charitable Foundation of Canada), Nov 2001 and Sept 2002

## **12. AWARDS AND DISTINCTIONS**

(a) *Awards for Teaching (indicate name of award, awarding organizations, date)*

(b) *Awards for Scholarship (indicate name of award, awarding organizations, date)*

Friends of the Hebrew University Scholarship, 1971-1973  
 Hebrew University Graduate Scholarship, 1974-1976, 1978-1979  
 Killam Postdoctoral Fellowship, 1979-1981  
 NIH Postdoctoral Fellowship, 1981-1983  
 BC Heath Research Scholarship, 1988-1992  
 William Evans Visiting Fellowship, Otago University, Dunedin, NZ, 2005

(c) *Awards for Service (indicate name of award, awarding organizations, date)*

39 Brigade Commander's Commendation, 1999  
 Militia Staff College, 2003  
 Canada Decoration, 2004  
 Queen's Jubilee Medal, 2004

(d) *Other Awards*

## **OTHER RELEVANT INFORMATION (Maximum One Page)**

My research has focused on two key areas, neuroplasticity and neuropathology, and studies of these areas are the basis for the publications highlighted in the attached list of publications.

When I first arrived at UBC, most of my work was directed at understanding the mechanisms underlying receptor regulation. These studies resulted in numerous research publications and reviews (e.g., Lanius et al., 1993; Shaw et al., 1994; Shaw, 1996; Pasqualotto and Shaw, 1996, 2000). The outcome of these studies laid the groundwork for a reevaluation of current theories of 'neuroplastic' phenomenon such as long-term potentiation (see McEachern and Shaw, 1996, 1999, 2000), and the role that abnormal receptor regulation may play in some neurodegenerative disorders (Bains and Shaw, 1997; Shaw and Bains, 2000). This focus also led to an evaluation of the various complex roles played in normal and abnormal synaptic function by the antioxidant molecule glutathione (see Janaky et al., 1999; Shaw et al., 1996; Shaw, 1996). Such studies were also seminal to our recent attempt to provide a unified theory to encompass the vast and diverse subject termed "neuroplasticity" (Shaw and McEachern, 2000).

My current research focus is on ALS-parkinsonism dementia complex (ALS-PDC), a complex neurological disorder of the Western Pacific. The ongoing studies in my laboratory are devoted to an animal model of the disease and include the following subjects: isolation and mechanism of action of the putative environmental toxin, a detailed time course of the behavioural, morphological, and biochemical events that occur from initial insult to neural cell death, interactions with genetic susceptibility factors, and the roles of age and gender (see Shaw and Wilson, 2003).

My laboratory now hosts 3 graduate students, 2 research technicians, and various undergraduate honours/work study students. Our studies on the ALS-PDC model involve numerous past and current UBC collaborators as well as international collaborators.

## **NOTE ON PUBLICATIONS:**

## 1. Impact Factor

The following section contains my list of publications. Note that the journals chosen span a number of areas within the neurosciences and include pharmacology and experimental medicine. These journals were assessed on the basis of journal impact assessment rating of 461 journals (Journal Citation Reports, 1998, CD ROM, IRC, UBC). While this is a highly artificial rating, it does provide a basis for comparison of the various journals. Note also that many other factors determined my choice of journal for particular articles. (For example, I was encouraged to support Canadian neuroscience by sending an article to the *Canadian Journal of Physiology and Pharmacology* even though this journal does not score within the upper 25% based on impact assessment. Similarly, articles such as that published in *Medical Hypotheses* were aimed at a specific target audience irrespective of impact factor.

Overall, based on the Journal Citation Report, 71% of my research articles up to 2004 (date of promotion to full professor) scored in the upper 25% of journals rated by impact factor. For primarily review articles, 67% were in the upper 25% by impact factor.

## 2. Citation Index

From 1993 (date of last promotion) through 2004, various of my papers were cited approx. 610 times (based on a comprehensive search of the ISI Web of Science website).

## 3. Policy on Authorship

My policy on authorship is the following: Each author should have made a material contribution to either the basic research and/or the analysis and interpretation of the data. A contribution to writing and/or editing resulting manuscripts is also essential. Order of authors is based on two factors: (i) the amount of contribution of each author and (ii) the stage of professional development and hence the relative 'need' for more or less recognition in cases where contributions have been equivalent (note that many journals now allow authors to be listed as 'equal co-authors'). For the latter, I give the example of the manuscript by Janaky et al. 1999 (listed as # 46 in the following list of publications) on which I am listed as the senior author. In this case, the original idea for the review was mine, I wrote the vast bulk of the manuscript, did all the revisions, formatted and reformatted all figures, etc. However, the three primary authors (me, Dr. R. Janaky, and Dr. K. Ogita) each contributed approximately equal amounts of primary data. In this circumstance it was viewed as best to advance the career development of Dr. Janaky who was then in the promotion process. This principle has applied to virtually all review papers and chapters submitted from my laboratory since arriving at UBC.

## 4. Note on Key Publications

Single asterisks below indicate those publications that I consider to be my most significant contributions to the literature. Those publications preceded by double asterisks have their abstracts appended to this document. These three documents are reviews that illustrate the range of my contributions to three key areas in the neurosciences: neurodegenerative disease, neuroplasticity, and glutathione in the CNS.

**THE UNIVERSITY OF BRITISH COLUMBIA**  
***Publications Record***

SURNAME: Shaw

FIRST NAME: Christopher

Date: December 6, 2016

MIDDLE NAME(S): Ariel

Initials: CAS

**Total Full Publications: 150 (refereed, 12 reviews)**  
**3 (non-refereed)**  
**20 book chapters**

**Total Abstracts: 178****Books: 6****1. REFEREED PUBLICATIONS**Journals

1. Crepeaux G, Eidi H, David MO, Baba-Amer Y, Tzavara E, giros B, authier FJ, Exley C, Shaw CA, Cadusseau J, Gherardi RK. Non-linear dose-response of aluminium hydroxide adjuvant particles: Selective dose neurotoxicity. *Toxicology*. 375:48-57. (2016).
2. Morrice JR, Gregory-Evans CY, Shaw CA. Necroptosis in amyotrophic lateral sclerosis and other neurological disorders. *Biochimica et Biophysica Acta*. 1863: 347-353. (2017).
3. Van Kampen J, Baranowski DC, Robertson HA, Shaw CA, Kay DK. The progressive BSSG rat model of Parkinson's : recapitulating multiple key features of the human disease. *PLOS*. <http://dx.doi.org>.insert details. (2015).
4. Zwiegers P, Shaw CA. Disparity of outcomes: the limits of modeling amyotrophic lateral sclerosis in murine models and translating results clinically. *J. Controversies in BioMed Res*. 1(1):4-22. (2015).
5. Crepeaux G, Eidi H, David M-O, Tzavara E, Giros B, Exley C, Curmi PA, Shaw CA, Gherardi RK, Cadusseau J. Highly delayed systemic translocation of aluminium-based adjuvant in CD1 mice following intramuscular injections. *J. Inorg. Biochem*. 152:199-205. (2015).  
<http://dx.doi.org/10.1016/j.jinogbio.2015.07.004>
6. Shaw CA, Li D, Tomljenovic L. Are there negative CNS impacts of aluminum adjuvants in vaccines and immunotherapy? *Immunotherapy*. 6 (10):1055-1071. (2014).
7. Van Kampen, J.M., Baranowski, D.C., Shaw, C.A., and D.G Kay. Panax ginseng is neuroprotective in a novel progressive model of Parkinson's disease. *Exp Gerontol*, 50(2014):95-105. (2014).
8. Shaw CA, Seneff S, Kette SD, Tomljenovic L, Oller Jr JW, Davidson RM. Aluminum-induced entropy in biological systems: Implications for neurological disease. *J Toxicology*. Volume 2014, Article ID 491316. <http://dx.doi.org/10.1155/2014/401316>. (2014).
9. Shaw CA, Sheth S, Li D, Tomljenovic L. Etiology of autism spectrum disorders: Genes, environment, or both? *OA Autism*. 10:2(2):11. (2014).
10. Zwiegers P, Lee G, and Shaw CA. Reduction in hSOD1 copy number significantly impacts ALS phenotype presentation in G37R (line 29) mice: implications for the assessment of putative therapeutic agents. *Journal of Negative Results in BioMedicine*. 13:14. Doi:10.1186/1477-5751-13-14. (2014).
11. Shaw CA, Kette SD, Davidson RM, Seneff S. Aluminum's role in CNS-immune system interactions leading to neurological disorders. *Immunome Res*. 9:1. <http://dx.doi.org/10.4172/1745-7580.1000069>.(2013).
12. Colafrancesco S, Perricone C, Tomljenovic L, Shoenfeld Y. Human papilloma virus vaccine and primary ovarian failure: Another facet of the Autoimmune/Inflammatory Syndrome Induced by Adjuvants. *Am J Reproductive Immunology*. 70(4):309-16. (2013).



13. Shaw CA, Marler TE. Aluminum and the human diet revisited. In: *Communicative & Integrative Biology; Landes Bioscience*. 6:e26369. (2013).
14. Tomljenovic L, Wilyman J, Vanamee E, Bark T, Shaw CA. (Letter) HPV vaccine and cancer prevention, science versus activism. *Infectious Agents and Cancer*. 8(1):6. (2013).
15. Shaw CA, Tomljenovic L. Aluminum in the central nervous system (CNS): toxicity in humans and animals, vaccine adjuvants, and autoimmunity. *Immunol Res*. DOI 10.1007/s12026-013-8403-1. (2013).
16. Shaw CA, Li Y, Tomljenovic L. Administration of aluminum to neonatal mice in vaccine in vaccine-relevant amounts is associated with adverse long term neurological outcomes. *J Inorg Chem*. <http://dx.doi.org/10.1016/j.jinorgbio.2013.07.022>. (2013).
17. Tomljenovic L, Spinoso JP, Shaw CA. Human Papillomavirus (HPV) Vaccines an option for preventing cervical malignancies: (How) Effective and safe? *Current Pharm Design*. 19(8):1466-87. (2013).
18. Tomljenovic L, Shaw AC. Too Fast or Not Too Fast: The FDA's Approval of Merck's HPV Vaccine Gardasil. *J Law Med Ethics*. Fall: 673-681. (2012).
19. Tomljenovic L, Dorea JG, Shaw AC. Commentary: A link between mercury exposure, autism spectrum disorder and other neurological disorders? Implications for thimerosal-containing vaccines. *JoDD*. 18(1):34-42. (2012).
20. Tomljenovic L, Shaw CA. Death after quadrivalent human papillomavirus (HPV) vaccination: causal or coincidental? *Pharma Reg Affairs*. S12:001. Doi:10.4172/2167-7689.S12-001. (2012).
21. Tomljenovic L, Shaw CA. Mechanisms of aluminum adjuvant toxicity and autoimmunity in pediatric populations. *Lupus*. 21:223-230. (2012)
22. Lee G, Shaw AC. Early exposure to environmental toxin contributes to neuronal vulnerability and axonal pathology in a model of familial ALS. *Neuroscience Medicine*. Doi:10.4236/nm.2012. (2012).
23. Tomljenovic L and Shaw CA. Who profits from uncritical acceptance of biased estimates of vaccine efficacy and safety? *AJPH*. Doi: 10.2105/AJPH.2012.300827. (2012).
24. Tomljenovic L and Shaw CA. No autoimmune safety signal after vaccination with quadrivalent HPV vaccine Gardasil? *J Internal Medicine*. Doi:10.1111/j.1365-2796.2012.025551.x. (2012).
25. Tomljenovic L and Shaw CA. Editorial, Special Issue: The Biochemistry/Toxicity of Aluminum. *Current Inorganic Chemistry*. 2(1): 1-2. (2012).
26. Tomljenovic L and Shaw CA. Mandatory HPV vaccination. [Letter to Editor]. *JAMA*. 307(3): 254; Author reply 254-5. (2012).
27. Tomljenovic L and Shaw CA. Microglia-mediated immunoexcitotoxicity, a key player in traumatic brain injury? [Commentary]. *Surg Neuro Int*. 2(107). (2011).
28. Tomljenovic L and Shaw CA. Human papillomavirus (HPV) vaccine policy and evidence-based medicine: Are they at odds? *Annals of Medicine*. 1 – 12, DOI: 10.3109/07853890.2011.645353. (2011).
29. Tomljenovic L and Shaw CA. One size fits all? *Vaccine*. Doi:10.1016/j.vaccine.2011.11.053. (2011).
30. Tomljenovic L and Shaw CA. Do aluminum vaccine adjuvants contribute to the rising prevalence of autism? *J Inorg Biochem*. 105(11):1489-99. (2011).
31. Tomljenovic L and Shaw CA. Aluminum vaccine adjuvants: Are they safe? *Current Medicinal Chemistry*. 18:2630 – 2637. (2011).
32. Panov A, Kubalik N, Brooks BR, and Shaw CA. In vitro effects of cholesterol  $\beta$ -D-glucoside, cholesterol and cycad glucosides on respiration and reactive oxygen species generation in brain mitochondria. *J. Membrane Biol*. DOI 10.1007/s00232- 10-9307-7. (2010).
33. Marler TE, Snyder LR, and Shaw CA. Cycas micronesica (Cycadales) plants devoid of endophytic cyanobacteria increase in b-methylamino-L-alanine. *Toxicon*. 56: 563-568. (2010).

34. Shen W-B, McDowell KA, Siebert AA, Clark SM, Dugger NV, Valentino KM, Jinnah A, Sztalryd C, Fishman PS, Shaw CA, Jafri MS, and Yarowsky PJ. Environmental neurotoxin-induced progressive model of parkinsonism in rats. *Annals of Neurology*. 68(1):70-80. (2010).
35. Tasker RA, Adams-Marriott AL, and Shaw CA. New animal models of progressive neurodegeneration: tools for identifying presymptomatic therapeutic targets. *The EMPA Journal*. DOI: 10.1007/S13167-010-0019-0. (2010).
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37. Ryan CL, Baranowski DC, Chitramuthu BP, Malik S, Li Z, Cao M, Minotti S, D Durham HD, Kay DG, Shaw CA, Bennett HPJ, and Bateman A. Progranulin is expressed within motor neurons and promotes neuronal cell survival. *BMC Neuroscience*. Doi.10.1186/1471-2202-10-130. (2009).
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42. Lee G, Chau T, and Shaw CA. The primary locus of motor neuron death in an ALS-PDC mouse model. *NeuroReport*. 20 (14): 1284-1289. (2009).
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### 3. **BOOKS**

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#### 4. **PATENTS**

1. Mosquito attractor (US patent, 1979).
2. Sterol Glucoside Toxins (Canada, European Patent Convention, USA; 2012); patents held by Neurodyn Corp. Inc.

#### 5. **SPECIAL COPYRIGHTS**

3. *Honeymoon 1-10* (original screenplay)
4. *Digoxin Lullaby* (short novel)
5. The 'neural net' (cover design for *Toward a Theory of Neuroplasticity*)
6. *Five Ring Circus: Myths and Realities of the Olympic Games*, New Society Publishers, 2008.
7. Numerous political essays/articles

#### 6. **ARTISTIC WORKS, PERFORMANCES, DESIGNS**

N/A

#### 7. **OTHER WORKS**

N/A

#### 8. **WORK SUBMITTED** (including publisher and date of submission)

N/A

#### 9. **CONTRIBUTING AUTHOR IN OTHER ARTICLES**

1. *The Republic of East Vancouver*. 2003-2007.

2. *The Tyee*. 2007 to present.
3. *The Vancouver Sun*. 2005, 2009.
4. *Vancouver Observer*, 2008 to 2011
5. *Rabble.ca*, 2008 to present.
6. Briarpatch Magazine, 2008 to present.

June 15, 2017

United States Department of Health & Human Services  
National Institutes of Health  
Food & Drug Administration  
Centers for Disease Control & Prevention  
200 Independence Avenue, S.W.  
Washington, D.C. 20201

Re: *Aluminum Adjuvants*

Dear Directors:

I am an expert in the field of aluminum adjuvants toxicity in humans and animal models. I have been working in this field since the initial description of the Al vaccine-induced macrophagic myofasciitis in 1998. Since that time I have written 40 peer-reviewed scientific publications and one book on this subject.

I strongly support the contention that aluminum adjuvants in vaccines may have a role in the etiology of autism spectrum disorder (ASD). My view is founded on a significant and burgeoning body of peer-reviewed scientific evidence which makes the link between ASD and exposure to aluminum through vaccinations and other sources. Examples of this literature from my own group are detailed below and I urge the HHS to take them into consideration in forming any future opinion on the safety of aluminum adjuvants in vaccines.

The Center for Disease Control's claim on its website that "Vaccines Do Not Cause Autism" is unsupported with respect to aluminum adjuvants and this claim stifles the important research to determine the safety of aluminum adjuvants used in vaccines. As an expert in the field of aluminum adjuvants and aluminum toxicity I solemnly declare that more research on the role of aluminum adjuvant in vaccines and neurological disorders, including ASD, is essential and urgently required.

Yours very sincerely



Romain K. Gherardi  
Professor, Neuromuscular Pathology Expert Centre  
University Paris-Est, INSERM U955-E10,  
Henri Mondor hospital, Créteil France  
Contact at the hospital  
Tel 00 (33) 1 49812746  
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UMR U955 INSERM / UPEC

Team 10

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system »

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**Selection of significant publications from our group in the field**

Gherardi R. Toxic Story: deux ou trois vérités embarrassantes sur les adjuvants des vaccins. **Actes Sud** (publisher), Paris, 2016, 250 pages

Crépeaux G, Eidi H, David MO, Baba-Amer Y, Tzavara E, Giros B, Authier FJ, Exley C, Shaw CA, Cadusseau J, Gherardi RK. Non-linear dose-response of aluminium hydroxide adjuvant particles: Selective low dose neurotoxicity. **Toxicology**. 2017 Jan 15;375:48-57.

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Authier FJ, Sauvat S, Champey J, Drogou I, Coquet M, Gherardi RK. Chronic fatigue syndrome in patients with macrophagic myofascitis. **Arthritis Rheum**. 2003 Feb;48(2):569-70.

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Authier FJ, Cherin P, Creange A, Bonnotte B, Ferrer X, Abdelmoumni A, Ranoux D, Pelletier J, Figarella-Branger D, Granel B, Maisonnobe T, Coquet M, Degos JD, Gherardi RK. Central nervous system disease in patients with macrophagic myofascitis. **Brain**. 2001 May;124(Pt 5):974-83.

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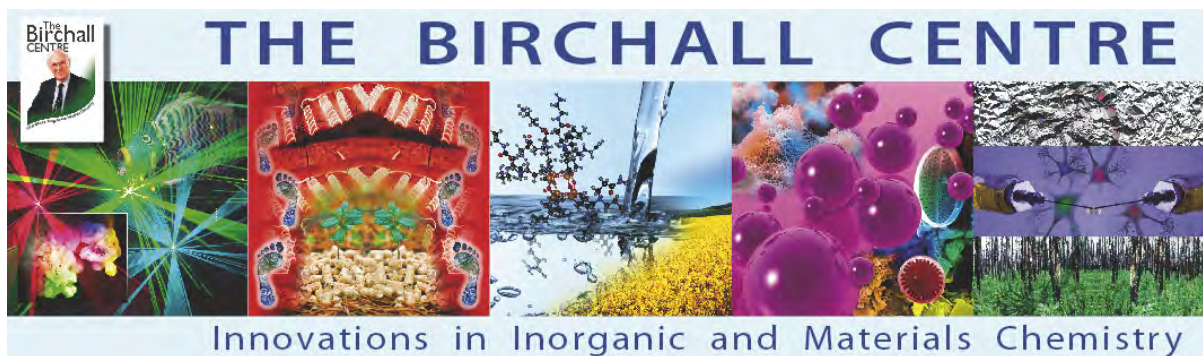
Gherardi RK, Coquet M, Chérin P, Authier FJ, Laforêt P, Bélec L, Figarella-Branger D, Mussini JM, Pellissier JF, Fardeau M. Macrophagic myofascitis: an emerging entity. **Lancet**. 1998 Aug 1;352(9125):347-52.

## BRIEF CURRICULUM VITAE

### Romain K. Gherardi MD

<b>Date of birth:</b>	20 April 1952
<b>Marital Status:</b>	Married, 3 children
<b>Nationality:</b>	French
<b>Qualifications:</b>	Medical doctor, Paris VI University. - CES of Neurology CES of Pathology CES of Forensic Medicine-Toxicology DESS of Health Law DEA of Methodology in History of Ideas AEA of Medical Expertise
<b>Awards</b>	Bonus for scientific excellence constantly obtained
<b>Medical &amp; Scientific career:</b>	
1976-1982	Fellowship at APHP (Interne des hôpitaux de Paris 59/310).
1985-1990	Assistant Professor in Forensic Medicine-Toxicology, Paris-Est
1991-now	Professor in Histology (neuropathology), Paris-Est University Head of Neuromuscular Pathology Expert Centre, Henri Mondor APHP hospital, Créteil, France
1992-2000	Head of research unit at the University (ER, EA)
2000- 2014	Head of research U955 team 10, INSERM & Paris-Est University
2001-2012	Member of INSERM boards (AVENIR, CSS2)
2003-now	Member of the National University Council (section 42.02)
<b>Publications:</b>	Approaching 350 peer-reviewed publications mainly dedicated to neuromuscular inflammatory and toxic diseases, and to muscle stem cells and their niche (New England Journal of Medicine, Lancet, Cell Stem Cell, J Exp Med, JAMA, Blood, Brain, Ann Neurol, J Cell Biol, Development etc) <b>H index:</b> 50
<b>Phone &amp; Email:</b>	+ (33) 1 49 81 27 46, <a href="mailto:romain.gherardi@aphp.fr">romain.gherardi@aphp.fr</a>





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June 15, 2017

United States Department of Health & Human Services  
National Institutes of Health  
Food & Drug Administration  
Centers for Disease Control & Prevention  
200 Independence Avenue, S.W.  
Washington, D.C. 20201

*Re: Aluminum Adjuvants*

Dear Directors:

I am an expert in the field of aluminum adjuvants and aluminum toxicity. I have been working in this field for more than 30 years during which time I have written in excess of 150 peer-reviewed scientific publications on this subject.

I strongly support the contention that aluminum adjuvants in vaccines may have a role in the etiology of autism spectrum disorder (ASD). My view is founded on a significant and burgeoning body of peer-reviewed scientific evidence which makes the link between ASD and exposure to aluminum through vaccinations and other sources. Examples of this literature from my own group are detailed below and I urge the HHS to take them into consideration in forming any future opinion on the safety of aluminum adjuvants in vaccines.

The Center for Disease Control's claim on its website that "Vaccines Do Not Cause Autism" is unsupported with respect to aluminum adjuvants and this claim stifles the important research to determine the safety of aluminum adjuvants used in vaccines. As an expert in the field of aluminum adjuvants and aluminum toxicity I solemnly declare that more research on the role of aluminum adjuvant in vaccines and neurological disorders, including ASD, is essential and urgently required.

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Yours faithfully



Christopher Exley PhD  
Professor in Bioinorganic Chemistry

Honorary Professor, University of the Highlands and Islands

**List of Recent, Relevant and Significant Publications From Our Group**

Exley C, Siesjö P & Eriksson H (2010) The immunobiology of aluminium adjuvants: how do they really work? Trends in Immunology 31, 103-109.

Exley C and House E (2011) Aluminium in the human brain. Monatshefte für Chemie - Chemical Monthly 142, 357-363.

House E, Esiri M, Forster G, Ince PG and Exley C (2012) Aluminium, iron and copper in human brain tissues donated to the medical research council's cognitive function and ageing study. Metallomics 4, 56-65.

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Khan Z, Combadière C, Authier FJ, Itier V, Lux F, Exley C, Mahrouf-Yorgov M, Decrouy X, Moretto P, Tillement O, Gherardi RK, and Cadusseau J (2013) Slow CCL2-dependent translocation of biopersistent particles from muscle to brain. BMC Medicine 11:99.

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Mold M, Eriksson H, Siesjö P, Darabi A, Shardlow E and Exley C (2014) Unequivocal identification of intracellular aluminium adjuvant in a monocytic THP-1 cell line. Scientific Reports 4, 6287.

Telephone number +44 (01782) 584211  
Fax +44 (01782) 712378

Exley C (2014) Why industry propaganda and political interference cannot disguise the inevitable role played by human exposure to aluminium in neurodegenerative diseases, including Alzheimer's disease. *Frontiers in Neurology* 5:212. doi: 10.3389/fneur.2014.00212.

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Exley C (2016) The toxicity of aluminium in humans. *Morphologie* 100, 51-55.

Mirza A, King A, Troakes C and Exley C (2016) The identification of aluminium in human brain tissue using lumogallion and fluorescence microscopy. *Journal of Alzheimer's Disease* 54, 1333-1338.

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Mirza A, King A, Troakes C and Exley C (2017) Aluminium in brain tissue in familial Alzheimer's disease. *Journal of Trace Elements in Medicine and Biology* 40, 30-36.

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## BRIEF CURRICULUM VITAE

Christopher Exley PhD

<b>Date of birth:</b>	17th April 1963
<b>Marital Status:</b>	Married
<b>Nationality:</b>	British
<b>Qualifications:</b>	BSc 2i Biology, University of Stirling. PhD, Institute of Aquaculture, University of Stirling.
<b>Awards:</b>	2015 – Fellow of the Royal Society of Biology
<b>Scientific career:</b>	
1989-1992	ICI Postdoctoral Research Fellow, Institute of Aquaculture, University of Stirling.
1992-1994	ICI Postdoctoral Research Fellow, Department of Chemistry, Keele University.
1994-2002	Royal Society University Research Fellow, Senior Research Fellow & Lecturer School of Chemistry and Physics , Keele University.
2002-2011	Reader in Bioinorganic Chemistry, Life Sciences, Keele University
2011-Present	Professor in Bioinorganic Chemistry, Life Sciences, Keele University
2009-Present	Honorary Professor, University of the Highland and Islands
2015-Present	Fellow of The Royal Society of Biology
<b>Funding:</b>	Approximately £4.5M over 25 years at Keele
<b>Publications:</b>	Approaching 150 peer-reviewed publications to-date.
<b>Website:</b>	<a href="http://www.keele.ac.uk/aluminium/">http://www.keele.ac.uk/aluminium/</a>
<b>Email:</b>	<a href="mailto:c.exley@keele.ac.uk">c.exley@keele.ac.uk</a>

### ANALYSIS OF TAYLOR 2014

Taylor 2014 illustrates the deficiencies in vaccine-autism studies, and how they have been misused. It is a meta-analysis of studies of the MMR vaccine and thimerosal, in relation to autism. It looks at no other vaccines, and no other vaccine ingredients. Even with this limited scope, it is often presented as evidence for the safety of all vaccines and all vaccine ingredients, and as evidence for the safety of the vaccine schedule in aggregate. This characterization is simply wrong.

With one exception, there has never been a study comparing neurological health outcomes (e.g. autism) among the fully-vaccinated and completely unvaccinated. The exception is a survey study (Mawson 2017) reporting a 4.2 odds ratio (OR) for autism among children fully vaccinated according to the CDC schedule, compared to completely unvaccinated children. Mawson 2017 also reported greatly elevated ORs for other neurological and immune disorders. These effects are biologically plausible in view of the proven neuro- and immuno-toxicity of aluminum adjuvants.

Taylor 2014 includes 6 studies of MMR and 4 studies of thimerosal/Hg. None of these studies included control subjects with low or no vaccine exposure. In Taylor 2014, the term “unvaccinated” refers to children missing only the MMR vaccine. They may have received all other CDC-recommended vaccines, which amount to 21 vaccines in the first 12 months of life.

The included cohort studies use controls that likely received all (or nearly all) recommended vaccines except MMR. In other words, the cohort study controls were unvaccinated only with respect to MMR. Similarly, the control groups in the thimerosal/Hg studies received the same or similar vaccine exposure as the Hg-exposed groups. For example, the Verstraeten 2003 and Hviid 2003 studies were designed to isolate the effect of thimerosal. Hence, these studies do not provide safety evidence for anything other than thimerosal.

### **Cohort Studies in Taylor 2014**

<b>Study</b>	<b>Focus</b>	<b>Design</b>	<b>Subjects</b>	<b>Exposed Group</b>	<b>Control group</b>
Madsen 2002	MMR	Cohort	440,655 MMR 96,648 no MMR	Vaccinated including the MMR	Vaccinated except for MMR
Uchiyama 2007	MMR	Cohort	904 autistics= 292 MMR 612 no MMR	Vaccinated including the MMR	Vaccinated except for MMR
Andrews 2004	Hg	Cohort	109,863, with varying thimerosal exposures	Vaccinated with higher level of Hg exposure.	Vaccinated with lower level of Hg exposure.
Hviid 2003	Hg	Cohort	467,450, with varying thimerosal exposures	Vaccinated with Hg-containing pertussis vaccine.	Vaccinated with Hg-free pertussis vaccine.

Verstraeten 2003	Hg	Cohort	124,170, with varying thimerosal exposures	Vaccinated with higher level of Hg exposure.	Vaccinated with lower level of Hg exposure.
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The case-control studies have similar problems. Only one (Uno 2012) looks at any vaccine exposure other than MMR. So the case-control studies in general are not relevant to any vaccines other than MMR.

All of the case-control studies obtained control subjects from a population with almost universal vaccine uptake (e.g., typical vaccine exemption rates in the US are about 1-2%). Obviously, if vaccination is universal (or nearly so), then it is impossible or difficult to observe differences in vaccine exposure in cases and controls. This is because universal vaccination causes cases and controls to have the same vaccine exposure. So, in order for case-control studies to detect vaccine adverse effects, there must be a substantial number of unvaccinated individuals in the population being studied.

#### Case-Control Studies in Taylor 2014

Study	Design	Subjects	Exposures Tested	Control Group Features
DeStefano 2004	Case-Control	624 cases (autism) 1824 controls	Age at first MMR receipt. No other vaccines or ingredients considered	Controls obtained from population with almost universal vaccination.
Mrozek-Budzyn 2010	Case-Control	96 cases (autism) 192 controls	MMR or measles vaccine. No other vaccines or ingredients considered	Controls obtained from population with almost universal vaccination.
Smeeth 2004	Case-Control	1294 cases (autism or PDD) 4469 controls	MMR. No other vaccines or ingredients considered	Controls obtained from population with almost universal vaccination.
Uno 2012	Case-Control	189 cases (autism) 224 controls	MMR, and other vaccines. No vaccine ingredients considered.	Controls obtained from population with almost universal vaccination. Study is from Japan, which had far fewer vaccines in the schedule in the study period (1984-1992), compared to the CDC schedule of today.
Price 2010	Case-Control	256 cases (ASD) 752 controls	Thimerosal dose.	Controls obtained from population with almost universal vaccination.

The 10 studies included in Taylor 2014 are thus plainly not comparing vaccinated with unvaccinated children. The 10 studies tell us virtually nothing about the relationship of other (i.e. non-MMR) vaccines to autism. They also tell us nothing about the safety of the CDC schedule as a whole. Since MMR does not contain aluminum, the Taylor 2014 paper also tells us nothing about the safety of aluminum adjuvants. Taylor 2014 is relevant to one vaccine (MMR) and one vaccine ingredient (thimerosal), and nothing else.

Additionally, the MMR-autism studies might be wrong.. All the MMR studies are likely affected by healthy user bias (HUB). HUB is a type of selection bias, created when vaccines are not given to children displaying signs of poor health or developmental delay. Evidence for HUB is present in Mrozek-Budzyn 2010 and Smeeth 2004 in the form of inverse associations between autism and MMR. Both studies explain the inverse associations may be caused by withholding vaccines from already-sick children. So, children injured by vaccines at 0-, 2-, 4-, or 6- months are less likely to receive MMR, and are therefore used as MMR-unvaccinated controls. Obviously, HUB is a highly misleading phenomenon in the context of MMR-autism research. Note that HUB occurs even if parents are wrong in believing that the 0-, 2-, 4-, or 6- month vaccines caused injury. Fine and Chen, 1992 describes the problem of healthy user bias.

Vaccines other than MMR have not been studied in relation to autism. For example, the IOM stated in 2011 that there isn't a single study that supports the assertion that DTaP (injected at 2 months, 4 months, 6 months, etc.) does not cause autism, concluding that "*The evidence is inadequate to accept or reject a causal relationship between diphtheria toxoid-, tetanus toxoid-, or acellular pertussis-containing vaccine and autism.*" Attached is an excerpt regarding autism and DTaP from the 2011 IOM report (there have been no studies on DTaP and autism since 2011). As another example, the only study on Hepatitis B vaccine and autism reported that neonatal Hep B vaccination is associated with a three-fold increase in autism risk. (Gallagher and Goodman 2010.) The DTaP and Hepatitis B vaccines contain aluminum adjuvant.

## References

Fine and Chen 1992, Confounding in Studies of Adverse Reactions to Vaccines, American Journal of Epidemiology, 136(2): 121-135.

Gallagher and Goodman Hepatitis B vaccination of male neonates and autism diagnosis, NHIS 1997-2002. J Toxicol Environ Health A. 73(24):1665-77.

Mawson et al 2017, Pilot comparative study on the health of vaccinated and unvaccinated 6- to 12-year-old U.S. children, J Transl Sci., 3(3): 1-12.

Taylor et al. 2014 Vaccines are not associated with autism: An evidence-based meta-analysis of case-control and cohort studies, Vaccine 32: 3623-3629.

**From:** [Gordon, Joshua \(NIH/NIMH\) \[E\]](#)  
**To:** [Allen-Gifford, Patrice \(NIH/OD\) \[E\]](#)  
**Cc:** [Koeneman, Sandy \(NIH/OD\) \[E\]](#)  
**Subject:** Re: 1 selected item: 24814559 - PubMed  
**Date:** Friday, September 1, 2017 12:56:15 PM

---

Should I send the response then?

Josh

-----  
Joshua A Gordon, MD, PhD  
Director  
National Institute of Mental Health

On Sep 1, 2017, at 12:48 PM, Allen-Gifford, Patrice (NIH/OD) [E] <[patrice.allen-gifford@nih.gov](mailto:patrice.allen-gifford@nih.gov)> wrote:

Josh,

Thanks for so graciously working this through ES. We just received Francis' and Larry's approval of your draft.

Thanks and best wishes,

Patrice

---

**From:** Gordon, Joshua (NIH/NIMH) [E]  
**Sent:** Wednesday, August 30, 2017 9:25 PM  
**To:** Allen-Gifford, Patrice (NIH/OD) [E] <[patrice.allen-gifford@nih.gov](mailto:patrice.allen-gifford@nih.gov)>  
**Cc:** Koeneman, Sandy (NIH/OD) [E] <[sandra.koeneman@nih.gov](mailto:sandra.koeneman@nih.gov)>  
**Subject:** Re: 1 selected item: 24814559 - PubMed

Draft as follows:

Dear Aaron,

I appreciate you following up with me, and apologize for the delay in my response. I think the information you are seeking would be best obtained from the CDC.

Best,

Josh



-----  
Joshua A Gordon, MD, PhD  
Director  
National Institute of Mental Health

---

**From:** "Allen-Gifford, Patrice (NIH/OD) [E]" <[patrice.allen-gifford@nih.gov](mailto:patrice.allen-gifford@nih.gov)>  
**Date:** Wednesday, August 30, 2017 at 7:26 PM  
**To:** "M. Joshua Gordon" <[joshua.gordon@nih.gov](mailto:joshua.gordon@nih.gov)>  
**Cc:** "Koeneman, Sandy (NIH/OD) [E]" <[sandra.koeneman@nih.gov](mailto:sandra.koeneman@nih.gov)>  
**Subject:** FW: 1 selected item: 24814559 - PubMed

Dear Dr. Gordon –

I am writing to follow up on your emails with Drs. Collins and Tabak and NIMH's request for ES to reassign the response to this lawyer to NIAID. Follow that, Dr. Tabak had conversations with Dr. Fauci and Dr. Collins.

Yesterday, Larry asked me to request that you draft a response to the emails you have received from Mr. Siri by thanking him for his follow up and informing him that he can best obtain the studies and information he is seeking from the CDC. No further discussion of the issue. Please provide the draft to my office, and we will provide it to Larry and Francis for review.

Please let me know if there is anything I can do to assist.

With best regards,  
Patrice

**Patrice Allen-Gifford**  
Director  
Executive Secretariat  
301-496-3976

---

**From:** Collins, Francis (NIH/OD) [E]  
**Sent:** Tuesday, August 15, 2017 5:38 AM  
**To:** Gordon, Joshua (NIH/NIMH) [E] <[joshua.gordon@nih.gov](mailto:joshua.gordon@nih.gov)>  
**Cc:** Burklow, John (NIH/OD) [E] <[burklowj@od.nih.gov](mailto:burklowj@od.nih.gov)>; Tabak, Lawrence (NIH/OD) [E] <[lawrence.tabak@nih.gov](mailto:lawrence.tabak@nih.gov)>  
**Subject:** RE: 1 selected item: 24814559 - PubMed

Just got to this. Larry's advice is better. Please follow that instead of my message from a few minutes ago.

FC

---

**From:** Tabak, Lawrence (NIH/OD) [E]?  
**Sent:** Monday, August 14, 2017 10:01 PM  
**To:** Gordon, Joshua (NIH/NIMH) [E] <[joshua.gordon@nih.gov](mailto:joshua.gordon@nih.gov)>  
**Cc:** Collins, Francis (NIH/OD) [E] <[collinsf@od.nih.gov](mailto:collinsf@od.nih.gov)>  
**Subject:** Re: 1 selected item: 24814559 - PubMed

Please do not respond directly. Route through ES.  
Thanks

Sent from my iPhone

On Aug 14, 2017, at 9:22 PM, Gordon, Joshua (NIH/NIMH) [E] <[joshua.gordon@nih.gov](mailto:joshua.gordon@nih.gov)> wrote:

Francis,

The lawyer affiliated with the Kennedy group has continued to communicate with me. How do you suggest I respond? Should I route the email through OD ExecSec or respond directly? Do you want to see a draft first?

Josh

-----  
Joshua A Gordon, MD, PhD  
Director  
National Institute of Mental Health

---

**From:** Aaron Siri <[aaron@sirillp.com](mailto:aaron@sirillp.com)>  
**Date:** Monday, August 14, 2017 at 4:48 PM  
**To:** "M. Joshua Gordon" <[joshua.gordon@nih.gov](mailto:joshua.gordon@nih.gov)>  
**Subject:** RE: 1 selected item: 24814559 - PubMed

Dr. Gordon,

I hope all is well.

I have not received a response to the emails below of July 10 and July 24.

The July 10 email was in response to a review you provided indicating it compared vaccinated and unvaccinated children (but which actually compares vaccinated children with vaccinated children who, at most, were missing MMR). As discussed at our meeting, I would like to see a study which supports the claim that the nearly two dozen doses of vaccines given in the first year of life (which would not include MMR and

thimerosal) do not cause autism. I still await receipt of a study which supports same. Are you aware of any such study?

The July 24 email elaborated on my prior email and also sought to facilitate a meeting between with various experts in the field of aluminum adjuvant that do believe there is a connection between aluminum adjuvant in vaccines and autism. Are you willing to have this meeting?

Best regards,  
Aaron

---

**From:** Aaron Siri

**Sent:** Monday, July 24, 2017 6:18 PM

**To:** 'Gordon, Joshua (NIH/NIMH) [E]' <[joshua.gordon@nih.gov](mailto:joshua.gordon@nih.gov)>

**Subject:** RE: 1 selected item: 24814559 - PubMed

Good evening Joshua,

As promised in my email below, I am following up regarding the research that has been conducted regarding aluminum adjuvants and neuro/psychiatric disorders, and to facilitate a meeting with you and the scientists conducting this research.

In recent years researchers have discovered that injected aluminum adjuvant travels into the brain, where it causes long term chronic inflammation, damage to neurons and behavioral abnormalities. These adverse effects occur at dosages (mcg/Kg body weight) even lower than dosages received by infants according to the CDC vaccine schedule.

Additionally, it is now well established that autism and other neuro/psychiatric disorders are caused by early life inflammation (i.e. elevated cytokines) in the brain. I have seen your published papers on immune activation and brain development so I presume you are aware of the immune activation findings. Aluminum adjuvant can cause chronic brain inflammation, and this establishes a biologically-plausible and empirically-supported mechanism for how vaccines may cause autism and other neurological disorders. None of the vaccine-autism studies to date tell us anything about the safety of aluminum adjuvants. There are no epidemiological studies showing that aluminum adjuvants do not produce these effects in humans.

Attached is a detailed explanation of the proposed mechanism for how aluminum adjuvants may cause autism. The mechanism suggests that aluminum adjuvant may cause other brain and neurodevelopmental disorders as well. Attached are also supporting letters from experts in the fields of aluminum toxicity. (Finally, I have also attached a more detailed analysis of Taylor 2014.)

I invite you to consider the arguments in the attached document and respond with your observations. I also invite you to share the document with colleagues, particularly if they may have insightful comments or rebuttals.

I also hope to facilitate a meeting with you and a number of the experts studying aluminum adjuvant toxicity, letters from a number of which are attached to this email. Assuming you are open to having this discussion, kindly have your office provide suggested dates/times for such a meeting.

Best regards,  
Aaron

---

**From:** Aaron Siri

**Sent:** Monday, July 10, 2017 4:16 PM

**To:** 'Gordon, Joshua (NIH/NIMH) [E]' <[joshua.gordon@nih.gov](mailto:joshua.gordon@nih.gov)>

**Subject:** RE: 1 selected item: 24814559 - PubMed

Good afternoon Joshua,

Thank you for sending me the below abstract/review article and it was great meeting at NIH. Really appreciate the opportunity to dialogue on the issue of vaccines and autism.

The abstract/review article you sent me below highlights the concern raised that there has never been a study assessing the relative risk of autism between vaccinated and unvaccinated child. To be sure, this review (and its abstract) leave the impression that the studies it relies upon compare “unvaccinated” children (no vaccines) with vaccinated children. Unfortunately, this is misleading since all 10 of the underlying studies relied upon for this review compared highly vaccinated children with highly vaccinated children. The only difference typically between the study and control groups was a single MMR vaccine or thimerosal vs. non-thimerosal vaccines. (I would be happy to provide you with a breakdown of each of the 10 studies reflecting same.) Meaning, what this review considers “unvaccinated” are vaccinated children typically only missing the MMR vaccine. Assuming the control children in these studies followed the current CDC recommended vaccination schedule, they would each have received 21 vaccine injections during the first 12 months of life excluding the MMR vaccine. Hence, these studies tell us virtually nothing about the relationship of vaccines to autism because they are not comparing vaccinated and unvaccinated children.

For example, the IOM stated in 2011 that there isn't a single study

that supports the assertion that DTaP (injected at 2 months, 4 months, 6 months, etc.) does not cause autism, concluding that “The evidence is inadequate to accept or reject a causal relationship between diphtheria toxoid-, tetanus toxoid-, or acellular pertussis-containing vaccine and autism.” Attached is an excerpt of the discussion regarding autism and DTaP from the 2011 IOM report. (I am not aware of a single study regarding DTaP and autism that has been done since 2011.) As another example, the only study regarding Hepatitis B vaccine and autism I have located found a three-fold increase in the odds of an autism diagnosis for neonates that received the hepatitis B vaccine at birth compared that those that did not. (Gallagher CM, Goodman MS. 2010. Hepatitis B vaccination of male neonates and autism diagnosis, NHIS 1997-2002. J Toxicol Environ Health A. 73(24):1665-77.) There is simply no studies for the numerous other vaccines given to children during the first year of life with regard to their relationship with autism (except for the Mawson study which showed vaccination had an over 4 fold increase in autism risk but that study has some serious limitations).

As we discussed at the meeting, I really am open to seeing the evidence that the vaccination schedule, and in particular the cumulative impact of the 31 vaccine doses the CDC recommends a child receive in the first year of life, are not casually related to autism. I would gladly share that support with the community concerned with this issue with my personal endorsement. On the other hand, if that proof doesn't exist, that does not mean that vaccines cause autism. It just means that we need to really do the science necessary to rule out that possibility. (Seeking to assess the health outcomes of those receiving vaccines and those not receiving vaccines really is asking for nothing more than how all drugs are safety tested prior to licensure.)

I respected what appeared to be your thoughtful rather than reflexive reaction to the spirited discussion at NIH. Conducting a true study of the health outcomes between actually unvaccinated and vaccinated children (at least an initial quick and easy retrospective study) that shows no connection with autism should be something that everyone should want. If it shows no connection, it will likely provide the greatest relief to the portion of the autism community that thinks there may be a connection. Parents who think that it was their actions, in vaccinating their children, that lead to their child's condition would feel freed from that guilt by knowing it wasn't the vaccines.

I look forward to your response and being persuaded that the science on the question of whether vaccines cause autism really is settled.

Thanks again in advance for your time and thoughtful consideration of this issue.

Best regards,  
Aaron

p.s. I have had a number of discussions with various aluminum adjuvant experts around the globe who believe there is a connection between the aluminum adjuvants in vaccines given in large quantities during the first six months of life and autism; I hope to soon send you a write-up regarding same for your consideration.

---

**From:** Gordon, Joshua (NIH/NIMH) [E] [<mailto:joshua.gordon@nih.gov>]  
**Sent:** Wednesday, May 31, 2017 4:03 PM  
**To:** Aaron Siri <[aaron@sirillp.com](mailto:aaron@sirillp.com)>  
**Subject:** Fwd: 1 selected item: 24814559 - PubMed

-----  
Joshua A Gordon, MD, PhD  
Director  
National Institute of Mental Health

Begin forwarded message:

**From:** Sent by NCBI <[nobody@ncbi.nlm.nih.gov](mailto:nobody@ncbi.nlm.nih.gov)>  
**Date:** May 31, 2017 at 4:00:01 PM EDT  
**To:** <[joshua.gordon@nih.gov](mailto:joshua.gordon@nih.gov)>  
**Subject:** 1 selected item: 24814559 - PubMed

This message contains search results from the National Center for Biotechnology Information ([NCBI](#)) at the U.S. National Library of Medicine ([NLM](#)). Do not reply directly to this message

Sent on: Wed May 31 15:58:39 2017

1 selected item: 24814559

PubMed Results

Item 1 of 1 ([Display the citation in PubMed](#))

1. Vaccine. 2014 Jun 17;32(29):3623-9. doi: 10.1016/j.vaccine.2014.04.085. Epub 2014 May 9.

## Vaccines are not

# associated with autism: an evidence-based meta- analysis of case-control and cohort studies.

[Taylor LE](#)<sup>1</sup>, [Swerdfeger AL](#)<sup>1</sup>, [Eslick GD](#)<sup>2</sup>.

Author information:

1

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Discipline of Surgery, The University of  
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Australia.

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The Whiteley-Martin Research Centre,  
Discipline of Surgery, The University of  
Sydney, Nepean Hospital, Level 3, Clinical  
Building, PO Box 63, Penrith 2751, NSW,  
Australia. Electronic address:  
[guy.eslick@sydney.edu.au](mailto:guy.eslick@sydney.edu.au).

## Comment in

<!--[if !supportLists]-->? <!--[endif]--  
>[Autism and vaccination: The value  
of the evidence base of a recent meta-  
analysis.](#) [Vaccine. 2015]  
<!--[if !supportLists]-->? <!--[endif]--  
>[Answers regarding the link between  
vaccines and the development of  
autism: A question of appropriate  
study design, ethics, and bias.](#)  
[Vaccine. 2015]

## Abstract

There has been enormous debate regarding the possibility of a link between childhood vaccinations and the subsequent development of autism. This has in recent times become a major public health issue with vaccine preventable diseases increasing in the community due to the fear of a 'link' between

vaccinations and autism. We performed a meta-analysis to summarise available evidence from case-control and cohort studies on this topic (MEDLINE, PubMed, EMBASE, Google Scholar up to April, 2014). Eligible studies assessed the relationship between vaccine administration and the subsequent development of autism or autism spectrum disorders (ASD). Two reviewers extracted data on study characteristics, methods, and outcomes. Disagreement was resolved by consensus with another author. Five cohort studies involving 1,256,407 children, and five case-control studies involving 9,920 children were included in this analysis. The cohort data revealed no relationship between vaccination and autism (OR: 0.99; 95% CI: 0.92 to 1.06) or ASD (OR: 0.91; 95% CI: 0.68 to 1.20), nor was there a relationship between autism and MMR (OR: 0.84; 95% CI: 0.70 to 1.01), or thimerosal (OR: 1.00; 95% CI: 0.77 to 1.31), or mercury (Hg) (OR: 1.00; 95% CI: 0.93 to 1.07). Similarly the case-control data found no evidence for increased risk of developing autism or ASD following MMR, Hg, or thimerosal exposure when grouped by condition (OR: 0.90, 95% CI: 0.83 to 0.98;  $p=0.02$ ) or grouped by exposure type (OR: 0.85, 95% CI: 0.76 to 0.95;  $p=0.01$ ). Findings of this meta-analysis suggest that vaccinations are not associated with the development of autism or autism spectrum disorder. Furthermore, the components of the vaccines (thimerosal or mercury) or multiple vaccines (MMR) are not associated with the development of autism or autism spectrum disorder.

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PMID: 24814559 [Indexed for MEDLINE]

[<image001.png>](#)



**From:** [Gordon, Joshua \(NIH/NIMH\) \[E\]](#)  
**To:** [NIMH Executive Secretariat](#)  
**Subject:** FW: 1 selected item: 24814559 - PubMed  
**Date:** Thursday, September 14, 2017 3:14:18 PM  
**Attachments:** [image001.png](#)

---

Please send this up to OD ExecSec. More communications from the Kennedy-affiliated Lawyer.

Josh

-----  
Joshua A Gordon, MD, PhD  
Director  
National Institute of Mental Health

---

**From:** Aaron Siri <aaron@sirillp.com>  
**Date:** Thursday, September 14, 2017 at 1:44 PM  
**To:** "M. Joshua Gordon" <joshua.gordon@nih.gov>  
**Cc:** "dchristensen@cdc.gov" <dchristensen@cdc.gov>, "Shapira, Stuart (CDC/ONDIEH/NCBDDD)" <cso6@CDC.GOV>, "Christensen, Deborah (Daisy) (CDC/ONDIEH/NCBDDD)" <dq3@CDC.GOV>  
**Subject:** RE: 1 selected item: 24814559 - PubMed

Good afternoon Dr. Gordon,

Thank you for your response. The information I seek is nothing more than a simple reference to one study which supports HHS's claim that the vaccines it recommends children receive in the first year of life do not cause autism. I gather from our exchange below that you are not aware of any such study. Let me know if that is incorrect.

You are the Director of the Interagency Autism Coordinating Committee (IACC) which coordinates all efforts at HHS, including at the CDC, concerning autism. The IACC's members include the CDC itself, as well as the CDC's Chief Medical Officer & Associate Director for Science (Stuart K. Shapira, M.D., Ph.D.) and the CDC's Surveillance Team Lead, Developmental Disabilities Branch (Deborah Christensen, Ph.D.) Since you state below that the support I seek is best obtained from the CDC, I have cc'd the CDC members on your committee.

I am just trying to get a copy of a study supporting HHS's claim that the vaccines it recommends in the first year of life do not cause autism. I assume you have the best intentions and I would really like to drop this issue – but, as you can appreciate, I like to rely on data/science. I am just asking for a citation to a single study supporting HHS's claim that the vaccines it recommends children receive in the first year of life do not cause autism. I would think you too, as the Director of IACC and NIMH, would be interested in seeing such a study and its underlying data.

Also, can you kindly let me know one way or another if you are interested in meeting with the aluminum adjuvant experts whose letters and CVs were previously provided regarding the potential

connection between aluminum adjuvants and autism. Again, I would think you would be interested in hearing them out. (Docs relevant to same reattached.)

Best regards,  
Aaron

p.s. Btw, your response below reminds of me of what former House representative, Dr. Dave Weldon, wrote in 2007: "When I first tasked my staff with investigating federal vaccine safety research we got a lot of confused responses and blank stares from federal officials. The FDA told us to check in with the CDC, telling us that CDC did most of the vaccine safety research. The CDC referred us over to the NIH. Then, the NIH referred us back to the CDC." Happy to send you his full statement.

---

**From:** Gordon, Joshua (NIH/NIMH) [E] [mailto:joshua.gordon@nih.gov]  
**Sent:** Friday, September 1, 2017 3:40 PM  
**To:** Aaron Siri <aaron@sirillp.com>  
**Subject:** Re: 1 selected item: 24814559 - PubMed

Dear Aaron,

I appreciate you following up with me, and apologize for the delay in my response. I think the information you are seeking would be best obtained from the CDC.

Best,

Josh

-----  
Joshua A Gordon, MD, PhD  
Director  
National Institute of Mental Health

---

**From:** Aaron Siri <aaron@sirillp.com>  
**Date:** Monday, August 14, 2017 at 4:48 PM  
**To:** "M. Joshua Gordon" <joshua.gordon@nih.gov>  
**Subject:** RE: 1 selected item: 24814559 - PubMed

Dr. Gordon,

I hope all is well.

I have not received a response to the emails below of July 10 and July 24.

The July 10 email was in response to a review you provided indicating it compared vaccinated and unvaccinated children (but which actually compares vaccinated children with vaccinated children who, at most, were missing MMR). As discussed at our meeting, I would like to see a study which supports the claim that the nearly two dozen doses of vaccines given in the first year of life (which would not include MMR and thimerosal) do not cause autism. I still await receipt of a study which supports same. Are you aware of any such study?

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---

**From:** Aaron Siri  
**Sent:** Monday, July 24, 2017 6:18 PM  
**To:** 'Gordon, Joshua (NIH/NIMH) [E]' <[joshua.gordon@nih.gov](mailto:joshua.gordon@nih.gov)>  
**Subject:** RE: 1 selected item: 24814559 - PubMed

Good evening Joshua,

As promised in my email below, I am following up regarding the research that has been conducted regarding aluminum adjuvants and neuro/psychiatric disorders, and to facilitate a meeting with you and the scientists conducting this research.

In recent years researchers have discovered that injected aluminum adjuvant travels into the brain, where it causes long term chronic inflammation, damage to neurons and behavioral abnormalities. These adverse effects occur at dosages (mcg/Kg body weight) even lower than dosages received by infants according to the CDC vaccine schedule.

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---

**From:** Aaron Siri

**Sent:** Monday, July 10, 2017 4:16 PM

**To:** 'Gordon, Joshua (NIH/NIMH) [E]' <[joshua.gordon@nih.gov](mailto:joshua.gordon@nih.gov)>

**Subject:** RE: 1 selected item: 24814559 - PubMed

Good afternoon Joshua,

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For example, the IOM stated in 2011 that there isn't a single study that supports the assertion that DTaP (injected at 2 months, 4 months, 6 months, etc.) does not cause autism, concluding that “The evidence is inadequate to accept or reject a causal relationship between diphtheria toxoid-, tetanus toxoid-, or acellular pertussis-containing vaccine and autism.” Attached is an excerpt of the discussion regarding autism and DTaP from the 2011 IOM report. (I am not aware of a single study regarding DTaP and autism that has been done since 2011.) As another example, the only study regarding Hepatitis B vaccine and autism I have located found a three-fold increase in the odds of an autism diagnosis for neonates that received the hepatitis B vaccine at birth compared that those that did not. (Gallagher CM, Goodman MS. 2010. Hepatitis B vaccination of male neonates and autism diagnosis, NHIS 1997-2002. J Toxicol Environ Health A. 73(24):1665-77.) There is simply no studies for the numerous other vaccines given to children during the first year of life with regard to their relationship

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I look forward to your response and being persuaded that the science on the question of whether vaccines cause autism really is settled.

Thanks again in advance for your time and thoughtful consideration of this issue.

Best regards,  
Aaron

p.s. I have had a number of discussions with various aluminum adjuvant experts around the globe who believe there is a connection between the aluminum adjuvants in vaccines given in large quantities during the first six months of life and autism; I hope to soon send you a write-up regarding same for your consideration.

---

**From:** Gordon, Joshua (NIH/NIMH) [E] [<mailto:joshua.gordon@nih.gov>]

**Sent:** Wednesday, May 31, 2017 4:03 PM

**To:** Aaron Siri <[aaron@sirillp.com](mailto:aaron@sirillp.com)>

**Subject:** Fwd: 1 selected item: 24814559 - PubMed

-----  
Joshua A Gordon, MD, PhD  
Director  
National Institute of Mental Health

Begin forwarded message:

**From:** Sent by NCBI <[nobody@ncbi.nlm.nih.gov](mailto:nobody@ncbi.nlm.nih.gov)>

**Date:** May 31, 2017 at 4:00:01 PM EDT

**To:** <[Joshua.gordon@nih.gov](mailto:Joshua.gordon@nih.gov)>

**Subject:** 1 selected item: 24814559 - PubMed

This message contains search results from the National Center for Biotechnology Information ([NCBI](#)) at the U.S. National Library of Medicine ([NLM](#)). Do not reply directly to this message

Sent on: Wed May 31 15:58:39 2017

1 selected item: 24814559

#### PubMed Results

Item 1 of 1 ([Display the citation in PubMed](#))

1. Vaccine. 2014 Jun 17;32(29):3623-9. doi: 10.1016/j.vaccine.2014.04.085. Epub 2014 May 9.

## Vaccines are not associated with autism: an evidence-based meta-analysis of case-control and cohort studies.

[Taylor LE](#)<sup>1</sup>, [Swerdfeger AL](#)<sup>1</sup>, [Eslick GD](#)<sup>2</sup>.

Author information:

1

The Whiteley-Martin Research Centre, Discipline of Surgery, The University of Sydney, Nepean Hospital, Level 3, Clinical Building, PO Box 63, Penrith 2751, NSW, Australia.

2

The Whiteley-Martin Research Centre, Discipline of Surgery, The University of Sydney, Nepean Hospital, Level 3, Clinical Building, PO Box 63, Penrith 2751, NSW, Australia. Electronic address: [guy.eslick@sydney.edu.au](mailto:guy.eslick@sydney.edu.au).

### Comment in

- [Autism and vaccination: The value of the evidence base of a](#)

- [recent meta-analysis](#). [Vaccine. 2015]  
• [Answers regarding the link between vaccines and the development of autism: A question of appropriate study design, ethics, and bias](#). [Vaccine. 2015]

## Abstract

There has been enormous debate regarding the possibility of a link between childhood vaccinations and the subsequent development of autism. This has in recent times become a major public health issue with vaccine preventable diseases increasing in the community due to the fear of a 'link' between vaccinations and autism. We performed a meta-analysis to summarise available evidence from case-control and cohort studies on this topic (MEDLINE, PubMed, EMBASE, Google Scholar up to April, 2014). Eligible studies assessed the relationship between vaccine administration and the subsequent development of autism or autism spectrum disorders (ASD). Two reviewers extracted data on study characteristics, methods, and outcomes. Disagreement was resolved by consensus with another author. Five cohort studies involving 1,256,407 children, and five case-control studies involving 9,920 children were included in this analysis. The cohort data revealed no relationship between vaccination and autism (OR: 0.99; 95% CI: 0.92 to 1.06) or ASD (OR: 0.91; 95% CI: 0.68 to 1.20), nor was there a relationship between autism and MMR (OR: 0.84; 95% CI: 0.70 to 1.01), or thimerosal (OR: 1.00; 95% CI: 0.77 to 1.31), or mercury (Hg) (OR: 1.00; 95% CI: 0.93 to 1.07). Similarly the case-control data found no evidence for increased risk of developing autism or ASD following MMR, Hg, or thimerosal exposure when grouped by condition (OR: 0.90, 95% CI: 0.83 to 0.98;  $p=0.02$ ) or grouped by exposure type (OR: 0.85, 95% CI: 0.76 to 0.95;  $p=0.01$ ). Findings of this meta-analysis suggest that vaccinations are not associated with the development of autism or autism spectrum disorder. Furthermore, the components of the vaccines (thimerosal or mercury) or multiple vaccines (MMR) are not associated with the development of autism or autism spectrum disorder.

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PMID: 24814559 [Indexed for MEDLINE]



From: [Gordon, Joshua \(NIH/NIMH\) \[E\]](#)  
To: [NIMH Executive Secretariat](#)  
Subject: Fwd: 1 selected item: 24814559 - PubMed  
Date: Monday, November 13, 2017 4:10:00 PM  
Attachments: [image002.png](#)  
[ATT00001.htm](#)  
[Dr. Gordon Response.pdf](#)  
[ATT00002.htm](#)  
[DTaP-Autism - 2011 IOM Report.pdf](#)  
[ATT00003.htm](#)

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Joshua A Gordon, MD, PhD  
Director  
National Institute of Mental Health

Begin forwarded message:

**From:** Aaron Siri <[aaron@sirillp.com](mailto:aaron@sirillp.com)>  
**Date:** November 13, 2017 at 3:27:39 PM EST  
**To:** "Gordon, Joshua (NIH/NIMH) [E]" <[joshua.gordon@nih.gov](mailto:joshua.gordon@nih.gov)>  
**Cc:** "[dchristensen@cdc.gov](mailto:dchristensen@cdc.gov)" <[dchristensen@cdc.gov](mailto:dchristensen@cdc.gov)>, "[cs06@cdc.gov](mailto:cs06@cdc.gov)" <[cs06@cdc.gov](mailto:cs06@cdc.gov)>, "[dqc3@cdc.gov](mailto:dqc3@cdc.gov)" <[dqc3@cdc.gov](mailto:dqc3@cdc.gov)>  
**Subject:** RE: 1 selected item: 24814559 - PubMed

Good afternoon Dr. Gordon,

I received the attached email from HHS which I assume is your official response to the simple request to provide at least one study which supports that DTaP and the other vaccines HHS recommends during the first year of life do not cause autism.

This response provides a link to the HHS webpage which claims "Vaccines Do Not Cause Autism" and lists a number of reviews/studies to support this assertion. Sadly, not a single one of these reviews/studies (which all related to either one vaccine, MMR, and/or one vaccine ingredient, thimerosal) provides a shred of support that the 29 doses of 9 different vaccines CDC recommends children receive by six months of age do not cause autism. Ironically, the very first study/review listed on this webpage is the 2011 IOM report, paid for by HHS, which looked at the most commonly claimed vaccine reactions, including that DTaP causes autism, and the IOM could not find a single study that supports the assertion that DTaP (injected at 2 months, 4 months, 6 months, etc.) does not cause autism. (See excerpt from the IOM report attached.) Your response therefore makes it clear you do not have a single study to share which supports that the vaccines given to children in the first year of life do not cause autism.

It is understandable that you thought the study you sent me below actually contained unexposed controls (unvaccinated children) given its misleading title. But now that you



know the reality that there is no study supporting the claim that vaccines given during the first year of life do not contribute to the incidence of autism, are you going to take action to conduct an appropriate study that would either support or reject this claim?

I understand this is a difficult and controversial topic but I hope the National Institute of Mental Health and the IACC do not shy away from a scientific study because of fear of what it may show. There are a number of plausible reasons for how 29 doses of 9 different vaccines given during pregnancy, 1 day, 2 months, 4 months, and 6 months can cause autism, including immune activation, aluminum adjuvant being carried to the brain by macrophages, MCP-1 signaling, molecular mimicry, etc. Vaccines are intended to create a permanent change in the body's immune system often using adjuvants intended to generate a sustained and significant immune event which modern science is not even close to fully understanding; there is also a growing understanding of the connections between the immune and nervous systems. But no need to make this complicated since all you need to do is what is done for every drug pre-licensure. Compare the rates of neurological and immune disorders between an exposed group (vaccinated) and unexposed group (unvaccinated) – this study can even be done retrospectively to avoid supposed ethical concerns.

You are in the unfortunate position of defending vaccine safety because, unlike drugs, most pediatric vaccines currently on the market have been approved based on studies with inadequate follow-up periods of only a few days or weeks (and no saline placebo control). You however are in the fortunate position to remedy this deficiency. In that regard, I have attempted as best as I can to engage with you in a constructive manner on this topic, giving you many months since our meeting to provide the support you were adamant existed during our meeting (a study of vaccinated versus unvaccinated children). Absent a response in the coming days with such support or firm plans to openly conduct such a study, I am left with the conclusion that you (directly or by order of your superiors) don't care to know the real answer to the question of whether giving 29 doses of 9 different vaccines by six months of life contributes to the incidence of autism (and other neurological and immune issues).

Dr. Collins asked during our meeting to consider the implications if Mr. Kennedy was wrong about his concerns regarding vaccine safety. Given your station, I ask you the same question. What if you are wrong about the safety profile of the first year vaccination schedule? What if it is a major contributor to the rising incidence of various neurological and immune (including immune mediated neurological) disorders that have risen in tandem with the increase in HHS's recommended vaccine schedule. If you conduct the desperately needed vaccine safety science noted above the worst that will have happened is that you will have the science to prove what you now can only assume. However, if you don't conduct this study and it eventually turns out your belief (and this email chain makes clear it is a belief) regarding vaccine safety is incorrect, I hope you can live with knowing you could have avoided these harms (and provided the basis to finally begin the desperately needed science of identifying the children susceptible to serious vaccine injury) but chose instead to sit on your hands...

Very truly yours,  
Aaron

---

**From:** Aaron Siri  
**Sent:** Thursday, September 14, 2017 1:41 PM  
**To:** 'Gordon, Joshua (NIH/NIMH) [E]' <[joshua.gordon@nih.gov](mailto:joshua.gordon@nih.gov)>  
**Cc:** '[dchristensen@cdc.gov](mailto:dchristensen@cdc.gov)' <[dchristensen@cdc.gov](mailto:dchristensen@cdc.gov)>; '[cso6@cdc.gov](mailto:cso6@cdc.gov)' <[cso6@cdc.gov](mailto:cso6@cdc.gov)>; '[dqc3@cdc.gov](mailto:dqc3@cdc.gov)' <[dqc3@cdc.gov](mailto:dqc3@cdc.gov)>  
**Subject:** RE: 1 selected item: 24814559 - PubMed

Good afternoon Dr. Gordon,

Thank you for your response. The information I seek is nothing more than a simple reference to one study which supports HHS's claim that the vaccines it recommends children receive in the first year of life do not cause autism. I gather from our exchange below that you are not aware of any such study. Let me know if that is incorrect.

You are the Director of the Interagency Autism Coordinating Committee (IACC) which coordinates all efforts at HHS, including at the CDC, concerning autism. The IACC's members include the CDC itself, as well as the CDC's Chief Medical Officer & Associate Director for Science (Stuart K. Shapira, M.D., Ph.D.) and the CDC's Surveillance Team Lead, Developmental Disabilities Branch (Deborah Christensen, Ph.D.) Since you state below that the support I seek is best obtained from the CDC, I have cc'd the CDC members on your committee.

I am just trying to get a copy of a study supporting HHS's claim that the vaccines it recommends in the first year of life do not cause autism. I assume you have the best intentions and I would really like to drop this issue – but, as you can appreciate, I like to rely on data/science. I am just asking for a citation to a single study supporting HHS's claim that the vaccines it recommends children receive in the first year of life do not cause autism. I would think you too, as the Director of IACC and NIMH, would be interested in seeing such a study and its underlying data.

Also, can you kindly let me know one way or another if you are interested in meeting with the aluminum adjuvant experts whose letters and CVs were previously provided regarding the potential connection between aluminum adjuvants and autism. Again, I would think you would be interested in hearing them out. (Docs relevant to same reattached.)

Best regards,  
Aaron

p.s. Btw, your response below reminds of me of what former House representative, Dr.

Dave Weldon, wrote in 2007: "When I first tasked my staff with investigating federal vaccine safety research we got a lot of confused responses and blank stares from federal officials. The FDA told us to check in with the CDC, telling us that CDC did most of the vaccine safety research. The CDC referred us over to the NIH. Then, the NIH referred us back to the CDC." Happy to send you his full statement.

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**From:** Gordon, Joshua (NIH/NIMH) [E] [<mailto:joshua.gordon@nih.gov>]

**Sent:** Friday, September 1, 2017 3:40 PM

**To:** Aaron Siri <[aaron@sirillp.com](mailto:aaron@sirillp.com)>

**Subject:** Re: 1 selected item: 24814559 - PubMed

Dear Aaron,

I appreciate you following up with me, and apologize for the delay in my response. I think the information you are seeking would be best obtained from the CDC.

Best,

Josh

-----  
Joshua A Gordon, MD, PhD  
Director  
National Institute of Mental Health

---

**From:** Aaron Siri <[aaron@sirillp.com](mailto:aaron@sirillp.com)>

**Date:** Monday, August 14, 2017 at 4:48 PM

**To:** "M. Joshua Gordon" <[joshua.gordon@nih.gov](mailto:joshua.gordon@nih.gov)>

**Subject:** RE: 1 selected item: 24814559 - PubMed

Dr. Gordon,

I hope all is well.

I have not received a response to the emails below of July 10 and July 24.

The July 10 email was in response to a review you provided indicating it compared vaccinated and unvaccinated children (but which actually compares vaccinated children with vaccinated children who, at most, were missing MMR). As discussed at our meeting, I would like to see a study which supports the claim that the nearly two dozen doses of vaccines given in the first year of life (which would not include MMR and thimerosal) do not cause autism. I still await receipt of a study which supports same.

Are you aware of any such study?

The July 24 email elaborated on my prior email and also sought to facilitate a meeting between with various experts in the field of aluminum adjuvant that do believe there is a connection between aluminum adjuvant in vaccines and autism. Are you willing to have this meeting?

Best regards,  
Aaron

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**From:** Aaron Siri  
**Sent:** Monday, July 24, 2017 6:18 PM  
**To:** 'Gordon, Joshua (NIH/NIMH) [E]' <[joshua.gordon@nih.gov](mailto:joshua.gordon@nih.gov)>  
**Subject:** RE: 1 selected item: 24814559 - PubMed

Good evening Joshua,

As promised in my email below, I am following up regarding the research that has been conducted regarding aluminum adjuvants and neuro/psychiatric disorders, and to facilitate a meeting with you and the scientists conducting this research.

In recent years researchers have discovered that injected aluminum adjuvant travels into the brain, where it causes long term chronic inflammation, damage to neurons and behavioral abnormalities. These adverse effects occur at dosages (mcg/Kg body weight) even lower than dosages received by infants according to the CDC vaccine schedule.

Additionally, it is now well established that autism and other neuro/psychiatric disorders are caused by early life inflammation (i.e. elevated cytokines) in the brain. I have seen your published papers on immune activation and brain development so I presume you are aware of the immune activation findings. Aluminum adjuvant can cause chronic brain inflammation, and this establishes a biologically-plausible and empirically-supported mechanism for how vaccines may cause autism and other neurological disorders. None of the vaccine-autism studies to date tell us anything about the safety of aluminum adjuvants. There are no epidemiological studies showing that aluminum adjuvants do not produce these effects in humans.

Attached is a detailed explanation of the proposed mechanism for how aluminum adjuvants may cause autism. The mechanism suggests that aluminum adjuvant may cause other brain and neurodevelopmental disorders as well. Attached are also supporting letters from experts in the fields of aluminum toxicity. (Finally, I have also attached a more detailed analysis of Taylor 2014.)

I invite you to consider the arguments in the attached document and respond with your observations. I also invite you to share the document with colleagues, particularly if they may have insightful comments or rebuttals.

I also hope to facilitate a meeting with you and a number of the experts studying aluminum adjuvant toxicity, letters from a number of which are attached to this email. Assuming you are open to having this discussion, kindly have your office provide suggested dates/times for such a meeting.

Best regards,  
Aaron

---

**From:** Aaron Siri  
**Sent:** Monday, July 10, 2017 4:16 PM  
**To:** 'Gordon, Joshua (NIH/NIMH) [E]'; <[joshua.gordon@nih.gov](mailto:joshua.gordon@nih.gov)>  
**Subject:** RE: 1 selected item: 24814559 - PubMed

Good afternoon Joshua,

Thank you for sending me the below abstract/review article and it was great meeting at NIH. Really appreciate the opportunity to dialogue on the issue of vaccines and autism.

The abstract/review article you sent me below highlights the concern raised that there has never been a study assessing the relative risk of autism between vaccinated and unvaccinated child. To be sure, this review (and its abstract) leave the impression that the studies it relies upon compare “unvaccinated” children (no vaccines) with vaccinated children. Unfortunately, this is misleading since all 10 of the underlying studies relied upon for this review compared highly vaccinated children with highly vaccinated children. The only difference typically between the study and control groups was a single MMR vaccine or thimerosal vs. non-thimerosal vaccines. (I would be happy to provide you with a breakdown of each of the 10 studies reflecting same.) Meaning, what this review considers “unvaccinated” are vaccinated children typically only missing the MMR vaccine. Assuming the control children in these studies followed the current CDC recommended vaccination schedule, they would each have received 21 vaccine injections during the first 12 months of life excluding the MMR vaccine. Hence, these studies tell us virtually nothing about the relationship of vaccines to autism because they are not comparing vaccinated and unvaccinated children.

For example, the IOM stated in 2011 that there isn't a single study that supports the assertion that DTaP (injected at 2 months, 4 months, 6 months, etc.) does not cause autism, concluding that “The evidence is inadequate to accept or reject a causal relationship between diphtheria toxoid-, tetanus toxoid-, or acellular pertussis-containing vaccine and autism.” Attached is an excerpt of the discussion regarding autism and DTaP from the 2011 IOM report. (I am not aware of a single study regarding DTaP and autism that has been done since 2011.) As another example, the only study regarding Hepatitis B vaccine and autism I have located found a three-fold increase in the odds of an autism diagnosis for neonates that received the hepatitis B vaccine at birth compared that those that did not. (Gallagher CM, Goodman MS. 2010. Hepatitis B vaccination of male neonates and autism diagnosis, NHIS 1997-2002. J Toxicol Environ

Health A. 73(24):1665-77.) There is simply no studies for the numerous other vaccines given to children during the first year of life with regard to their relationship with autism (except for the Mawson study which showed vaccination had an over 4 fold increase in autism risk but that study has some serious limitations).

As we discussed at the meeting, I really am open to seeing the evidence that the vaccination schedule, and in particular the cumulative impact of the 31 vaccine doses the CDC recommends a child receive in the first year of life, are not casually related to autism. I would gladly share that support with the community concerned with this issue with my personal endorsement. On the other hand, if that proof doesn't exist, that does not mean that vaccines cause autism. It just means that we need to really do the science necessary to rule out that possibility. (Seeking to assess the health outcomes of those receiving vaccines and those not receiving vaccines really is asking for nothing more than how all drugs are safety tested prior to licensure.)

I respected what appeared to be your thoughtful rather than reflexive reaction to the spirited discussion at NIH. Conducting a true study of the health outcomes between actually unvaccinated and vaccinated children (at least an initial quick and easy retrospective study) that shows no connection with autism should be something that everyone should want. If it shows no connection, it will likely provide the greatest relief to the portion of the autism community that thinks there may be a connection. Parents who think that it was their actions, in vaccinating their children, that lead to their child's condition would feel freed from that guilt by knowing it wasn't the vaccines.

I look forward to your response and being persuaded that the science on the question of whether vaccines cause autism really is settled.

Thanks again in advance for your time and thoughtful consideration of this issue.

Best regards,  
Aaron

p.s. I have had a number of discussions with various aluminum adjuvant experts around the globe who believe there is a connection between the aluminum adjuvants in vaccines given in large quantities during the first six months of life and autism; I hope to soon send you a write-up regarding same for your consideration.

---

**From:** Gordon, Joshua (NIH/NIMH) [E] [<mailto:joshua.gordon@nih.gov>]

**Sent:** Wednesday, May 31, 2017 4:03 PM

**To:** Aaron Siri <[aaron@sirillp.com](mailto:aaron@sirillp.com)>

**Subject:** Fwd: 1 selected item: 24814559 - PubMed

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Joshua A Gordon, MD, PhD  
Director  
National Institute of Mental Health

Begin forwarded message:

**From:** Sent by NCBI <[nobody@ncbi.nlm.nih.gov](mailto:nobody@ncbi.nlm.nih.gov)>  
**Date:** May 31, 2017 at 4:00:01 PM EDT  
**To:** <[Joshua.gordon@nih.gov](mailto:Joshua.gordon@nih.gov)>  
**Subject:** 1 selected item: 24814559 - PubMed

This message contains search results from the National Center for Biotechnology Information (NCBI) at the U.S. National Library of Medicine (NLM). Do not reply directly to this message

Sent on: Wed May 31 15:58:39 2017

1 selected item: 24814559

#### PubMed Results

Item 1 of 1 ([Display the citation in PubMed](#))

1. Vaccine. 2014 Jun 17;32(29):3623-9. doi:  
10.1016/j.vaccine.2014.04.085. Epub 2014 May 9.

## Vaccines are not associated with autism: an evidence- based meta-analysis of case- control and cohort studies.

[Taylor LE](#)<sup>1</sup>, [Swerdfeger AL](#)<sup>1</sup>, [Eslick GD](#)<sup>2</sup>.

Author information:

1

The Whiteley-Martin Research Centre, Discipline of Surgery, The University of Sydney, Nepean Hospital, Level 3, Clinical Building, PO Box 63, Penrith 2751, NSW, Australia.

2

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NSW, Australia. Electronic address:  
[guy.eslick@sydney.edu.au](mailto:guy.eslick@sydney.edu.au).

## Comment in

<!--[if !supportLists]-->• <!--[endif]-->[Autism and vaccination: The value of the evidence base of a recent meta-analysis.](#) [Vaccine. 2015]  
<!--[if !supportLists]-->• <!--[endif]-->[Answers regarding the link between vaccines and the development of autism: A question of appropriate study design, ethics, and bias.](#) [Vaccine. 2015]

## Abstract

There has been enormous debate regarding the possibility of a link between childhood vaccinations and the subsequent development of autism. This has in recent times become a major public health issue with vaccine preventable diseases increasing in the community due to the fear of a 'link' between vaccinations and autism. We performed a meta-analysis to summarise available evidence from case-control and cohort studies on this topic (MEDLINE, PubMed, EMBASE, Google Scholar up to April, 2014). Eligible studies assessed the relationship between vaccine administration and the subsequent development of autism or autism spectrum disorders (ASD). Two reviewers extracted data on study characteristics, methods, and outcomes. Disagreement was resolved by consensus with another author. Five cohort studies involving 1,256,407 children, and five case-control studies involving 9,920 children were included in this analysis. The cohort data revealed no relationship between vaccination and autism (OR: 0.99; 95% CI: 0.92 to 1.06) or ASD (OR: 0.91; 95% CI: 0.68 to 1.20), nor was there a relationship between autism and MMR (OR: 0.84; 95% CI: 0.70 to 1.01), or thimerosal (OR: 1.00; 95% CI: 0.77 to 1.31), or mercury (Hg) (OR: 1.00; 95% CI: 0.93 to 1.07). Similarly the case-control data found no evidence for increased risk of developing autism or ASD following MMR, Hg, or thimerosal exposure when grouped by condition (OR: 0.90, 95% CI: 0.83 to 0.98;  $p=0.02$ ) or grouped by exposure type (OR: 0.85, 95% CI: 0.76 to 0.95;  $p=0.01$ ). Findings of this meta-analysis suggest that vaccinations are not associated with the development of autism or autism spectrum disorder. Furthermore, the components of the vaccines (thimerosal or mercury) or multiple vaccines (MMR) are not



associated with the development of autism or autism spectrum disorder.

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PMID: 24814559 [Indexed for MEDLINE]

## Aaron Siri

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**From:** CDCExecSec (CDC) <CDCExecSec@cdc.gov>  
**Sent:** Wednesday, October 25, 2017 8:19 AM  
**To:** Aaron Siri  
**Subject:** Vaccine Inquiry

Dear Mr. Siri:

Thank you for your inquiry. The Centers for Disease Control and Prevention (CDC) information on vaccines and autism can be found here, [www.cdc.gov/vaccinesafety/concerns/autism.html](http://www.cdc.gov/vaccinesafety/concerns/autism.html).

Please send any future correspondence to [CDCExecSec@cdc.gov](mailto:CDCExecSec@cdc.gov).

Sincerely,

Sandra Cashman, MS  
Executive Secretary  
Office of the Chief of Staff, CDC

# Adverse Effects of Vaccines

## Evidence and Causality

Committee to Review Adverse Effects of Vaccines

Board on Population Health and Public Health Practice

Kathleen Stratton, Andrew Ford, Erin Rusch, and Ellen Wright Clayton,  
*Editors*

INSTITUTE OF MEDICINE  
*OF THE NATIONAL ACADEMIES*

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*Weight of Epidemiologic Evidence*

*The epidemiologic evidence is insufficient or absent to assess an association between diphtheria toxoid-, tetanus toxoid-, or acellular pertussis-containing vaccine and ataxia.*

**Mechanistic Evidence**

The committee identified one publication reporting the development of ataxia after the administration of DTaP vaccine. Kubota and Takahashi (2008) did not provide evidence of causality beyond a temporal relationship of 2 days between vaccine administration and development of cerebellar symptoms leading to a diagnosis of acute cerebellar ataxia. The publication did not contribute to the weight of mechanistic evidence.

*Weight of Mechanistic Evidence*

*The committee assesses the mechanistic evidence regarding an association between diphtheria toxoid-, tetanus toxoid-, or acellular pertussis-containing vaccine and ataxia as lacking.*

**Causality Conclusion**

**Conclusion 10.5:** The evidence is inadequate to accept or reject a causal relationship between diphtheria toxoid-, tetanus toxoid-, or acellular pertussis-containing vaccine and ataxia.

**AUTISM****Epidemiologic Evidence**

The committee reviewed one study to evaluate the risk of autism after the administration of DTaP vaccine. This one study (Geier and Geier, 2004) was not considered in the weight of epidemiologic evidence because it provided data from a passive surveillance system and lacked an unvaccinated comparison population.

*Weight of Epidemiologic Evidence*

*The epidemiologic evidence is insufficient or absent to assess an association between diphtheria toxoid-, tetanus toxoid-, or acellular pertussis-containing vaccine and autism.*

### Mechanistic Evidence

The committee did not identify literature reporting clinical, diagnostic, or experimental evidence of autism after the administration of vaccines containing diphtheria toxoid, tetanus toxoid, and acellular pertussis antigens alone or in combination.

#### *Weight of Mechanistic Evidence*

*The committee assesses the mechanistic evidence regarding an association between diphtheria toxoid–, tetanus toxoid–, or acellular pertussis–containing vaccine and autism as lacking.*

### Causality Conclusion

**Conclusion 10.6:** The evidence is inadequate to accept or reject a causal relationship between diphtheria toxoid–, tetanus toxoid–, or acellular pertussis–containing vaccine and autism.

## ACUTE DISSEMINATED ENCEPHALOMYELITIS

### Epidemiologic Evidence

No studies were identified in the literature for the committee to evaluate the risk of acute disseminated encephalomyelitis (ADEM) after the administration of vaccines containing diphtheria toxoid, tetanus toxoid, or acellular pertussis antigens alone or in combination.

#### *Weight of Epidemiologic Evidence*

*The epidemiologic evidence is insufficient or absent to assess an association between diphtheria toxoid–, tetanus toxoid–, or acellular pertussis–containing vaccines and ADEM.*

### Mechanistic Evidence

The committee identified five publications of ADEM developing after the administration of vaccines containing diphtheria toxoid and tetanus toxoid antigens alone or in combination. Four publications did not provide evidence beyond temporality, one of which was deemed too short based on the possible mechanisms involved (Abdul-Ghaffar and Achar, 1994; Bolukbasi and Ozmenoglu, 1999; Hamidon and Raymond, 2003; Rogalewski et al., 2007). In addition, Rogalewski et al. (2007) reported the administration of vaccines against hepatitis B, hepatitis A, and poliovirus in

From: [Aaron Siri](#)  
To: [Gordon, Joshua \(NIH/NIMH\) \[E\]](#); [Chris Shaw; Birnbaum, Linda \(NIH/NIEHS\) \[E\]](#)  
Cc: [Bianchi, Diana \(NIH/NICHD\) \[E\]](#)  
Subject: RE: 1 selected item: 24814559 - PubMed  
Date: Tuesday, May 1, 2018 2:39:40 PM  
Attachments: Gordon letter (1).docx  
aluminum adjuvant file.pdf

---

Dear Dr. Birnbaum,

It was a pleasure meeting at NIH. As you may recall, I briefly mentioned to you the potential issues with aluminum adjuvants. Dr. Gordon stated below that NIEHS is very interested in the role of metals and autism. In that regard, I am pleased to introduce to you Dr. Shaw, who is cc'd on this email, and attach a letter from him and the studies he compiled regarding the potential negative impact of aluminum adjuvants on the developing CNS.

I would also like to separately provide you a link to a more "mainstream" article that discusses the connection between aluminum adjuvants and autism: <http://icandecide.org/white-papers/ICAN-AluminumAdjuvant-Autism.pdf>

Best regards,  
Aaron

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**From:** Gordon, Joshua (NIH/NIMH) [E] [<mailto:joshua.gordon@nih.gov>]  
**Sent:** Tuesday, May 1, 2018 1:36 PM  
**To:** Aaron Siri <[aaron@sirillp.com](mailto:aaron@sirillp.com)>; Chris Shaw <[cashawlab@gmail.com](mailto:cashawlab@gmail.com)>  
**Cc:** Bianchi, Diana (NIH/NICHD) [E] <[diana.bianchi@nih.gov](mailto:diana.bianchi@nih.gov)>  
**Subject:** Re: 1 selected item: 24814559 - PubMed

Oh, my apologies.

Dr. Shaw, please see below. IN particular, NIEHS is very interested in the role of metals and autism. I don't know what they've already funded in the area of aluminum but it is worth enquiring there perhaps first.

Best

Josh

-----  
Joshua A Gordon, MD, PhD  
Director  
National Institute of Mental Health

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**From:** Aaron Siri <[aaron@sirillp.com](mailto:aaron@sirillp.com)>

**Date:** Tuesday, May 1, 2018 at 1:34 PM

**To:** "M. Joshua Gordon" <[joshua.gordon@nih.gov](mailto:joshua.gordon@nih.gov)>, Chris Shaw <[cashawlab@gmail.com](mailto:cashawlab@gmail.com)>

**Cc:** "Bianchi, Diana (NIH/NICHD) [E]" <[diana.bianchi@nih.gov](mailto:diana.bianchi@nih.gov)>

**Subject:** RE: 1 selected item: 24814559 - PubMed

Dr. Gordon, I think you are thinking about Dr. Exley. This is the first time you have communicated with Dr. Shaw.

---

**From:** Gordon, Joshua (NIH/NIMH) [E] [<mailto:joshua.gordon@nih.gov>]

**Sent:** Tuesday, May 1, 2018 1:31 PM

**To:** Aaron Siri <[aaron@sirillp.com](mailto:aaron@sirillp.com)>; Chris Shaw <[cashawlab@gmail.com](mailto:cashawlab@gmail.com)>

**Cc:** Bianchi, Diana (NIH/NICHD) [E] <[diana.bianchi@nih.gov](mailto:diana.bianchi@nih.gov)>

**Subject:** Re: 1 selected item: 24814559 - PubMed

AS I have mentioned to Dr. Shaw and others, he is welcome to submit grants in this area. NIMH, NIEHS, and NICHD would each consider such grants and he would be welcome to contact relevant program staff for technical assistance.

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Joshua A Gordon, MD, PhD  
Director  
National Institute of Mental Health

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**From:** Aaron Siri <[aaron@sirillp.com](mailto:aaron@sirillp.com)>

**Date:** Tuesday, May 1, 2018 at 1:06 PM

**To:** "M. Joshua Gordon" <[joshua.gordon@nih.gov](mailto:joshua.gordon@nih.gov)>, Chris Shaw <[cashawlab@gmail.com](mailto:cashawlab@gmail.com)>

**Cc:** "Bianchi, Diana (NIH/NICHD) [E]" <[diana.bianchi@nih.gov](mailto:diana.bianchi@nih.gov)>

**Subject:** RE: 1 selected item: 24814559 - PubMed

Dr. Gordon,

Further to my last email below, attached is a letter from Dr. Shaw and the primary studies he compiled regarding the potential negative impact of aluminum adjuvants on the developing CNS. He requested I forward both to you directly.

Best regards,  
Aaron

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**From:** Aaron Siri

**Sent:** Thursday, March 22, 2018 3:55 PM

**To:** 'Gordon, Joshua (NIH/NIMH) [E]' <[joshua.gordon@nih.gov](mailto:joshua.gordon@nih.gov)>; 'Chris Shaw' <[cashawlab@gmail.com](mailto:cashawlab@gmail.com)>

**Cc:** Bianchi, Diana (NIH/NICHD) [E] <[diana.bianchi@nih.gov](mailto:diana.bianchi@nih.gov)>

**Subject:** RE: 1 selected item: 24814559 - PubMed

Good afternoon Dr. Gordon,

I hoped for a more constructive exchange with Dr. Exley and trust that, since American babies are typically injected with over 3,500 micrograms of alum adjuvant by six months of age (including appx. 245 mcg/kg of body weight at 2 months of age) and animal models have shown injected alum adjuvants travel to the brain, there continues to be a shared interest in generating the science needed to support the safety of this practice.

In that regard -- as you are probably one of the few people in the world with access to the resources to undertake such a study -- would NIH be willing to compare the aluminum deposits (both location and quantity) in brains of ASD versus healthy children that have died prematurely for non-medical reasons?

In order to help understand the importance of this study, I have added Dr. Chris Shaw (Faculty of Medicine, University of British Columbia) to this chain who has conducted studies in which newborn lab animals were injected with the proportionate amount of alum adjuvant given to newborn humans and found that the alum traveled to the brain of the lab animals and that the lab animals exhibited developmental and social deficits (as compared to controls), including features which resemble ASD. I am certain Dr. Shaw would gladly email you some of these studies (as a follow-up to this email) and hopefully a short constructive and friendly exchange with Dr. Shaw will help place in context why it is important to understand the safety profile of alum adjuvants injected into babies during critical brain development stages.

Best regards,  
Aaron

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**From:** Gordon, Joshua (NIH/NIMH) [E] [<mailto:joshua.gordon@nih.gov>]  
**Sent:** Wednesday, March 21, 2018 1:54 PM  
**To:** Christopher Exley <[c.exley@keele.ac.uk](mailto:c.exley@keele.ac.uk)>  
**Cc:** Aaron Siri <[aaron@sirillp.com](mailto:aaron@sirillp.com)>; Bianchi, Diana (NIH/NICHD) [E] <[diana.bianchi@nih.gov](mailto:diana.bianchi@nih.gov)>  
**Subject:** Re: 1 selected item: 24814559 - PubMed

I refer you to our NeuroBioBank:

<https://neurobiobank.nih.gov>

Josh

-----  
Joshua A Gordon, MD, PhD  
Director  
National Institute of Mental Health

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**From:** Christopher Exley <[c.exley@keele.ac.uk](mailto:c.exley@keele.ac.uk)>



**Date:** Wednesday, March 21, 2018 at 1:38 PM

**To:** "M. Joshua Gordon" <[joshua.gordon@nih.gov](mailto:joshua.gordon@nih.gov)>

**Cc:** Aaron Siri <[aaron@sirillp.com](mailto:aaron@sirillp.com)>, "Bianchi, Diana (NIH/NICHD) [E]" <[diana.bianchi@nih.gov](mailto:diana.bianchi@nih.gov)>

**Subject:** Re: 1 selected item: 24814559 - PubMed

Hope is one thing but there are no such control brain tissues in the UK. I think you will find that the ethical approval processes in the UK are at least as stringent as they are in the US.

However, if you can get such from the US and ship them to me then I would be very grateful. We very much want to do these measurements.

Regarding the microscopy. We have looked at and identified aluminium in many human brains including individuals with AD, fAD, MS and some older controls and we have never seen the same distribution and location of Al as we saw in ALL 10 autism brains. This is a unique and standout observation for ASD.

Best wishes

Chris

On 21 March 2018 at 17:27, Gordon, Joshua (NIH/NIMH) [E] <[joshua.gordon@nih.gov](mailto:joshua.gordon@nih.gov)> wrote:

Ad hominem simply means directed at the person rather than the subject matter. I believe my comments made to Mr. Siri were reflective of the content of the paper rather than its authors.

NIMH has several brain banks where I would hope appropriate control subjects could be found, if you wish to request them. So does the Simons Foundation collection. But yes, controls are necessary, regardless of the rigor with which the experimental group was treated. And NIMH and others have clear guidelines about the inclusion of controls, sample size calculations, and other such issues.

Thank you for providing your response to the concerns raised by another scientist. While it may indeed be surprising that you found intracellular aluminum in the autism subjects, and that may increase your confidence in your own work, without parallel treatment of control tissue you cannot expect others to be similarly convinced. Unfortunately, history is not your side, as the psychiatric disease literature is rife with similar rigorously conducted but uncontrolled studies that subsequently fail to replicate.

I do not mean to suggest that the topic is not worthy of your continued pursuit. If you remain confident in your results then I respectfully suggest you consider a well-powered, appropriately controlled study on the matter.

Josh

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Joshua A Gordon, MD, PhD  
Director  
National Institute of Mental Health

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**From:** Christopher Exley <[c.exley@keele.ac.uk](mailto:c.exley@keele.ac.uk)>  
**Date:** Wednesday, March 21, 2018 at 12:52 PM  
**To:** "M. Joshua Gordon" <[joshua.gordon@nih.gov](mailto:joshua.gordon@nih.gov)>  
**Cc:** Aaron Siri <[aaron@sirillp.com](mailto:aaron@sirillp.com)>, "Bianchi, Diana (NIH/NICHD) [E]" <[diana.bianchi@nih.gov](mailto:diana.bianchi@nih.gov)>, Dirk Schaumlöffel <[dirk.schaumloeffel@univ-pau.fr](mailto:dirk.schaumloeffel@univ-pau.fr)>, Tanja Schwerdtle <[taschwer@uni-potsdam.de](mailto:taschwer@uni-potsdam.de)>  
**Subject:** Re: 1 selected item: 24814559 - PubMed

You make me laugh! Ad hominem attacks!! Your comments on our research and the journal it was published in were perfectly reasonable in their nature, of course!

So, only now do you think to ask this question? Perhaps you might have guessed that it was also brought up during the process of peer review.

What if I told you that tissue for 'non-affected control subjects' was not available through the Oxford Autism Brain Bank (or anywhere else within the UK). Would that mean that the research shouldn't be carried out?

An inquiring and respectful scientist did write to ask me a similar question and I am pasting my reply to him below for your information.

Best wishes

Chris

When the subject of your research is aluminium and human health you expect rigorous peer review upon submission of a manuscript. Actually many journals do not review the 'science' only the 'subject' and return your manuscript without the opportunity of peer review. Indeed even when your research is sent for peer review, anonymous reviewers also simply review the subject and not the science.

In many ways I consider our recent research on aluminium and autism to be some of our strongest research (out of approaching 200 peer-reviewed scientific publications). It is certainly some of our most unequivocal and worrying in terms of what we have found. Please do note that we do not work on autism, we do not work on vaccines, we work on aluminium.

The autism research took about 2 years to complete, not including obtaining ethical review and the tissues through the autism brain bank.

The ABB, part of the Oxford Brain Bank, only has brain tissue from 10 donors. They only have frozen tissue for 5 donors. Quantitative analyses for AI require frozen tissue. They had fixed tissue (extremely

limited as indicated in the paper) for 10 donors.

So, we had access to all the autism brain tissue available in the UK.

As part of ethical review and approval we had to consider appropriate control brain tissues. The Oxford Brain Bank were not able to provide age-matched controls (they identified 5 donors with an average age of about 50). In addition none of this group were appropriate controls as they all died of some form of condition, disease or mental illness. We needed age-matched controls who were 'healthy' when they died, for example, killed in a car accident or similar. Nothing similar was available. Clearly healthy donors of a young age are rare. However, we do not consider this too much of an issue as explained below.

We are the world's leading laboratory for the measurement of Al in human tissue. We have developed the most stringent procedures and quality assurance ever in this field and we have done this because we are well aware of the often-used defence of the Al Industry and others that tissue samples are routinely contaminated with Al. All of the above are published in our landmark paper in Metallomics (House et al., 2012).

We now have brain Al data for about 100 human brains though the majority of these are from donors who were at least 70 years of age. However, what these data afford us is a very good understanding of how much aluminium is in human brain tissue.

You may not have noticed that over the last 10 years or so we have always insisted that all data for all tissue measurements are included in our published papers. We see very little value in data expressed as means or medians, since such an approach has little biological significance when trying to understand the toxicity of a non-essential metal in any tissue. (This is a long and interesting discussion which I cannot really elaborate upon easily in an email!)

We do include 'average' data in our papers but nearly always on the insistence of reviewers in peer review. There seems to be an obsession for such statistics among scientists!

So, in the autism paper we give all the quantitative data and we express averages as requested through peer review. When we discuss these data we point out that they are some of the highest single point measurements that we have made in any human tissue. We can say this because we have many other tissue measurements made in the identical manner to compare with.

Of course, we point out that the stand-out observation in autism is not actually these high values but the location of the Al. The observation that the majority of Al as imaged by fluorescence is intracellular and non-neuronal is striking and this seems to have been glossed over by many reading our paper. Perhaps because we saw this in 10 out of 10 donors and even the most critical of observer could not suggest that our observations of intracellular Al were a consequence of some form of contamination!

These, combined with the quantitative data, are the observations which changed my mind about a possible role for Al in autism and, importantly, that aluminium adjuvant could be transported to the brain from a vaccine injection site. As I have said already several times in interviews given following the publication of our study, before we began this research I could not see any strong science to support a role for Al in autism and/or a role for Al adjuvants in autism and/or transport of Al to the brain.

I am very proud of this research and as a scientist of some repute I know that it is a game-changer in the field of human exposure to Al.

On 21 March 2018 at 16:32, Gordon, Joshua (NIH/NIMH) [E] <[joshua.gordon@nih.gov](mailto:joshua.gordon@nih.gov)> wrote:

I would prefer to engage in a scientific discussion rather than receive ad hominem attacks. If there is an argument to be made that would obviate the need for non-affected control subjects, and that justifies the use of a smaller sample size than is generally agreed upon for clinical research, by all means make it. Otherwise I consider this matter closed.

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Joshua A Gordon, MD, PhD  
Director  
National Institute of Mental Health

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**From:** Christopher Exley <[c.exley@keele.ac.uk](mailto:c.exley@keele.ac.uk)>  
**Date:** Wednesday, March 21, 2018 at 12:25 PM  
**To:** "M. Joshua Gordon" <[joshua.gordon@nih.gov](mailto:joshua.gordon@nih.gov)>  
**Cc:** Aaron Siri <[aaron@sirillp.com](mailto:aaron@sirillp.com)>, "Bianchi, Diana (NIH/NICHD) [E]" <[diana.bianchi@nih.gov](mailto:diana.bianchi@nih.gov)>, Dirk Schaumlöffel <[dirk.schaumloeffel@univ-pau.fr](mailto:dirk.schaumloeffel@univ-pau.fr)>, Tanja Schwerdtle <[taschwer@uni-potsdam.de](mailto:taschwer@uni-potsdam.de)>  
**Subject:** Re: 1 selected item: 24814559 - PubMed

So, you clearly believe that you know better than the authors, the referees and the journal. How lucky we are to have such a high-brow input as yours into this important subject area. Good luck.  
Chris

On 21 March 2018 at 15:56, Gordon, Joshua (NIH/NIMH) [E] <[joshua.gordon@nih.gov](mailto:joshua.gordon@nih.gov)> wrote:

Dear Professor Exley,

I did read the paper and stand by my comments. Of course this is just my opinion from reading this paper and I don't pretend to be an expert on trace metals. But the fact remains that the paper includes no control group and utilizes a very small number of cases.

Other than this paper, I have no knowledge regarding the journal and did not meant to convey a particular opinion on its standards.

Note that I have not made these comments in a public forum but rather in a direct communication with Mr. Siri, in which I was trying to help him evaluate the impact of the work in my capacity as a public servant.

Josh

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Joshua A Gordon, MD, PhD  
Director  
National Institute of Mental Health

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**From:** Christopher Exley <[c.exley@keele.ac.uk](mailto:c.exley@keele.ac.uk)>  
**Date:** Wednesday, March 21, 2018 at 11:40 AM  
**To:** Aaron Siri <[aaron@sirillp.com](mailto:aaron@sirillp.com)>  
**Cc:** "M. Joshua Gordon" <[joshua.gordon@nih.gov](mailto:joshua.gordon@nih.gov)>, "Bianchi, Diana (NIH/NICHD) [E]" <[diana.bianchi@nih.gov](mailto:diana.bianchi@nih.gov)>, Dirk Schaumlöffel <[dirk.schaumloeffel@univ-pau.fr](mailto:dirk.schaumloeffel@univ-pau.fr)>, Tanja Schwerdtle <[taschwer@uni-potsdam.de](mailto:taschwer@uni-potsdam.de)>  
**Subject:** Re: 1 selected item: 24814559 - PubMed

Dear Dr Gordon,

I have to say that I am utterly dismayed that someone in an esteemed a position as yourself would make such comments (see the below email trail) so openly about both our research and, alarmingly, the journal that published the research. While one might expect to find ill-informed statements such as these across social media one would expect so much more of individuals purporting to be scientists and to be supporters of science.

If you had read the paper and even perhaps taken the time to verify the expertise of its authors (never mind the first class reputation of the journal) and you still had questions concerning the research then I would have been (and still am) happy to answer your questions.

Instead by your actions you have brought yourself and that of the NIH into disrepute.

I remain willing to ignore your malicious comments should you wish to discuss our research with us and give it the respect it deserves. I am not sure that the editors of the Journal of Trace Elements in Medicine and Biology (copied in) will be so forgiving.

Best wishes

Professor Christopher Exley PhD FRSB

On 21 March 2018 at 15:11, Aaron Siri <[aaron@sirillp.com](mailto:aaron@sirillp.com)> wrote:

Dear Dr. Gordon,

I have added Dr. Exley to this chain so that the two of you can communicate directly regarding his study related to aluminum in the brains of individuals with ASD and your comments below regarding this study.

Best regards,  
Aaron

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**From:** Gordon, Joshua (NIH/NIMH) [E] [mailto:[joshua.gordon@nih.gov](mailto:joshua.gordon@nih.gov)]  
**Sent:** Saturday, December 9, 2017 8:09 PM  
**To:** Aaron Siri <[aaron@sirillp.com](mailto:aaron@sirillp.com)>  
**Cc:** Bianchi, Diana (NIH/NICHD) [E] <[diana.bianchi@nih.gov](mailto:diana.bianchi@nih.gov)>  
**Subject:** Re: 1 selected item: 24814559 - PubMed

Dear Mr. Siri,

I took a look at the paper you mention. Unfortunately the science is extremely poorly done for many reasons, the most disturbing of which is there is no control comparison group, so there is no way of verifying the authors' claims that the findings in autism cases differ from controls. This is one of many serious flaws in the paper which would preclude publication in any responsible peer-reviewed journal.

I hope you find this helpful.

Best,

Josh

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Joshua A Gordon, MD, PhD  
Director  
National Institute of Mental Health

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**From:** Aaron Siri <[aaron@sirillp.com](mailto:aaron@sirillp.com)>  
**Date:** Thursday, December 7, 2017 at 8:57 AM  
**To:** "M. Joshua Gordon" <[joshua.gordon@nih.gov](mailto:joshua.gordon@nih.gov)>  
**Cc:** "[dchristensen@cdc.gov](mailto:dchristensen@cdc.gov)" <[dchristensen@cdc.gov](mailto:dchristensen@cdc.gov)>, "Shapira, Stuart (CDC/ONDIEH/NCBDDD)" <[cso6@CDC.GOV](mailto:cso6@CDC.GOV)>, "Christensen, Deborah (Daisy) (CDC/ONDIEH/NCBDDD)" <[dqc3@CDC.GOV](mailto:dqc3@CDC.GOV)>  
**Subject:** RE: 1 selected item: 24814559 - PubMed

Good morning Dr. Gordon,

As an addendum to my last email below, the following is a study, released a few days ago, finding significant concentrations of alum in the brains of autistic individuals:  
<http://www.sciencedirect.com/science/article/pii/S0946672X17308763> And here is a short interview with Dr. Exley: <https://www.youtube.com/watch?v=SmkVv8pcVhc>

While you have not yet responded to the numerous overtures seeking a meeting between relevant NIMH scientists and the alum adjuvants scientists identified below, maybe the above study peaks your interest -- especially since it is just one in a large body of science connecting alum with neurological harm. This white paper discusses some of that science in the context of autism: <http://icandecide.com/white-papers/ICAN-AluminumAdjuvant-Autism.pdf>

Best regards,  
Aaron

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**From:** Aaron Siri

**Sent:** Monday, November 13, 2017 3:28 PM

**To:** 'Gordon, Joshua (NIH/NIMH) [E]' <[joshua.gordon@nih.gov](mailto:joshua.gordon@nih.gov)>

**Cc:** '[dchristensen@cdc.gov](mailto:dchristensen@cdc.gov)' <[dchristensen@cdc.gov](mailto:dchristensen@cdc.gov)>; '[cs06@cdc.gov](mailto:cs06@cdc.gov)' <[cs06@cdc.gov](mailto:cs06@cdc.gov)>; '[dqc3@cdc.gov](mailto:dqc3@cdc.gov)' <[dqc3@cdc.gov](mailto:dqc3@cdc.gov)>

**Subject:** RE: 1 selected item: 24814559 - PubMed

Good afternoon Dr. Gordon,

I received the attached email from HHS which I assume is your official response to the simple request to provide at least one study which supports that DTaP and the other vaccines HHS recommends during the first year of life do not cause autism.

This response provides a link to the HHS webpage which claims "Vaccines Do Not Cause Autism" and lists a number of reviews/studies to support this assertion. Sadly, not a single one of these reviews/studies (which all related to either one vaccine, MMR, and/or one vaccine ingredient, thimerosal) provides a shred of support that the 29 doses of 9 different vaccines CDC recommends children receive by six months of age do not cause autism. Ironically, the very first study/review listed on this webpage is the 2011 IOM report, paid for by HHS, which looked at the most commonly claimed vaccine reactions, including that DTaP causes autism, and the IOM could not find a single study that supports the assertion that DTaP (injected at 2 months, 4 months, 6 months, etc.) does not cause autism. (See excerpt from the IOM report attached.) Your response therefore makes it clear you do not have a single study to share which supports that the vaccines given to children in the first year of life do not cause autism.

It is understandable that you thought the study you sent me below actually contained unexposed controls (unvaccinated children) given its misleading title. But now that you know the reality that there is no study supporting the claim that vaccines given during the first year of life do not contribute to the incidence of autism, are you going to take action to conduct an appropriate study that would either support or reject this claim?

I understand this is a difficult and controversial topic but I hope the National Institute of Mental Health and the IACC do not shy away from a scientific study because of fear of

what it may show. There are a number of plausible reasons for how 29 doses of 9 different vaccines given during pregnancy, 1 day, 2 months, 4 months, and 6 months can cause autism, including immune activation, aluminum adjuvant being carried to the brain by macrophages, MCP-1 signaling, molecular mimicry, etc. Vaccines are intended to create a permanent change in the body's immune system often using adjuvants intended to generate a sustained and significant immune event which modern science is not even close to fully understanding; there is also a growing understanding of the connections between the immune and nervous systems. But no need to make this complicated since all you need to do is what is done for every drug pre-licensure. Compare the rates of neurological and immune disorders between an exposed group (vaccinated) and unexposed group (unvaccinated) – this study can even be done retrospectively to avoid supposed ethical concerns.

You are in the unfortunate position of defending vaccine safety because, unlike drugs, most pediatric vaccines currently on the market have been approved based on studies with inadequate follow-up periods of only a few days or weeks (and no saline placebo control). You however are in the fortunate position to remedy this deficiency. In that regard, I have attempted as best as I can to engage with you in a constructive manner on this topic, giving you many months since our meeting to provide the support you were adamant existed during our meeting (a study of vaccinated versus unvaccinated children). Absent a response in the coming days with such support or firm plans to openly conduct such a study, I am left with the conclusion that you (directly or by order of your superiors) don't care to know the real answer to the question of whether giving 29 doses of 9 different vaccines by six months of life contributes to the incidence of autism (and other neurological and immune issues).

Dr. Collins asked during our meeting to consider the implications if Mr. Kennedy was wrong about his concerns regarding vaccine safety. Given your station, I ask you the same question. What if you are wrong about the safety profile of the first year vaccination schedule? What if it is a major contributor to the rising incidence of various neurological and immune (including immune mediated neurological) disorders that have risen in tandem with the increase in HHS's recommended vaccine schedule. If you conduct the desperately needed vaccine safety science noted above the worst that will have happened is that you will have the science to prove what you now can only assume. However, if you don't conduct this study and it eventually turns out your belief (and this email chain makes clear it is a belief) regarding vaccine safety is incorrect, I hope you can live with knowing you could have avoided these harms (and provided the basis to finally begin the desperately needed science of identifying the children susceptible to serious vaccine injury) but chose instead to sit on your hands...

Very truly yours,  
Aaron

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**From:** Aaron Siri



**Sent:** Thursday, September 14, 2017 1:41 PM

**To:** 'Gordon, Joshua (NIH/NIMH) [E]' <[joshua.gordon@nih.gov](mailto:joshua.gordon@nih.gov)>

**Cc:** '[dchristensen@cdc.gov](mailto:dchristensen@cdc.gov)' <[dchristensen@cdc.gov](mailto:dchristensen@cdc.gov)>; '[cs06@cdc.gov](mailto:cs06@cdc.gov)' <[cs06@cdc.gov](mailto:cs06@cdc.gov)>; '[dqc3@cdc.gov](mailto:dqc3@cdc.gov)' <[dqc3@cdc.gov](mailto:dqc3@cdc.gov)>

**Subject:** RE: 1 selected item: 24814559 - PubMed

Good afternoon Dr. Gordon,

Thank you for your response. The information I seek is nothing more than a simple reference to one study which supports HHS's claim that the vaccines it recommends children receive in the first year of life do not cause autism. I gather from our exchange below that you are not aware of any such study. Let me know if that is incorrect.

You are the Director of the Interagency Autism Coordinating Committee (IACC) which coordinates all efforts at HHS, including at the CDC, concerning autism. The IACC's members include the CDC itself, as well as the CDC's Chief Medical Officer & Associate Director for Science (Stuart K. Shapira, M.D., Ph.D.) and the CDC's Surveillance Team Lead, Developmental Disabilities Branch (Deborah Christensen, Ph.D.) Since you state below that the support I seek is best obtained from the CDC, I have cc'd the CDC members on your committee.

I am just trying to get a copy of a study supporting HHS's claim that the vaccines it recommends in the first year of life do not cause autism. I assume you have the best intentions and I would really like to drop this issue – but, as you can appreciate, I like to rely on data/science. I am just asking for a citation to a single study supporting HHS's claim that the vaccines it recommends children receive in the first year of life do not cause autism. I would think you too, as the Director of IACC and NIMH, would be interested in seeing such a study and its underlying data.

Also, can you kindly let me know one way or another if you are interested in meeting with the aluminum adjuvant experts whose letters and CVs were previously provided regarding the potential connection between aluminum adjuvants and autism. Again, I would think you would be interested in hearing them out. (Docs relevant to same reattached.)

Best regards,  
Aaron

p.s. Btw, your response below reminds of me of what former House representative, Dr. Dave Weldon, wrote in 2007: "When I first tasked my staff with investigating federal vaccine safety research we got a lot of confused responses and blank stares from federal officials. The FDA told us to check in with the CDC, telling us that CDC did most of the vaccine safety research. The CDC referred us over to the NIH. Then, the NIH referred us back to the CDC." Happy to send you his full statement.

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**From:** Gordon, Joshua (NIH/NIMH) [E] [<mailto:joshua.gordon@nih.gov>]  
**Sent:** Friday, September 1, 2017 3:40 PM  
**To:** Aaron Siri <[aaron@sirillp.com](mailto:aaron@sirillp.com)>  
**Subject:** Re: 1 selected item: 24814559 - PubMed

Dear Aaron,

I appreciate you following up with me, and apologize for the delay in my response. I think the information you are seeking would be best obtained from the CDC.

Best,

Josh

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Joshua A Gordon, MD, PhD  
Director  
National Institute of Mental Health

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**From:** Aaron Siri <[aaron@sirillp.com](mailto:aaron@sirillp.com)>  
**Date:** Monday, August 14, 2017 at 4:48 PM  
**To:** "M. Joshua Gordon" <[joshua.gordon@nih.gov](mailto:joshua.gordon@nih.gov)>  
**Subject:** RE: 1 selected item: 24814559 - PubMed

Dr. Gordon,

I hope all is well.

I have not received a response to the emails below of July 10 and July 24.

The July 10 email was in response to a review you provided indicating it compared vaccinated and unvaccinated children (but which actually compares vaccinated children with vaccinated children who, at most, were missing MMR). As discussed at our meeting, I would like to see a study which supports the claim that the nearly two dozen doses of vaccines given in the first year of life (which would not include MMR and thimerosal) do not cause autism. I still await receipt of a study which supports same. Are you aware of any such study?

The July 24 email elaborated on my prior email and also sought to facilitate a meeting between with various experts in the field of aluminum adjuvant that do believe there is a connection between aluminum adjuvant in vaccines and autism. Are you willing to have this meeting?

Best regards,  
Aaron

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**From:** Aaron Siri

**Sent:** Monday, July 24, 2017 6:18 PM

**To:** 'Gordon, Joshua (NIH/NIMH) [E]' <[joshua.gordon@nih.gov](mailto:joshua.gordon@nih.gov)>

**Subject:** RE: 1 selected item: 24814559 - PubMed

Good evening Joshua,

As promised in my email below, I am following up regarding the research that has been conducted regarding aluminum adjuvants and neuro/psychiatric disorders, and to facilitate a meeting with you and the scientists conducting this research.

In recent years researchers have discovered that injected aluminum adjuvant travels into the brain, where it causes long term chronic inflammation, damage to neurons and behavioral abnormalities. These adverse effects occur at dosages (mcg/Kg body weight) even lower than dosages received by infants according to the CDC vaccine schedule.

Additionally, it is now well established that autism and other neuro/psychiatric disorders are caused by early life inflammation (i.e. elevated cytokines) in the brain. I have seen your published papers on immune activation and brain development so I presume you are aware of the immune activation findings. Aluminum adjuvant can cause chronic brain inflammation, and this establishes a biologically-plausible and empirically-supported mechanism for how vaccines may cause autism and other neurological disorders. None of the vaccine-autism studies to date tell us anything about the safety of aluminum adjuvants. There are no epidemiological studies showing that aluminum adjuvants do not produce these effects in humans.

Attached is a detailed explanation of the proposed mechanism for how aluminum adjuvants may cause autism. The mechanism suggests that aluminum adjuvant may cause other brain and neurodevelopmental disorders as well. Attached are also supporting letters from experts in the fields of aluminum toxicity. (Finally, I have also attached a more detailed analysis of Taylor 2014.)

I invite you to consider the arguments in the attached document and respond with your observations. I also invite you to share the document with colleagues, particularly if they may have insightful comments or rebuttals.

I also hope to facilitate a meeting with you and a number of the experts studying aluminum adjuvant toxicity, letters from a number of which are attached to this email. Assuming you are open to having this discussion, kindly have your office provide suggested dates/times for such a meeting.

Best regards,

Aaron

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**From:** Aaron Siri

**Sent:** Monday, July 10, 2017 4:16 PM

**To:** 'Gordon, Joshua (NIH/NIMH) [E]' <[joshua.gordon@nih.gov](mailto:joshua.gordon@nih.gov)>

**Subject:** RE: 1 selected item: 24814559 - PubMed

Good afternoon Joshua,

Thank you for sending me the below abstract/review article and it was great meeting at NIH. Really appreciate the opportunity to dialogue on the issue of vaccines and autism.

The abstract/review article you sent me below highlights the concern raised that there has never been a study assessing the relative risk of autism between vaccinated and unvaccinated child. To be sure, this review (and its abstract) leave the impression that the studies it relies upon compare “unvaccinated” children (no vaccines) with vaccinated children. Unfortunately, this is misleading since all 10 of the underlying studies relied upon for this review compared highly vaccinated children with highly vaccinated children. The only difference typically between the study and control groups was a single MMR vaccine or thimerosal vs. non-thimerosal vaccines. (I would be happy to provide you with a breakdown of each of the 10 studies reflecting same.) Meaning, what this review considers “unvaccinated” are vaccinated children typically only missing the MMR vaccine. Assuming the control children in these studies followed the current CDC recommended vaccination schedule, they would each have received 21 vaccine injections during the first 12 months of life excluding the MMR vaccine. Hence, these studies tell us virtually nothing about the relationship of vaccines to autism because they are not comparing vaccinated and unvaccinated children.

For example, the IOM stated in 2011 that there isn't a single study that supports the assertion that DTaP (injected at 2 months, 4 months, 6 months, etc.) does not cause autism, concluding that “The evidence is inadequate to accept or reject a causal relationship between diphtheria toxoid-, tetanus toxoid-, or acellular pertussis-containing vaccine and autism.” Attached is an excerpt of the discussion regarding autism and DTaP from the 2011 IOM report. (I am not aware of a single study regarding DTaP and autism that has been done since 2011.) As another example, the only study regarding Hepatitis B vaccine and autism I have located found a three-fold increase in the odds of an autism diagnosis for neonates that received the hepatitis B vaccine at birth compared that those that did not. (Gallagher CM, Goodman MS. 2010. Hepatitis B vaccination of male neonates and autism diagnosis, NHIS 1997-2002. J Toxicol Environ Health A. 73(24):1665-77.) There is simply no studies for the numerous other vaccines given to children during the first year of life with regard to their relationship with autism (except for the Mawson study which showed vaccination had an over 4 fold increase in autism risk but that study has some serious limitations).

As we discussed at the meeting, I really am open to seeing the evidence that the vaccination schedule, and in particular the cumulative impact of the 31 vaccine doses the CDC recommends a child receive in the first year of life, are not casually related to autism. I would gladly share that support with the community concerned with this issue with my personal endorsement. On the other hand, if that proof doesn't exist, that does not mean that vaccines cause autism. It just means that we need to really do the science necessary to rule out that possibility. (Seeking to assess the health outcomes of those receiving vaccines and those not receiving vaccines really is asking for nothing more than how all drugs are safety tested prior to licensure.)

I respected what appeared to be your thoughtful rather than reflexive reaction to the spirited discussion at NIH. Conducting a true study of the health outcomes between actually unvaccinated and vaccinated children (at least an initial quick and easy retrospective study) that shows no connection with autism should be something that everyone should want. If it shows no connection, it will likely provide the greatest relief to the portion of the autism community that thinks there may be a connection. Parents who think that it was their actions, in vaccinating their children, that lead to their child's condition would feel freed from that guilt by knowing it wasn't the vaccines.

I look forward to your response and being persuaded that the science on the question of whether vaccines cause autism really is settled.

Thanks again in advance for your time and thoughtful consideration of this issue.

Best regards,  
Aaron

p.s. I have had a number of discussions with various aluminum adjuvant experts around the globe who believe there is a connection between the aluminum adjuvants in vaccines given in large quantities during the first six months of life and autism; I hope to soon send you a write-up regarding same for your consideration.

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**From:** Gordon, Joshua (NIH/NIMH) [E] [<mailto:joshua.gordon@nih.gov>]

**Sent:** Wednesday, May 31, 2017 4:03 PM

**To:** Aaron Siri <[aaron@sirillp.com](mailto:aaron@sirillp.com)>

**Subject:** Fwd: 1 selected item: 24814559 - PubMed

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Joshua A Gordon, MD, PhD  
Director  
National Institute of Mental Health

Begin forwarded message:

**From:** Sent by NCBI <[nobody@ncbi.nlm.nih.gov](mailto:nobody@ncbi.nlm.nih.gov)>

**Date:** May 31, 2017 at 4:00:01 PM EDT

**To:** <[Joshua.gordon@nih.gov](mailto:Joshua.gordon@nih.gov)>

**Subject: 1 selected item: 24814559 - PubMed**

This message contains search results from the National Center for Biotechnology Information ([NCBI](http://ncbi.nlm.nih.gov)) at the U.S. National Library of Medicine ([NLM](http://nlm.nih.gov)). Do not reply directly to this message

Sent on: Wed May 31 15:58:39 2017

1 selected item: 24814559

#### PubMed Results

Item 1 of 1 ([Display the citation in PubMed](#))

1. Vaccine. 2014 Jun 17;32(29):3623-9. doi: 10.1016/j.vaccine.2014.04.085. Epub 2014 May 9.  
Vaccines are not associated with autism: an evidence-based meta-analysis of case-control and cohort studies.  
[Taylor LE](#)<sup>1</sup>, [Swerdfeger AL](#)<sup>1</sup>, [Eslick GD](#)<sup>2</sup>.  
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Comment in  
  - [Autism and vaccination: The value of the evidence base of a recent meta-analysis](#). [Vaccine. 2015]
  - [Answers regarding the link between vaccines and the development of autism: A question of appropriate study design, ethics, and bias](#). [Vaccine. 2015]  
Abstract  
There has been enormous debate regarding the possibility of a link between childhood vaccinations and the subsequent development of autism. This has in recent times become a major public health issue with vaccine preventable diseases increasing in the community due to the fear of a 'link' between vaccinations and autism. We performed a meta-analysis to summarise available evidence from case-control and cohort studies on this topic (MEDLINE, PubMed, EMBASE,

Google Scholar up to April, 2014). Eligible studies assessed the relationship between vaccine administration and the subsequent development of autism or autism spectrum disorders (ASD). Two reviewers extracted data on study characteristics, methods, and outcomes. Disagreement was resolved by consensus with another author. Five cohort studies involving 1,256,407 children, and five case-control studies involving 9,920 children were included in this analysis. The cohort data revealed no relationship between vaccination and autism (OR: 0.99; 95% CI: 0.92 to 1.06) or ASD (OR: 0.91; 95% CI: 0.68 to 1.20), nor was there a relationship between autism and MMR (OR: 0.84; 95% CI: 0.70 to 1.01), or thimerosal (OR: 1.00; 95% CI: 0.77 to 1.31), or mercury (Hg) (OR: 1.00; 95% CI: 0.93 to 1.07). Similarly the case-control data found no evidence for increased risk of developing autism or ASD following MMR, Hg, or thimerosal exposure when grouped by condition (OR: 0.90, 95% CI: 0.83 to 0.98;  $p=0.02$ ) or grouped by exposure type (OR: 0.85, 95% CI: 0.76 to 0.95;  $p=0.01$ ). Findings of this meta-analysis suggest that vaccinations are not associated with the development of autism or autism spectrum disorder. Furthermore, the components of the vaccines (thimerosal or mercury) or multiple vaccines (MMR) are not associated with the development of autism or autism spectrum disorder. Copyright © 2014 Elsevier Ltd. All rights reserved. PMID: 24814559 [Indexed for MEDLINE]



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April 11, 2018

Joshua A Gordon, MD, PhD

Director

National Institute of Mental Health

Re: Aluminum adjuvants

Dear Dr. Gordon:

I am writing to follow up on the correspondence between you and Mr. Aaron Siri concerning aluminum adjuvants.

In particular, my letter to HHS that you were copied on cited a number of articles that point to the potential negative impact of aluminum adjuvants on the developing CNS. While most of our work has been done in an *in vivo* mouse model, I believe, as do colleagues who also wrote to HHS, that the issue deserves further investigation as these experimental outcomes may apply to humans as well.

Some have said that the "science is settled" in regard to aluminum adjuvants. However, such is rarely the case for any field in science and is certainly not true, in my opinion, in regard to aluminum either in general or specifically as a vaccine adjuvant.

While some studies of aluminum adjuvants have indeed been conducted to date to examine the issue, much more can be done if NIH and other governmental organizations were to deliberately allocate funds for this purpose. Having grant applications for such work go through formal peer-reviewed study sections would serve to guarantee that appropriate methods and controls would be used. In addition, applicants would enormously benefit from the expertise on such panels which would also insure the quality of any NIH-funded studies.

In regard to this last point, I note that all of us who do such work recognize the limitations of any model systems approach, of ecological human studies, and the issues involved in human post-mortem studies. Such limitations apply as well to any studies of neurological disease conditions, including in my own field of ALS. However, as with ALS research, having the NIH involved in peer review and possible funding is the best guarantor of studies being done rigorously.

I therefore urge you to consider creating a special program to investigate the issue of the potential impact of aluminum adjuvants in CNS development. If this were done, a number of investigators could look at the issue dispassionately such that resulting publications might serve to clarify the actual impacts of aluminum, if any.

Such investigations would have one of two main outcomes: 1. These studies might confirm that a problem with aluminum adjuvants exists, in which case efforts could be directed at discovering less-toxic vaccine adjuvants; 2. Negative outcomes would further strengthen conventional views that aluminum adjuvants are not harmful to children or adults.

Regardless of the outcome, the benefits for the scientific/medical community would be enormous.

I would be quite happy to discuss this with you by email or telephone at your convenience.

Some of our relevant publications are attached.

Sincerely yours,

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ORIGINAL ARTICLE

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## Aluminum Adjuvant Linked to Gulf War Illness Induces Motor Neuron Death in Mice

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### Abstract

Gulf War illness (GWI) affects a significant percentage of veterans of the 1991 conflict, but its origin remains unknown. Associated with some cases of GWI are increased incidences of amyotrophic lateral sclerosis and other neurological disorders. Whereas many environmental factors have been linked to GWI, the role of the anthrax vaccine has come under increasing scrutiny. Among the vaccine's potentially toxic components are the adjuvants aluminum hydroxide and squalene. To examine whether these compounds might contribute to neuronal deficits associated with GWI, an animal model for examining the potential neurological impact of aluminum hydroxide, squalene, or aluminum hydroxide combined with squalene was developed. Young, male colony CD-1 mice were injected with the adjuvants at doses equivalent to those given to US military service personnel. All mice were subjected to a battery of motor and cognitive-behavioral tests over a 6-mo period postinjections. Following sacrifice, central nervous system tissues were examined using immunohistochemistry for evidence of inflammation and cell death. Behavioral testing showed motor deficits in the aluminum treatment group that expressed as a progressive decrease in strength measured by the wire-mesh hang test (final deficit at 24 wk; about 50%). Significant cognitive deficits in water-maze learning were observed in the combined aluminum and squalene group (4.3 errors per trial) compared with the controls (0.2 errors per trial) after 20 wk. Apoptotic neurons were identified in aluminum-injected animals that showed significantly increased activated caspase-3 labeling in lumbar spinal cord (255%) and primary motor cortex (192%) compared with the controls. Aluminum-treated groups also showed significant motor neuron loss (35%) and increased numbers of astrocytes (350%) in the lumbar spinal cord. The findings suggest a possible role for the aluminum adjuvant in some neurological features associated with GWI and possibly an additional role for the combination of adjuvants.

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**Index Entries:** Adjuvant; ALS; aluminum hydroxide; anthrax; Gulf War illness; neurotoxicity; squalene; vaccine.

## Introduction

Gulf War illness (GWI), popularly termed “Gulf War syndrome,” is a spectrum of disorders among veterans of the Persian Gulf War (1990–1991) characterized by a group of variable and nonspecific symptoms such as fatigue, muscle and joint pains, emotional disorders, posttraumatic stress reactions, headaches, and memory loss (Haley et al., 1997; Fukuda et al., 1998). Previous studies conducted on Gulf War veterans by the US Department of Defense (DOD), the US Department of Veteran Affairs, and the UK Gulf War Research Illness Unit have established a strong link between Gulf War-era service and the occurrence of GWI (Hom et al., 1997; Unwin et al., 1999; Kang et al., 2002; Wolfe et al., 2002; Dyer, 2004).

Recent studies have also established a correlation between Gulf War service and a neurological cluster of amyotrophic lateral sclerosis (ALS)–Gulf War illness (ALS–GWI; Charatan, 2002; Horner et al., 2003; Weisskopf et al., 2005). GWI can be partially described as a neurological illness that might carry an ALS component because of the overlapping symptomatology seen in ALS–GWI and classical ALS. According to a nationwide study by the Department of Veteran Affairs, deployed veterans of the Persian Gulf War are twice more likely to develop ALS than nondeployed veterans and the civilian population (Samson, 2002). Overall, GWI, however, does not appear to distinguish between troops who were deployed to the Gulf against those who were not (Steele, 2000). The most unique feature of this new ALS cluster is that the victims are younger than typical ALS patients (Haley, 2003). The only other known ALS cluster involves various geographical loci in the western Pacific expressing as a spectrum of neurological disorders termed ALS–parkinsonism dementia complex (Kurland, 1988; Murakami, 1999). ALS–parkinsonism dementia complex has been linked to environmental factors (Shaw and Wilson, 2003).

Both ALS clusters offer the possibility to identify causal environmental and/or genetic factors involved in sporadic ALS. Regarding ALS–GWI and GWI in general, epidemiological studies have suggested several potential environmental factors such

as exposure to depleted uranium (Fulco et al., 2000; Shawky, 2002), nerve gas (Sartin, 2000; Kalra et al., 2002), organophosphates (Abou-Donia et al., 1996; Kurt, 1998), vaccines (Hotopf et al., 2000), heavy metals (Ferguson and Cassaday, 2001–2002), and bacterial infections (Taylor et al., 1997; Nicolson et al., 2002).

In recent years, increased scrutiny has focused on vaccines, in particular the anthrax vaccine absorbed (AVA; Nass, 1999), largely owing to the observation that nondeployed but vaccinated US troops have developed GWI symptoms identical to those who were deployed (Steele, 2000). Soldiers from the United Kingdom who also received AVA showed increased psychological distress and chronic fatigue compared with control cohorts (Unwin et al., 1999). In contrast, Hunter et al. (2004) released a study that examined health effects of Canadian soldiers postanthrax vaccination but found no apparent link to the AVA vaccine and its adverse health effects. Notably, however, the study only monitored health outcomes for a maximum of 8-mo postvaccination; typically, patients with GWI did not express symptoms until years after the war. French soldiers participating in the war did not receive the AVA vaccine but did show some GWI-related disorders (respiratory, neurocognitive, psychological, and musculoskeletal), but no ALS symptoms were reported (Salamon et al., 2006).

The anthrax vaccine, in common with many other vaccines in wide usage, contains one chemical of particular interest from a neurological perspective: aluminum hydroxide. A second chemical, the lipid polymer squalene (a precursor to cholesterol), has been found in some lots of AVA (Plaisier, 2000); however, manufacturers of the AVA vaccine along with the DOD and other government agencies, deny that squalene was ever part of the formulation of AVA during the period in question. Antibodies to squalene have been demonstrated in many personnel expressing GWI (Asa et al., 2000). The origin of presumed squalene acting to trigger antibody formation remains uncertain.

Aluminum in various forms is the most common and currently licensed adjuvant and is generally regarded by industry and regulatory agencies as safe. Previous studies have found no adverse or long-term health effects (Baylor et al., 2002; Kanra et al., 2003; Jefferson et al., 2004) and the Food and Drug

Administration agency has continued its long-standing approval. However, aluminum in general has been shown to be neurotoxic under some conditions (Crapper et al., 1973; Kawahara et al., 2001) and adjuvants in particular have previously been implicated in neurological disease (Garruto et al., 1989; Wagner-Rrecio et al., 1991; Bilkei-Gorzo, 1993). Squalene has been intensively investigated as a potential adjuvant with some reports failing to find any significant health outcomes (Benisek et al., 2004; Suliet al., 2004; Gabutti et al., 2005). The potential toxicity of squalene is controversial; however, some reports have demonstrated both neuropathology (Gajkowska et al., 1999) and inflammatory responses (Carlson et al., 2000) in animal tests, albeit at very high concentrations. Median lethal dose<sub>50</sub> values (for subcutaneous injection) for either aluminum hydroxide or squalene have not been published to date to the best of our knowledge (J.T. Baker Material Safety Data Sheets).

The AVA vaccine has been criticized on both safety and efficacy grounds (Nass, 2002; Schumm et al., 2002a; Nass et al., 2005) and concerns have been raised that the Institute of Medicine ignored evidence from studies that implicate vaccine involvement in the epidemiology of GWI (Schumm et al., 2002b), and a recent publication has raised additional concerns about the long-term safety of the anthrax vaccine (Schumm et al., 2005).

Given the controversies surrounding AVA and its known and suspected vaccine adjuvants, the experiments described in this article were designed in order to provide an accurate multilevel analysis of the potential impact of aluminum hydroxide and squalene on the nervous system over extended time periods in an outbred strain of young male mice. The conditions chosen in the model system were intended to mimic the administration of AVA to young, predominantly male, US and other coalition military service personnel.

## Methods

### **Experimental Animals, Diet, and Tissue Collection**

Young adult CD-1 male mice were used in the study (3 mo old and weight approx 35 g at experiment onset). Younger animals were deliberately chosen to mimic the age of service during the Gulf War (Haley, 2003). Four treatment groups were used; control ( $n = 10$ )

injected with saline/phosphate-buffered saline (PBS), aluminum hydroxide ( $n = 11$ ), squalene ( $n = 10$ ), and aluminum hydroxide + squalene ( $n = 10$ ). All animals were housed solitarily at the Jack Bell Research Center animal care facility in Vancouver, BC, Canada. An ambient temperature of 22°C and a 12/12 h light cycle were maintained throughout the experiment. All mice were fed Purina™ mouse chow *ad libitum*. Mice were subjected at regular intervals to specific behavioral tests, including wire-mesh hang (twice a week), open field (once a week), and water maze (once a week) over a period of 6-mo postinjection. The order in which the animals were tested was randomized for each trial. Mice were sacrificed with an overdose of halothane and perfused with 4% paraformaldehyde. Central nervous system (CNS) tissues were collected for histological examination. Fixed brains and spinal cords from all mice were transferred to a 30% sucrose/phosphate-buffered saline (PBS) solution for overnight incubation and then frozen and stored at -80°C until sectioning. The CNS sections were cryoprotected in 30% ethylene glycol with 20% glycerol-dibasic and monobasic sodium phosphate solution and kept frozen at -20°C until use. All brain tissue blocks were mounted in Tissue-Tek optimum cutting temperature (O.C.T) compound (Sakura, Zoeterwoude, Netherlands), and then sectioned by cryostat into 30-μm coronal slices. Spinal cords were sectioned at 25 μm in the transverse plane.

### **Adjuvants**

Alhydrogel™, an aluminum hydroxide (Al[OH]<sub>3</sub>) gel suspension, was used as a source of aluminum hydroxide. Alhydrogel is manufactured by Superfos Biosector a/s (Denmark). MPL™ + TDM + CWS (Monophosphoryl Lipid A, synthetic Trehalose Dicorynomycolate, and cell wall skeleton of *Mycobacteria*), is a commercial squalene (C<sub>30</sub>H<sub>50</sub>)-containing adjuvant was manufactured by Corixa Corporation (Seattle, WA). Both adjuvants were supplied by Sigma, Canada.

### **Aluminum**

To calculate the approximate human dosages of aluminum hydroxide and squalene for the experiments the following information was used. The AVA vaccine for human use is made by Bioport Corporation, Lansing, MI. According to product data sheets from the Michigan Biologic Products



Institute (MBPI, Lansing, MI; Bioport's predecessor) a single dose of AVA vaccine contains 2.4 mg of aluminum hydroxide (equivalent to 0.83 mg of aluminum). Based on an average human body weight of 70–80 kg, the amount per kilogram body weight is approx 30–34  $\mu\text{g}/\text{kg}$ . Soldiers or civilians receiving the vaccine would have received between 30 and 34  $\mu\text{g}/\text{kg}$  (one injection) up to 120–136  $\mu\text{g}/\text{kg}$  if four injections were received.

### *Squalene*

As noted earlier, both Bioport Corporation (Lansing, MI) and the MBPI deny the addition of squalene in AVA formulation. Therefore, MF59 was calculated based on current vaccines in use outside the United States that employs a squalene-containing adjuvant oil emulsion. This adjuvant in experimental influenza vaccines (Chiron Corporation Emeryville, CA) uses a concentration of 5% squalene. Based on the total volume of the MF59 injection (0.5 mL), this would be equivalent to 0.025 mL of squalene. Again, based on an average 70–80 kg human, the amount per injection would be approx 0.31–0.35  $\mu\text{g}/\text{kg}$  for one injection, as much as 1.24–1.40  $\mu\text{g}/\text{kg}$  for a full series of four injections. The adjuvant injections in the mice were calibrated based on average animal weight for 3-mo-old male CD-1 mice (approx 35 g). Performing two injections as an average (range 1–4) based on US DOD usage during the Gulf War in 1991 was chosen. Based on the human values cited earlier, mice receiving aluminum hydroxide received two doses of 50  $\mu\text{g}/\text{kg}$  (suspension) in a total volume of 200- $\mu\text{L}$  sterile PBS (0.9%). The mice in this experiment would, therefore, have received 100  $\mu\text{g}/\text{kg}$  against a probable 68  $\mu\text{g}/\text{kg}$  in humans. Mice receiving squalene got the equivalent dose of 2% squalene suspension (MPL + TDM + CWS) in PBS for a total of 0.24–0.28  $\mu\text{g}/\text{kg}$  over two injections compared with the likely human dose of 0.62–0.71  $\mu\text{g}/\text{kg}$  at 5% squalene over two injections. Mice in the aluminum hydroxide + squalene group had both adjuvants administered in the same PBS volume. Controls were injected with 200- $\mu\text{L}$  PBS.

### **Immunization**

The injection site for human administration is typically subcutaneous over the deltoid muscle. For injections in mice, a subcutaneous injection into the loose skin behind the neck (the "scruff") was used for ease of injection and to minimize discomfort.

Animals received two injections (2 wk apart) of aluminum hydroxide, squalene, aluminum hydroxide + squalene, or PBS. This immunization protocol mimicked the anthrax vaccine dose schedule set by the Anthrax Vaccine Immunization Program except for the route of administration.

## **Behavioral Tests**

In all behavioral tests and histological assays, the experimenters were blind to the identity of treatment groups of the animals or samples.

### **Wire-Mesh Hang**

A wire-mesh hang test was used three times a week to test for muscular strength and endurance (Crawley, 2000). The wire-mesh hang consisted of a 6-in. wire mesh that was suspended 40-cm in front of a padded surface. Mice were placed onto the wire grid and inverted for a maximum period of 60 s. Latency to fall was measured and recorded.

### **Open Field**

An open-field test was used to evaluate anxiety (DeFries et al., 1974). The open-field arena consisted of a brightly lit open-field pool, 1.3 m in diameter, 30-cm high containing mouse bedding approx 5-cm thick. An overhead video camera was used to record mouse locomotion. The number of squares crossed in a measured area (outside, inside, and center perimeters) over a 5-min period was counted. Anxiety, or fear-related behavior, is seen when the mouse remains in the corners or near the edges of the arena (thigmotaxis) rather than moving into the center of the arena (Crawley et al., 1997). Testing was conducted once a week for the duration of the experiment.

### **Water Maze**

The water maze was used to evaluate spatial and reference memory, both forms of long-term memory (Morris, 1984). The water-maze setup included a pool, 1.3 m in diameter (Everts and Koolhaas, 1999), five radial arms 30-cm high, and a rescue platform 5 mm above the water level. The mice were trained for 4 d, at three trials per day before the injection regime. Mice were placed into the pool at the same start location

for each trial and were allowed to explore the pool for a maximum of 60 s, after which they were guided to the platform using a ruler. At 90 s, the handler placed mice on the platform if they still had not reached it on their own. Training was terminated when the mice consistently found the platform within 25 s on four consecutive trials. Testing was conducted once a week for the duration of the experiment. During testing, an error was scored if the mouse fully entered an incorrect arm of the maze.

## Immunohistochemistry

### Neuronal Nuclei and Activated Caspase-3 Labeling

Mouse neuronal nuclei (NeuN) antibody (Chemicon International, Temecula, CA, 1:300) a DNA-binding and neuron-specific nuclear protein was used to identify neurons (Mullen et al., 1992; Wolf et al., 1996). Mounted sections were rinsed in 10% Tris-ethylene diamine tetraacetic acid (EDTA) buffer and microwaved for 10 min. After heating, sections were allowed to cool for 20 min and were then incubated in working solution of mouse on mouse (MOM™) immunoglobulin (Ig) blocking reagent (MOM kit, Vector Laboratories) for 1 h. Sections were immersed in MOM diluent solution for 5 min and incubated in primary NeuN antibody for 30 min at room temperature. Sections were then incubated in MOM Biotinylated Anti-mouse immunoglobulin (Ig)G reagent for 10 min and incubated with fluorescein-avidin DCS for 5 min, then blocked with 10% NGS for 1 h. Sections were incubated with rabbit-antiactivated caspase-3 antibody (Promega; Madison, WI, 1:250) for overnight and AlexaFluor 546™ for 30 min at room temperature (Molecular Probes; Eugene, OR, 1:500) to detect cells undergoing apoptosis (Duan et al., 2003). Sections were mounted with fluorescent DAPI (4',6-diamidino-2-phenylindole, Vector Laboratories). A serial approach was used for double-fluorescence labeling because of having the use of Vector MOM kit for NeuN. All steps were performed at room temperature unless specified otherwise.

### Choline Acetyltransferase Labeling

Choline acetyltransferase (ChAT) antibody (AB144P, Chemicon International; Temecula, CA, 1:100) was used to identify cholinergic neurons in

the brain and spinal cord. It is used as a specific marker for spinal motor neurons (Wetts and Vaughn, 1996; Maatkamp et al., 2004). Fluorescent immunolabeling was performed on mounted sections pretreated with 0.5% Triton X-100 in buffer (PBST) twice for 15 min. Sections were then blocked in 5% normal goat serum (NGS) with 5% bovine serum albumin (BSA) for 3 h, then incubated in goat anti-ChAT IgG antibody (in PBS with 5% NGS + 1% BSA, 1:100) overnight at 4°C. The sections were incubated for 2 h each in rabbit anti-goat IgG antibody (DuoLuX™, Elite ABC Kit, Vector Laboratories; 1:200) at room temperature and mounted with fluorescent DAPI.

### Glial Fibrillary Acidic Protein Labeling

Glial fibrillary acidic protein (GFAP) is a member of the class III intermediate filament protein family and stains reactive rodent and normal human brain astrocytes as well as those induced by a variety of CNS injuries (Lee et al., 1984; Tohyama et al., 1991). Antigial fibrillary acidic protein rat monoclonal antibody (345860, Calbiochem, San Diego, CA, 1:100) was used to identify astrocytes in lumbar segment of animal spinal cord. Fluorescent immunolabeling was performed on slide-mounted sections and pretreated in PBST twice for 5 min. Sections were then blocked in 10% NGS + 1% BSA in PBST for 2 h, then incubated with primary antibody rat anti-GFAP (in PBST with 1% NGS + 1% BSA) at 10 µg/mL (1:100) in a humidified chamber at room temperature (23°C) overnight. Sections were then incubated for 1 h in anti-rat fluorescein isothiocyanate antibody (1:200 dilutions in PBS, Serotec Laboratories, Raleigh, NC) incubate for at room temperature and mounted with fluorescent DAPI.

### Microscopy

Brain and spinal cord sections processed with fluorescent materials were viewed with a Zeiss Axiovert (Carl Zeiss Canada Ltd., Toronto, ON) microscope zoom at ×40 and ×100 (under oil) magnification. DAPI (blue fluorescence) was viewed with a 359/461 nm absorption/emission filter. Alexa Fluor 546™ (red), and rabbit IgG DuoLuX (red) were viewed with 556,557/572,573 nm filter; fluorescein isothiocyanate antibody was viewed with a 490,494/520,525 nm filter. Images were captured using AxioVision 4.3 software.



## Histological Measurements

### *Neuronal Nuclei and Active Caspase-3*

Multiple brain ( $n = 3$ ) and lumbar spinal cord ( $n = 8$ ) sections from each mouse were examined. Five mice from each treatment group were used for assays of both lumbar spinal cord and brain. Fluorescent intensity levels of NeuN and activated caspase-3 were used to identify specific antibody labeling. Stained sections included tissue from lumbar spinal cord, primary motor cortex, the red nucleus, substantia nigra, and the dentate gyrus of the hippocampus. Regions of interest (ROI) were defined using landmarks from mouse brain and spinal cord stereotaxic atlases (Sidman et al., 1971; Paxinos and Franklin, 2001). All sections were counted in an unbiased manner. Cell counts included the total number of cells labeled with either NeuN, activated caspase-3, or both (double labeling) counted under a  $\times 40$  objective lens.

### *Choline Acetyltransferase*

Lumbar spinal cord sections ( $n = 8$ ) from each mouse were captured and ROIs defined using the methods described earlier. Eight mice from each treatment group were used for the assay of lumbar spinal cord. Ventral root motor neurons were counted under a  $\times 40$  objective lens. All motor neurons in the field of view were counted.

### *Glial Fibrillary Acidic Protein*

Lumbar spinal cord sections ( $n = 8$ ) from each mouse were captured and ROIs defined as mentioned earlier. Eight mice from each treatment group were used for the assay of lumbar spinal cord. Counts were conducted under a  $\times 40$  objective lens, including all astrocytic cells in the field of view.

### *Squalene Antibody Assay*

Serum was collected from animals through tail bleed and sent to Tulane University Health Sciences Center for Analysis. Squalene was diluted 10–10<sup>4</sup>-fold in distilled water, applied to nitrocellulose membranes using a cotton-tipped applicator, and allowed to air-dry. The nitrocellulose membranes were then cut into 4-mm-wide strips, placed in 20-well trays, and rinsed in wash buffer (tris-buffered saline

containing 0.3% polyoxyethylene sorbitan monolaurate and 0.005% thimerosal, pH 7.4). The strips were incubated in 2-mL blocking buffer (tris-buffered saline containing 5% powdered instant milk, 4% goat serum, and 0.008% thimerosal, pH 7.4) for 45 min before the addition of 5  $\mu$ L of mouse serum samples (1:100–400 dilution) followed by a further 90 min incubation. All incubations and washes were carried out at room temperature on a rocking platform. The blocking buffer was then removed and the strips were washed with washing buffer (three times for 5 min each). After the strips were washed, 2 mL of blocking buffer containing biotin conjugated to goat antimouse IgG (Sigma, St Louis, Mo), diluted 1:1000, was added. After 60 min incubation, the strips were again washed as above, and 2 mL of blocking buffer containing avidin-conjugated horseradish peroxidase (Jackson Immuno Research, West Grove, PA), diluted 1:500, was added. Following another 60 min incubation, the strips were washed and 2-mL buffered saline containing 30% methanol and the substrate 0.6 mg/mL 4-chloro-1-naphthol, 0.03% hydrogen peroxide (pH 7.4) was added. The reaction was allowed to proceed for 15 min and was stopped by rinsing the strips in distilled water. The strips were allowed to air-dry, then qualitatively scored on a scale of 0–4 (see Asa et al., 2002).

## Statistics

Values for each mouse on the individual tasks and in the cell counts were used to calculate mean  $\pm$  S.E.M. for each group and condition. Behavioral scores and cell counts were normalized to the mean value of controls. The means were compared using one-way ANOVA (Statistica, Statsoft Inc., Tulsa, OK; GraphPad Prism, San Diego, CA).

## Results

### *Behavioral Effects*

The greatest overall effects were seen in mice injected with aluminum hydroxide.

These mice showed a progressive and significant decrease in muscular strength and endurance (50% at time of sacrifice) compared with the controls (100% for all data; Fig. 1A). Squalene-injected mice

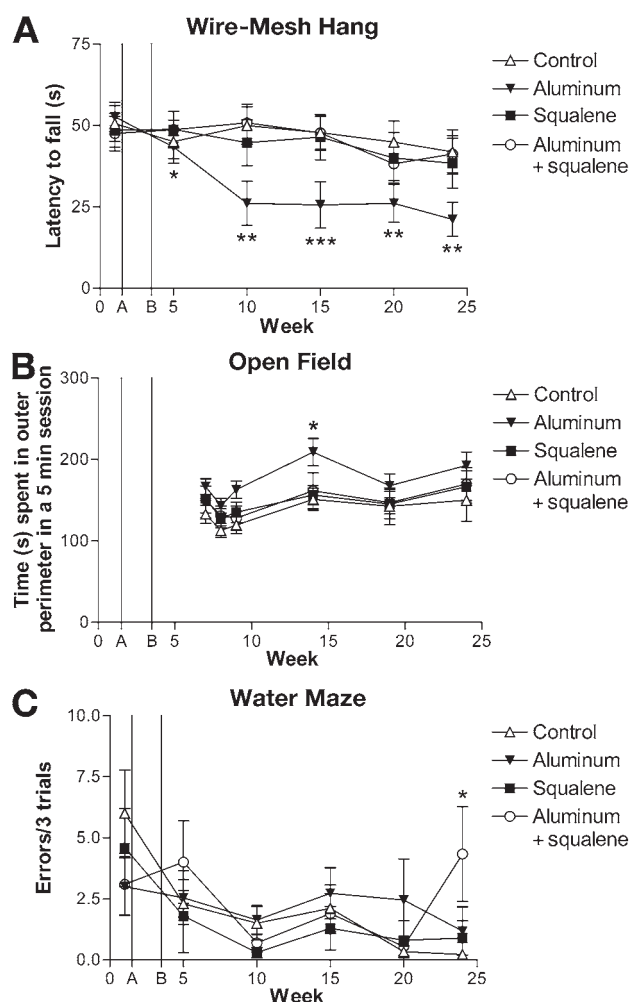


Fig. 1. Motor and cognitive effects of known and presumed AVA adjuvants. **(A)** Wire-mesh hang test. Mice injected with aluminum hydroxide showed a significant decrease in muscular strength and endurance (50%) compared with the controls (100%). Mice injected with squalene or both adjuvants did not show a significant decrease in muscular strength. **(B)** Open-field tests (during weeks 7–24). Mice injected with aluminum hydroxide show a significant increase in anxiety (138%) compared with the controls. Mice injected with squalene or both adjuvants did not show any significant effect. **(C)** The radial arm water maze (five arms). Mice injected with aluminum hydroxide (1.2 errors) or squalene (0.9 errors) did show increased errors after week 20 but these values did not reach statistical significance. Mice injected with both adjuvants showed a significant increase in errors after week 20 (4.3 errors), whereas, controls achieved 0.2 errors. A = first injection, B = second injection. \* $p < 0.05$ , \*\* $p < 0.01$ , \*\*\* $p < 0.001$ ; one-way ANOVA.

showed a minor decrease in muscular strength that did not achieve significance. The aluminum hydroxide and squalene (combined) group did not show any statistically significant differences in muscle strength and endurance.

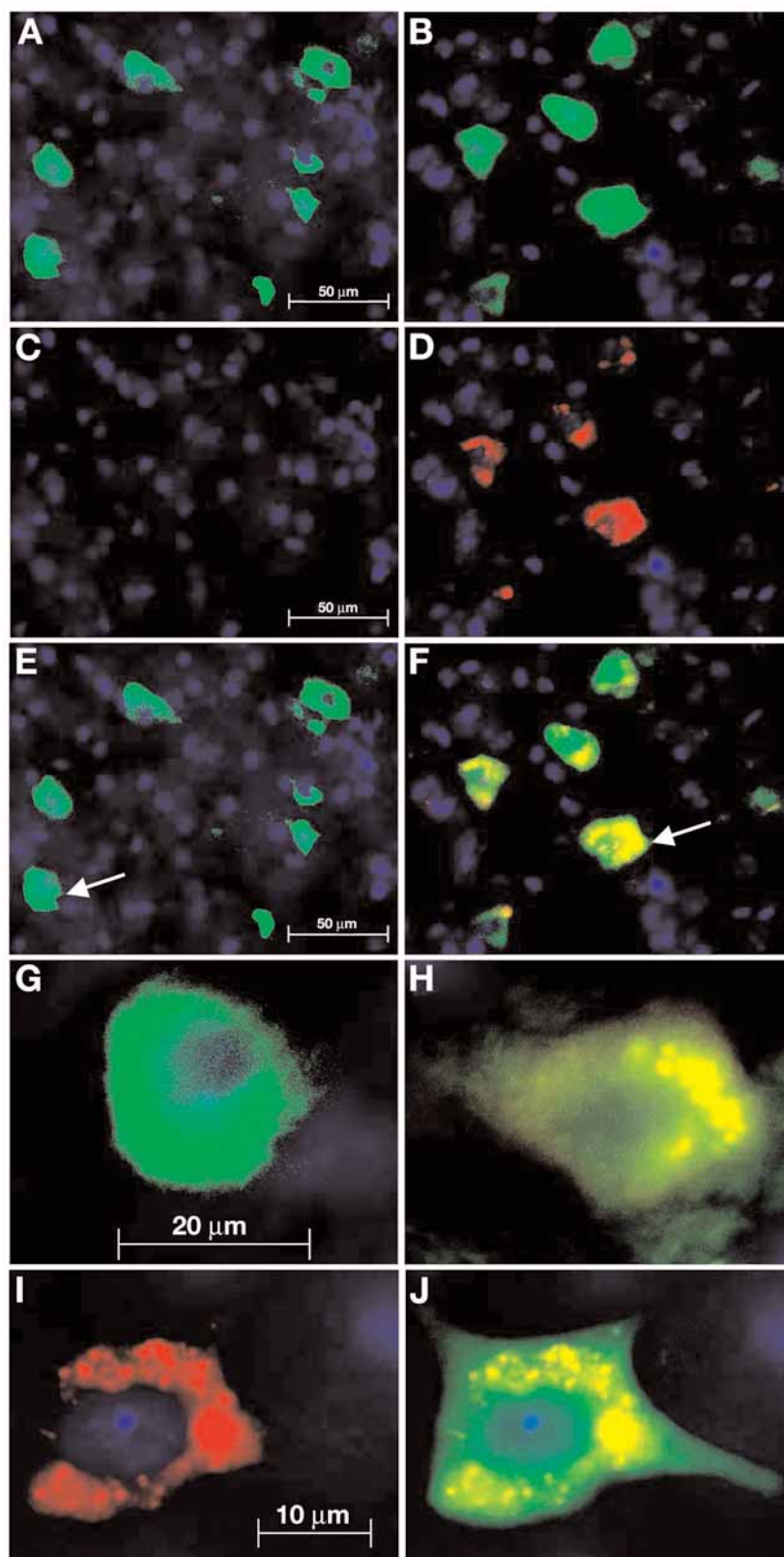
Aluminum-injected mice showed a significant increase in anxiety levels at week 14 (138%) as measured by the longer time spent in the outer perimeter during the open-field tests (Fig. 1B). After 14 wk, the aluminum group continued to show increased levels of anxiety compared with the controls but these values did not reach statistical significance ( $p = 0.018$  at week 24). The squalene group also showed a small increase in anxiety after week 20 but these results did not achieve statistical significance. There was no difference in anxiety levels between the combined group and controls.

Assessment of cognitive performance on the water maze showed that mice injected with aluminum hydroxide (1.2 errors) or squalene (0.9 errors) showed an increase in the number of errors after week 20, but these differences did not reach statistical significance. Mice injected with both adjuvants had significant late stage, long-term memory deficits with an increase in the number of errors after week 20 (4.3 errors) compared with the controls (0.2 errors; Fig. 1C).

## CNS Pathology

Mice injected with PBS showed little or no activated caspase-3 labeling in ventral lumbar spinal cord (Figs. 2C,E,G and 3A). In contrast, mice injected with aluminum hydroxide showed a significant 255% increase in activated caspase-3 labeling alone and a significant 233% increase in double labeling with NeuN (Figs. 2D,F,H-J and 3A). Activated caspase-3 was also increased in the squalene group as well as the combined aluminum and squalene group, but quantified cell counts did not reach statistical significance.

In addition to the spinal cord, other brain structures involved in motor function were also examined. NeuN and activated caspase-3 immunohistology was performed on the primary motor cortex, the red nucleus, substantia nigra, and hippocampus because these areas are affected in the human motor diseases such as ALS and Parkinson's disease (Sasaki et al., 1992; Eisen and Weber, 2001; Tsuchiya et al., 2002). Quantitative analysis of NeuN labeling showed comparable numbers of labeled neurons in all



treatment groups (Fig. 3A–E). Mice injected with aluminum hydroxide showed a significant increase in activated caspase-3 labeling (192%) and activated caspase-3/NeuN double labeling (185%) in the primary motor cortex compared with the controls (Fig. 3B). The squalene and combined group showed small increases in activated caspase-3 and activated caspase-3/NeuN double labeling but these did not reach statistical significance. Cell counts performed in the red nucleus show increased activated caspase-3 and double labeling in both aluminum groups, but these results were not significant (Fig. 3C). Analysis of the substantia nigra region did not reveal any differences in labeling between groups (Fig. 3D). In the hippocampus, cell counts conducted on the polymorphic layer of the dentate gyrus showed an increase in double labeling for squalene and combined groups but it did not reach statistical significance (Fig. 3E).

Only cells labeled with ChAT were included in the motor neuron counts of lumbar spinal cord. Aluminum-injected mice showed a significant reduction in motor neurons (35%) compared with the controls (Fig. 4A–C). The squalene and combined group also showed a reduction in motor neuron number that did not achieve statistical significance.

The aluminum-injected group showed a highly significant increase in the expression of GFAP-positive astrocytes (350%) greater than the controls (Fig. 5A–D). Animals treated with squalene or aluminum with squalene showed small increases in the number of astrocytes present when compared with the controls, but these differences were not statistically significant.

### Squalene-Antibodies Assay

Two out of ten control animals showed the presence of squalene antibodies (SA) in the first serum specimen taken at 4 wk (2 wk postsecond injection). A larger number of animals, 4/10, injected

with squalene possessed detectable levels of SA at this time-point; however, this difference was not statistically significant. Three out of the eleven animals injected with aluminum hydroxide and 1/10 injected with both adjuvants also showed increased SA. The presence of SA was generally stable over time in individual animals tested. However, one animal that had been injected with both adjuvants developed SA at a later time-point (24 wk).

### Non-CNS Features

In addition to behavioral changes and CNS pathology, various physiological changes were observed. Hair loss at the injection site (0.5–1.0-cm diameter region around the injections site) was common to all adjuvant treated groups; 2/10 from the aluminum hydroxide group, 4/10 from the squalene group, and 3/10 mice from the combined group. No control animals developed hair loss in the injection area. Four of the ten mice injected with both adjuvants developed an allergic skin reaction (dermatitis; inflammation of the skin characterized by itchiness and redness with scaling) showing in a 0.5-cm diameter region around the injection site.

### Discussion

Although, several animal studies using the anthrax vaccine have been published (Ivins et al., 1995; Fellows et al., 2001; Williamson et al., 2005), none of these experiments examined neurological outcomes or behavioral side-effects.

The present results indicate that anthrax vaccine adjuvants mimicking a minimal AVA administration regime (two injections) resulted in some neuropathological outcomes postinjection (Nass, personal communication). Aluminum hydroxide induced both behavioral and motor deficits, and the increased presence of apoptotic neurons and in various regions of

Fig. 2. (Opposite page) NeuN and activated caspase-3 fluorescent labeling in ventral horn of lumbar spinal cord. Green = NeuN; red = activated caspase-3; yellow = colocalization of NeuN and activated caspase-3; blue = nuclear DAPI. (A,B) NeuN labeling in control and aluminum hydroxide injected mouse lumbar spinal cord sections, respectively. (C,D) Control and aluminum hydroxide mouse lumbar spinal cord sections labeled with caspase-3. (E,F) Merge of NeuN and caspase. Magnification  $\times 40$  A–F. White arrow indicates neuron enlarged in (G,H). Enlargement of neurons E,F at  $\times 100$  magnification. (I,J) Enlargement of another activated caspase-3 positive motor neuron at  $\times 100$  magnification. J, Merged image of activated caspase-3 and NeuN. A–F; Scale bar = 50  $\mu$ m. G,H; Scale bar = 20  $\mu$ m. I,J, Scale bar = 10  $\mu$ m.



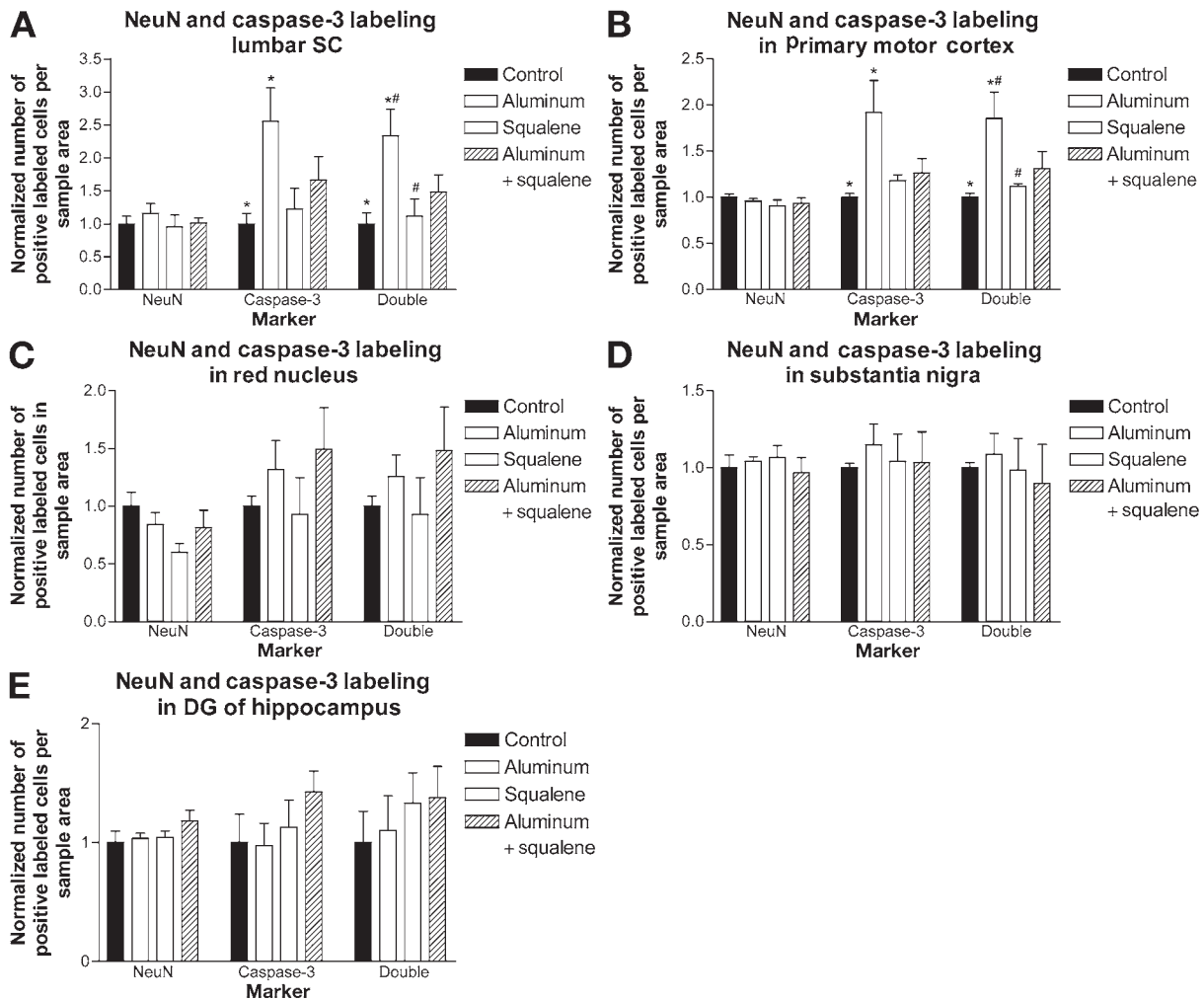


Fig. 3. (A) Cell counts for NeuN and activated caspase-3 labeling in ventral horn of lumbar spinal cord. NeuN counts between groups ( $n = 32$ , eight per group) show no significant differences indicating similar numbers of neuronal cells labeled in all groups. Activated caspase-3 marker shows significantly increased positive caspase-3 labeling (255%) in mice injected with aluminum hydroxide compared with the controls. NeuN and activated caspase-3 double labeling show significantly increased apoptotic neuronal cells (233%) in mice injected with aluminum hydroxide compared with the control and squalene injected groups. (B) NeuN counts ( $n = 20$ , five per group) in the primary motor cortex show no significant difference between groups. Animals injected with aluminum hydroxide show a significant increase in activated caspase-3 (192%) and double labeling (185%) in primary motor cortex compared with the controls. Aluminum hydroxide-injected mice showed a significant increase (165%) in double labeling when compared with the squalene-injected mice. (C) Cell counts ( $n = 20$ , five per group) performed in the red nucleus show a non significant increase in activated caspase-3 and double labeling in both aluminum groups compared with the controls. (D) SNpc; there was no significant difference in cell counts ( $n = 20$ , five per group) of NeuN and activated caspase-3 labeling between groups in the substantia nigra region. (E) Hippocampal cell counts ( $n = 20$ , five per group) performed on the polymorphic layer of the dentate gyrus show increased activated caspase-3 and double labeling in the squalene group, whereas, the combined group showed the greatest activated caspase-3 and double labeling. These results were not statistically significant. Histograms show means  $\pm$  S.E.M. \*, #  $p < 0.05$  vs control and squalene mice, \*\*  $p < 0.01$  vs control mice using one-way ANOVA.

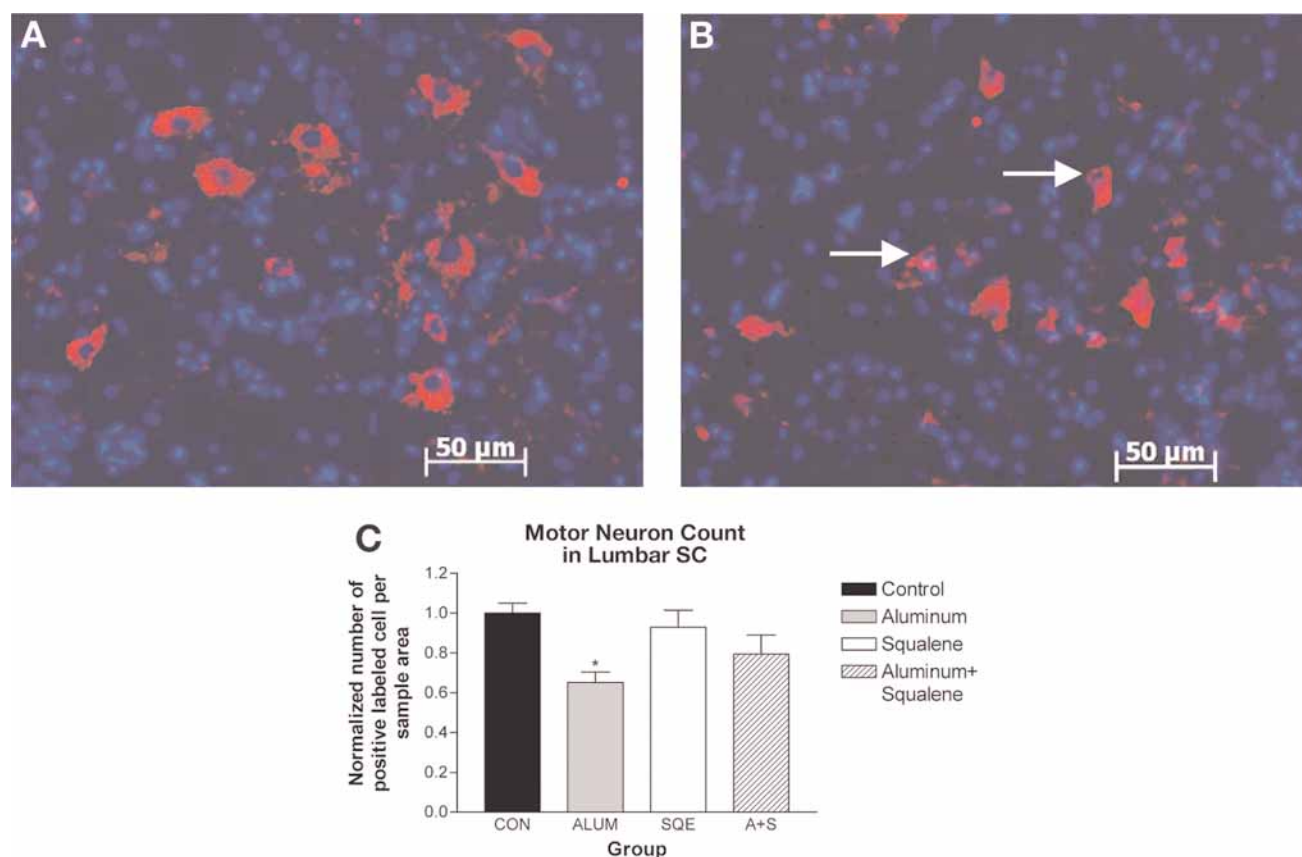


Fig. 4. Choline acetyltransferase (ChAT) fluorescent labeling in ventral horn of lumbar spinal cord. **(A)** Control section shows ChAT labeling of motor neurons ( $\times 20$  magnification). **(B)** Aluminum-injected animal shows decreased ChAT labeling and abnormal morphology of motor neurons (white arrows) compared with the controls ( $\times 20$  magnification). Scale bar = 50  $\mu$ m. **(C)** Only cells positively labeled with ChAT were counted as motor neurons ( $n = 32$ , eight per group). Mice injected with aluminum hydroxide showed a statistically significant decrease in motor neuron number (35%) compared with the controls. There was no significant difference in motor neuron counts between all other groups compared with the controls. Data are means  $\pm$  S.E.M. \*\*\* $p < 0.05$  vs control mice using one-way ANOVA.

CNS with significant motor neuron loss in the lumbar spinal cord. The presence of caspase-3 labeling in cells not labeled with NeuN suggests that non-neural cells also undergo apoptosis under these conditions.

These results are consistent with a potential role for aluminum in motor neuron death in ALS. In those CNS areas tested to date (spinal cord), reactive astrocytes were present in significant numbers, indicating an inflammatory response. Previous studies have shown the increased presence of reactive astrocytes in human ALS and animal models of the disease (Nagy et al., 1994; O'Reilly et al., 1995; Levine et al., 1999; Barbeito et al., 2004).

The squalene adjuvant alone produced a small change in locomotion and anxiety testing, but the differences in the cell counts of this group with respect to controls were not significant in any CNS region. The combination of both the adjuvants showed a significant long-term memory deficit with some indications of neuronal apoptosis in the red nucleus and DG region of the hippocampus. Thus, while squalene does not appear to have the same overall impact as aluminum at sacrifice, the change in cognitive function might suggest that possible longer-term squalene effects should be examined in future studies. Regarding to the SA

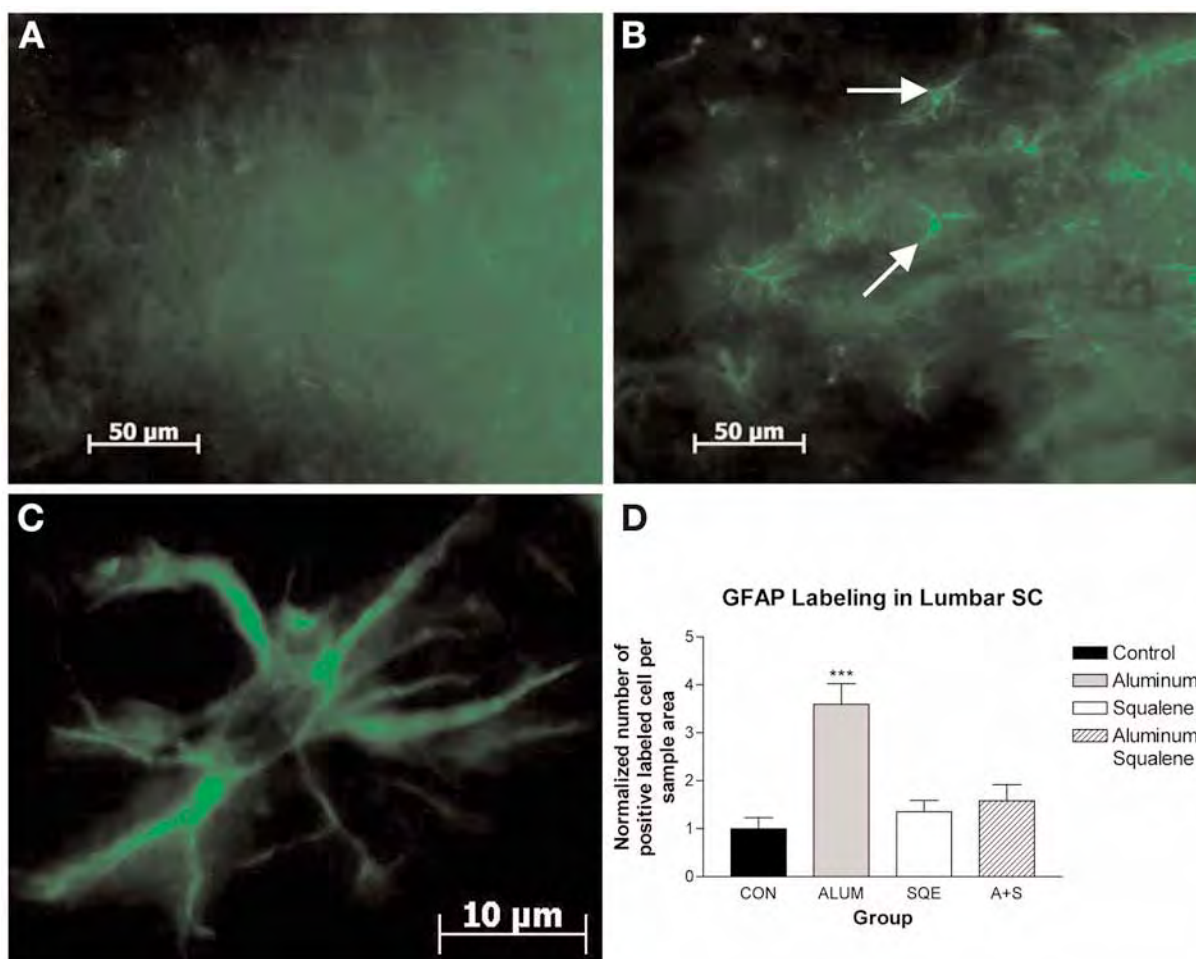


Fig. 5. GFAP-fluorescent labeling in ventral horn of lumbar spinal cord. **(A)** Control sections show little GFAP labeling. **(B)** Sections from mice injected with aluminum hydroxide show increased GFAP labeling and greater number of astrocytes (white arrows) compared with the controls (A,B  $\times 40$  magnification). Scale bar = 50  $\mu\text{m}$ . **(C)** Astrocyte from aluminum injected mouse observed under  $\times 100$  magnification. Scale bar = 10  $\mu\text{m}$ . **(D)** Normalized cell counts for GFAP-labeling of astrocytes in ventral horn of lumbar spinal cord ( $n = 32$ , eight per group). Squalene treated animals show a small increase in GFAP-labeled astrocytes when compared with the controls. Animals treated with both aluminum hydroxide and squalene showed a larger increase in astrocyte cell number whereas mice injected with aluminum showed the greatest increase in GFAP-labeled astrocytes (350%). Data are means  $\pm$  S.E.M \*\*\* $p < 0.001$  vs control mice using one-way ANOVA.

assays, we were able to detect antibodies in 40% of the mice injected with squalene. This outcome was the highest incidence level of all treatment groups; however, the other groups including the controls showed some SA-positive mice. Previous studies have suggested that naturally occurring antibodies against squalene develop in mice, as well as humans, during the aging process (Matyas et al., 2004). BALB/c, B10.Br, and C57BL/6 mice showed SA in

approx 12% of animals, a number qualitatively similar to the control and aluminum hydroxide injected CD-1 mice. The relatively low incidence of SA in squalene injected mice might reflect a transient antibody production. Future experiments with more specific antibodies may resolve this issue.

Aluminum can access CNS following injections with aluminum-adjuvanted vaccines (Wen and Wisniewski, 1985; Redhead et al., 1992; Sahin et al.,

1994). Various studies have clearly demonstrated that aluminum can be neurotoxic (Crapper et al., 1973; Banks and Kastin, 1989; Joshi, 1990; Kawahara et al., 2001). For example, aluminum-injected animals show severe anterograde degeneration of cholinergic terminals in cortex and hippocampus (Platt et al., 2001). Potential toxic mechanisms of action include interference with cholinergic projections, blockage of synaptic transmission, defective phosphorylation—dephosphorylation reactions, altered rate of transmembrane diffusion and selective changes in saturable transport systems in the blood–brain barrier (BBB), reduced glucose utilization, and site-specific damage inflicted by free radicals produced by altered iron metabolism. Aluminum has also been proposed as a factor in neurodegenerative diseases based on its demonstrated neurotoxic potential and its association with degenerating neurons in specific CNS areas (Perl et al., 1982; Perl and Pendlebury, 1986; Rao et al., 1998; Savory and Garruto, 1998).

Squalene has been shown to induce antibodies associated with lupus (Satoh et al., 2003) and to trigger chronic T-cell-mediated rheumatoid arthritis (Carlson et al., 2000). Its actions in the CNS have not been extensively investigated, but some studies using very high concentrations have demonstrated swelling of astrocytic processes (Gajkowska et al., 1999).

In addition to direct toxic actions on the CNS, aluminum, and squalene might act indirectly by stimulation of a generalized immune response. In fact, this is, what the adjuvants are placed in vaccines to do in the first place. Another possibility is that of an imbalanced immune response. Rook and Zumla (1997) hypothesize that multiple Th2 (T helper cell type-2)-inducing vaccinations, stressful circumstances, and the method of vaccine administration (oral vs subcutaneous vs intramuscularly) could lead to a shift from Th2 to Th1 (T-helper cell type-1) immunity (Rook and Zumla, 1997, 1998). Both aluminum hydroxide and squalene have previously been shown to stimulate a Th2-cytokine response (Valensi et al., 1994; Brewer et al., 1999). A recent study comparing inbred and outbred mouse strains injected with recombinant protective antigen (AVA) vaccine and challenged with *Bacillus anthracis*, found that both mouse strains displayed a predominantly Th2-based immune response (Flick-Smith et al., 2005). Such a Th1–Th2 shift could stimulate autoimmune

Table 1  
Summary of Human ALS and GWI Symptoms  
Compared With the Symptoms Observed  
in Aluminum-Injected Mice

Comparison of human ALS and GWI symptomology with symptoms observed in aluminum-injected mice			
Symptoms	ALS <sup>a</sup>	GWI <sup>b</sup>	Aluminum-injected mice
Muscular strength and endurance loss	+	+	+
Enhanced anxiety	+	+	+
Memory impairment	+	+	+
Dermatitis	–	+	+

This table also outlines the similarities between human ALS and Gulf War illness.

<sup>a</sup>Bromberg (2002); <sup>b</sup>Haley et al. (1997).

processes that target the neurons. Whereas a plausible mechanism, a recent study of blood samples from Gulf war veterans showed evidence for Th1 immune activation (Skowera et al., 2004).

Whereas significant behavioral and neuropathological outcomes with aluminum hydroxide and some additionally significant outcomes to the combination of adjuvants, it is important to recognize that these were achieved under *minimal* conditions was demonstrated. Table 1 shows a summary of human ALS and GWI symptoms compared with the symptoms observed in aluminum-injected mice. The likelihood that a synergistic effect exists between adjuvants and other variables such as stress, multiple vaccinations, and environmental toxic exposure is another possibility that cannot be ruled out. A recent study examining some of these combinations showed that stress, vaccination, and pyridostigmine bromide, a carbamate anticholinesterase inhibitor, may synergistically act on multiples stress-activated kinases in the brain to cause neurological impairments in GWI (Wang et al., 2005). In addition, genetic background might play a crucial role. Regarding to this last point, gene–toxin interactions remain a largely unexplored area in GWI and neurological disease in general.

However, interactions of various stressors or adjuvants does not have to be necessarily synergistic, for example, in the present study the combination of aluminum hydroxide and squalene seemed to



have less effect on motor behavior and anxiety than either aluminum hydroxide or squalene alone. The possibility of competing effects on immune response cannot be over ruled and deserves further investigation.

The current DOD immunization schedule requires a higher number of injections (six) than used in 1990–1991. The majority of those vaccinated with the AVA vaccine to date have been service personnel. As serious as this might be for the potential for adjuvant-associated complications in this population, legislation now before US Congress might mandate similar vaccination regimes for the civilian population as well (e.g., the Biodefense and Pandemic Vaccine and Drug Development Act of 2005). If a significant fraction of the military and civilians vaccinated were to develop neurological complications, then the impact on US society would be profound.

In addition, the continued use of aluminum adjuvants in various vaccines (i.e., Hepatitis A and B, DPT, and so on) for the general public may have even more widespread health implications. Until vaccine safety can be comprehensively demonstrated by controlled long-term studies that examine the impact on the nervous system in detail, many of those already vaccinated as well as those currently receiving injections may be at risk in the future. Whether the risk of protection from a dreaded disease outweighs the risk of toxicity is a question that demands urgent attention.

## Animal Ethics Committee Approval

Protocols governing the use of animals were approved by review committees of the University of British Columbia and were in compliance with guidelines published by the Canadian Council on Animal Care and are in accordance with the international guidelines including the NIH Guide for the Care and Use of Laboratory Animals, as well as the EEC Council Directive.

## Conflict of Interest Statement

None of the authors have received any grants or funding from Biopart, Chiron, and Corixa, nor any other pharmaceutical companies named in this article.

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## Aluminum hydroxide injections lead to motor deficits and motor neuron degeneration

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### Abstract

Gulf War Syndrome is a multi-system disorder afflicting many veterans of Western armies in the 1990–1991 Gulf War. A number of those afflicted may show neurological deficits including various cognitive dysfunctions and motor neuron disease, the latter expression virtually indistinguishable from classical amyotrophic lateral sclerosis (ALS) except for the age of onset. This ALS “cluster” represents the second such ALS cluster described in the literature to date. Possible causes of GWS include several of the adjuvants in the anthrax vaccine and others. The most likely culprit appears to be aluminum hydroxide. In an initial series of experiments, we examined the potential toxicity of aluminum hydroxide in male, outbred CD-1 mice injected subcutaneously in two equivalent-to-human doses. After sacrifice, spinal cord and motor cortex samples were examined by immunohistochemistry. Aluminum-treated mice showed significantly increased apoptosis of motor neurons and increases in reactive astrocytes and microglial proliferation within the spinal cord and cortex. Morin stain detected the presence of aluminum in the cytoplasm of motor neurons with some neurons also testing positive for the presence of hyper-phosphorylated tau protein, a pathological hallmark of various neurological diseases, including Alzheimer's disease and frontotemporal dementia. A second series of experiments was conducted on mice injected with six doses of aluminum hydroxide. Behavioural analyses in these mice revealed significant impairments in a number of motor functions as well as diminished spatial memory capacity. The demonstrated neurotoxicity of aluminum hydroxide and its relative ubiquity as an adjuvant suggest that greater scrutiny by the scientific community is warranted.

### Keywords

Aluminum hydroxide; Adjuvant; Neurotoxicity; Gulf War Syndrome; Amyotrophic lateral sclerosis

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## 1. Introduction

Various studies have established a correlation between Gulf War service (1990–1991) and a multi-system disorder commonly termed Gulf War Syndrome. Included in GWS are various neurological disorders, including an apparent cluster of cases of amyotrophic lateral sclerosis [1–4]. Haley [3] described classical ALS symptoms such as muscle weakness and wasting, impaired speech and swallowing, difficulty in breathing, and fasciculation in Gulf War veterans years after they first developed other symptoms of GWS. Seventeen of the 20 servicemen diagnosed with Gulf War illness and definite ALS were less than 45 years of age with the youngest of these 20 years old. All 20 of these patients presented with signs of upper (motor cortex or bulbar region) and lower (spinal cord) motor neuron degeneration. None of these patients had a family history of ALS or of other neurodegenerative disorders. Horner et al. [2] conducted a nationwide case study performed to identify incidence levels of ALS for the decade after August 1990 amongst active duty members of the military. One hundred and seven confirmed cases of ALS were identified from approximately 2.5 million eligible military personnel. When standardized to the average 1990 US general population, the average annual incidence of ALS among non-deployed military population was 1.4 per 100 000 persons per year compared to the generally accepted overall population incidence of 1.5 cases of ALS per 100 000. The incidence rate of ALS among the deployed military population was 3.6 per 100 000 persons/year. Weisskopf et al. [4] noted a general increase in ALS in US military populations going back a number of decades regardless of the conflict.

ALS–GWS is one of only two ALS disease clusters currently accepted as satisfying the definition of a cluster. The other is the Guamanian variant of ALS first described after World War 2 termed amyotrophic lateral sclerosis parkinsonism dementia complex (ALS–PDC). This spectrum of disorders, once present with an incidence levels hundreds of times higher than in the continental United States [5] (see Kurland, 1988, for review), expressed in one of two ways. The first was as a nearly classical form of ALS; the second was a form of parkinsonism associated with an Alzheimer's disease-like dementia (PDC). About 10% of the victims developed both disorders, with the ALS phenotype typically appearing first. Studies into potential etiologies focused on environmental factors with most attention eventually directed at the consumption of toxin-containing seeds of the local variety of cycad palm [6] and the presence of high aluminum in the soil on southern Guam [7].

In regard to the GWS-ALS AVA vaccine, attention has recently been directed at the anthrax vaccine adsorbed (AVA) and various vaccine ingredients, in particular the known and suspected adjuvants, aluminum hydroxide and squalene [8]. An adjuvant is a substance added during vaccine production designed to non-specifically increase the immune response to an antigen [9]. Aluminum compounds were first identified as adjuvants over 90 years ago. Currently aluminum, in various forms (aluminum hydroxide, aluminum phosphate and aluminum sulfate), is the most commonly licensed adjuvant whose use is generally regarded by both the pharmaceutical industry and the various governmental regulatory agencies as safe [10]. Various studies have found no adverse or long-term health effects due to aluminum adjuvants [11–13] and the Food and Drug Administration (FDA) has continued its longstanding approval for the use of aluminum in this fashion.

In spite of the long history of widespread use, the physicochemical interactions between aluminum compounds and antigens are relatively poorly understood and their underlying mechanisms remain relatively unstudied [14]. It also seems that there have been no rigorous animal studies of potential aluminum adjuvant toxicity. The absence of such studies is peculiar given the well known observation that aluminum in general can be neurotoxic under a number of conditions [15,16] and adjuvants in particular have previously been implicated in neurological disease [17–19]. Table 1 shows the results from previous studies that treated

animals with aluminum hydroxide, listing the resulting impacts on the nervous system. In context to the use of aluminum in vaccines, LD<sub>50</sub> values for aluminum hydroxide have not been published to date to the best of our knowledge (J.T. Baker Material Safety Data Sheets).

The potential for aluminum injections to induce macrophagic myofasciitis has also been noted in the literature [20–22].

A previous publication looked at the potential neurotoxicity of several known or suspected vaccine adjuvants [8]. In the current study, we will focus exclusively on the impact of aluminum hydroxide injections on motor and cognitive behaviours and on the expression of different forms of neuropathology in an *in vivo* mouse model.

## 2. Experimental procedures

### 2.1. Experimental animals

In our initial study [8], young adult (3 month old) CD-1 male mice were used (approx. 35 g at experiment onset). Younger animals were deliberately chosen to mimic the typical age of service during the Gulf War [3]. Four subcutaneous injection groups (two injections spaced 2 weeks apart) were used: control saline/phosphate buffered solution (PBS) ( $n = 10$ ); aluminum hydroxide ( $n = 11$ ); squalene ( $n = 10$ ); and aluminum hydroxide and squalene ( $n = 10$ ). The current study will report only on the aluminum treated and control groups from this experimental series. A second series of experiments was conducted on 9 month old CD-1 males that received six aluminum hydroxide injections over a 2 weeks period. These mice, along with controls and other treatment groups (to be reported elsewhere), were subjected to a more rigorous behavioural testing regime to be described below. Histological analyses of the spinal cords and brains of these mice are in progress.

All animals in both experiments were singly caged at the Jack Bell Research Centre animal care facility in Vancouver, B.C., Canada. An ambient temperature of 22 °C and a 12/12 h light cycle were maintained throughout the experiment. All mice were fed Purina® mouse chow and given access to both food and water *ad libitum*.

Mice from both studies were sacrificed with an overdose of halothane and transcardially perfused with 4% paraformaldehyde (PFA). CNS tissues were collected for histological examination. Fixed brains and spinal cords from all mice were transferred to a 30% sucrose/PBS solution overnight and then frozen and stored at –80 °C until sectioning. All brain/cord tissue blocks were mounted in Tissue-Tek optimum cutting temperature (O.C.T) compound (Sakura, Zoeterwoude, Netherlands), and then sectioned by cryostat into 30 µm coronal slices. Spinal cords were sectioned at 25 µm in the transverse plane. The sections were cryoprotected in 30% ethylene glycol–20% glycerol–dibasic and monobasic sodium phosphate solution and kept frozen at –20 °C until use.

### 2.2. Adjuvants

Alhydrogel®, an aluminum hydroxide (Al(OH)<sub>3</sub>) gel suspension, was used as a source of aluminum hydroxide. Alhydrogel is manufactured by Superfos Biosector a/s (Denmark) and was purchased from SIGMA Canada.

**2.2.1. Doses**—To calculate approximate human dosages of aluminum hydroxide for our experiments, we used the following information: The AVA vaccine for human use is made by Bioport Corporation, of Lansing, Michigan. According to product data sheets from the Michigan Biologic Products Institute (MBPI, Lansing, Michigan, USA; Bioport's predecessor), a single dose of AVA vaccine contains 2.4 mg of aluminum hydroxide (equivalent to 0.83 mg aluminum). Based on an assumed average human body weight of 70–80 kg, the amount per



kg body weight would be approximately 30–34  $\mu\text{g/kg}$ . Soldiers or civilians receiving the vaccine would have received between 30–34  $\mu\text{g/kg}$  (1 injection) and up to approx. 200  $\mu\text{g/kg}$  if six injections were received.

The adjuvant injections in the treated mice were calibrated based on average animal weight for both experiments. At 3-month-old male CD-1 mice weigh approx. 35 g; at 9 months, the weight is approx. 50 g. In Experiment 1, we performed two injections of a suspension of aluminum hydroxide of (50  $\mu\text{g/kg}$ ) in a total volume of 200  $\mu\text{L}$  sterile PBS (0.9%) spaced 2 weeks apart. The mice in this experiment would therefore have received 100  $\mu\text{g/kg}$  versus a probable 68  $\mu\text{g/kg}$  in humans. In Experiment 2, mice received six injections for a total of 300  $\mu\text{g/kg}$  aluminum hydroxide over 2 weeks. Controls in both studies were injected with 200  $\mu\text{L}$  PBS.

The injection site for human administration is typically subcutaneous over the deltoid muscle. For injections in mice we used a subcutaneous injection into the loose skin behind the neck (the “scruff”) to minimize discomfort and for ease of injection.

### 2.3. Behavioural tests

In the first study, mice were subjected at regular intervals to specific behavioral tests of motor and cognitive function, including wire mesh hang (2 $\times$ /week), open field (1 $\times$ /week), and water maze (1 $\times$ /week) over a 6 months post injection period (see [22]). The order in which the animals were tested was randomized for each trial. In the second study, we conducted a more detailed behavioural examination based on the automated EthoVision system (Noldus Information Technology, Seattle, WA) employing a video camera and tracking software (Noldus EthoVision<sup>®</sup> 3.1). Individual movements of the mice were tracked for 5 min in an open field at weekly intervals. The software allowed for quantitative measurements of a variety of motor functions, including distance moved, percentage of time moving, velocity, and a variety of others. These latter experiments continued for 28 weeks following the last injections.

### 2.4. Histological measurements (Experiment 1)

**2.4.1. NeuN and active caspase-3**—As cited in Petrik et al. [8], five mice were used from each treatment group. In each, multiple brain ( $n = 3$ ) and spinal cord ( $n = 8$ ) sections at different levels were examined. Fluorescent intensity levels of NeuN and activated caspase-3 were used to identify neurons and cells dying by apoptosis, respectively. Regions of interest were defined using landmarks from mouse brain and spinal cord stereotaxic atlases [23,24]. All sections were counted in an unbiased manner under a 40 $\times$  objective.

#### 2.4.2. Choline acetyltransferase (ChAT) and Glial fibrillary acidic protein (GFAP)

—As cited in Petrik et al. [8], the ChAT antibody was used to identify cholinergic motor neurons in the brain and spinal cord [25,26]. GFAP was used to label reactive astrocytes [27, 28].

**2.4.3. Iba-1**—A rabbit polyclonal antibody against the ionized calcium binding adapter molecule (Iba-1) (Wako, Richmond, VA, USA) was used to stain for activated microglia [29]. For Iba-1 fluorescent immunolabeling, staining followed the same protocol used for GFAP labeling except for the following modification: Sections were incubated with primary rabbit-anti-Iba-1 (in PBST with 1% NGS + 1% BSA; 1:1000 dilution) overnight at 4 °C. Sections were then incubated in anti-rabbit AlexaFluor 546<sup>™</sup> secondary antibody for 2 h at room temperature (Molecular Probes; Eugene, OR, 1:200).

**2.4.4. Morin (3,5,7,2',4'-pentahydroxyflavone, BDH)**—Morin (M4008-2G, Sigma) is a fluorochrome which forms a fluorescent complex with aluminum fluorescing green (with an

excitation wavelength of 420 nm) [15,30] when it does so. The aluminum-Morin fluorescence assay was used for the visualization and detection of aluminum in lumbar spinal cord and other CNS tissues in the present experiments. The Morin stain was used as a 0.2% solution in 85% ethyl alcohol containing 0.5% acetic acid. All mounted sections were first washed with PBS twice for 5 min. Sections were then pretreated for 10 min in a 1% aqueous solution of hydrochloric acid, rinsed in double distilled water (ddH<sub>2</sub>O) twice for 5 min, and immersed in 0.2% Morin stain for 10 min. The sections were then washed in ddH<sub>2</sub>O twice for 5 min, dehydrated in 70%, 90%, and 100% ethyl alcohol (EtOH), and cleared with 100% xylene. All sections were then mounted using Vectashield mounting medium (Vector Laboratories), sealed with clear nail polish, and allowed to air dry.

**2.4.5. Staining for hyper-phosphorylated tau protein**—Hyper-phosphorylated tau (Anti-Human PHF-Tau, Pierce Biotechnology, Inc., Rockford, IL) labeling was determined using the non-fluorescent diaminobenzidine (DAB) method. Slides containing mounted sections of lumbar spinal cord were first rinsed twice PBS (2× 5 min) before performing antigen unmasking. Endogenous peroxidase activity was quenched using 0.3% hydrogen peroxide in methanol for 20 min. The sections were rinsed twice in PBS (2× 5 min) before blocking at room temperature for 1 h in M.O.M. blocking reagent (M.O.M. Kit – peroxidase, cat # PK 2200, Vector Laboratories, Inc., Burlingame CA) followed by a quick rinse in PBS and a 5 min incubation in M.O.M. diluent solution. The primary PHF-Tau antibody was diluted 100× in M.O.M. diluent solution and incubation was conducted at room temperature for 1 h. After the primary antibody incubation step, the slides were rinsed twice in PBS, and then incubated in the M.O.M. biotinylated anti-mouse IgG reagent for 10 min. The sections were rinsed in PBS before incubating with the secondary antibody (Vectastain ABC Elite Kit, cat # PK-6101) for 1 h at room temperature followed by incubation in the Vectorstain ABC Elite Reagents for another 30 min. The slides were rinsed again in 1× PBS. Color development was achieved using the Vector ImmPACT™ DAB solution (cat # SK-4105). When the desired color was achieved, the slides were rinsed in ddH<sub>2</sub>O for 5 min and counter-stained in 0.1% methyl green for 5 min. After counter-staining, the slides were rinsed briefly in ddH<sub>2</sub>O, two changes of 95% ethanol and two changes of 100% ethanol. The slides were allowed to dry before they were mounted in Permount® (Fisher Scientific, Fair Lawn, NJ).

## 2.5. Microscopy

Brain and spinal cord sections processed with fluorescent antibodies or DAB were viewed with a Zeiss Axiovert 200 M (Carl Zeiss Canada Limited, Toronto, ON, Canada) microscope at 40× and 100× (under oil) magnification. DAPI (blue fluorescence) was viewed with a 359/461 nm absorption/emission filter. Alexa Fluor 546™ (red), and rabbit IgG DuoLuX™ (red) were viewed with 556 557/572 573 nm filter. FITC was viewed with a 490 494/520 525 nm filter. Brain and lumbar spinal cord sections for histology were chosen randomly for each group. When counting using 40× magnification two images were captured per spinal cord section: ventral left, ventral right. 40× images were 350 × 275 μm and 100× images were 50 × 115 μm. Images were captured using AxioVision 4.3 software.

## 2.6. Criteria for determination and quantification of labeled cells

For quantification, only cells that were in focus and completely within the field of view were counted. To eliminate the likelihood that the same cell would be counted twice, slices for each histological experiment were drawn from only one well of the collection dish to ensure that sections were at least 250 μm apart. Regions of interest for cell counts were defined using landmarks and reference points from mouse spinal cord and brain stereotaxic atlases [39,40]. In the spinal cord, only cells which were anterior to the central canal and deep apex where the grey and white matters meet were considered as part of the ventral horns; conversely, only cells which were posterior to the central canal and the posterior deep apex were considered as

part of the dorsal horns. These criteria applied regardless of the spinal segments examined. In the brain, only cells found within the corresponding brain structures were counted. All sections were counted in an unbiased manner (a code key was assigned to the animals for tracking purposes, but did not reveal the identity of treatment the animal was prescribed).

## 2.7. Statistics

Values for each mouse on the individual tasks and in the cell counts were used to calculate mean  $\pm$  S.E.M. for each group and condition. Behavioral scores and cell counts were normalized to the mean value of controls. The means were compared using one- or two-way ANOVA (Statistica, Statsoft Inc., Tulsa, OK; GraphPad Prism, San Diego, CA).

## 3. Results

Unlike the Petrik et al. [8] study which showed a loss of ChAT positive motor neurons in the lumbar cord of aluminum hydroxide treated mice, there was no significant difference in ChAT labeling or motor neuron counts in either the cervical or thoracic spinal cord segments (Fig. 1A and B). However, the aluminum injected group showed a highly significant increase in the expression of GFAP positive astrocytes (70%) are the control group (listed as 100% for all graphs; Fig. 1C) in the cervical segment of spinal cord. These GFAP results mirrored the outcomes previously reported in lumbar cord.

Iba-1 labeling demonstrated significantly increased levels of activated microglia in the lumbar spinal cord of animals injected with aluminum (111%) compared to controls (Fig. 1E). Other levels of cord were not tested for microglia in the present study.

Only mice injected with aluminum hydroxide showed significantly increased Morin labeling of cells in lumbar spinal cord compared to the other groups (Fig. 2A–E). Similarly, only aluminum-injected mice showed the presence of abnormal tau protein in motor neurons in lumbar cord (Fig. 3). Other regions of the cord were not tested in the current studies for either Morin or tau protein.

The multiple aluminum hydroxide injections of experiment 2 showed profound effects on motor and other behaviours as shown in Figs. 4 and 5. Multiple aluminum injections produced significant behavioural outcomes including changes in locomotive behaviour, (Fig. 4) and induced memory deficits on water maze tasks (Fig. 5). Other behavioural measures including muscle strength and endurance as measured by the wire hang and motor coordination and balance as measured by rotarod were not significantly affected.

## 4. Discussion

The current results extend the preliminary results reported by Petrik et al. [8] by showing that microglial activation is part of the underlying pathology in the lumbar cord. These data add to those previously reported, i.e., the loss of motor and other neurons and the activation of reactive astrocytes. Taken together with the current data, the overall activation of a glial inflammatory response in lumbar cord suggests that this process is a key early stage of the pathological events leading to motor neuron death. This interpretation is supported by an absence of motor neuron loss and astrocyte activation in the other levels of the spinal cord observed in the present study. In ALS and in animal models of the disease, glial activation followed by motor neuron death often appears to proceed in sequential manner along the ventral neuraxis with the first signs of pathology appearing first in lumbar cord [31]. Given this, it seems possible that an examination of later time points would show pathological responses in the thoracic and cervical cord as well. Alternatively, the aluminum shown to be present in lumbar cord motor neurons may not

have reached these other spinal cord segments. Studies now in progress will determine if motor neurons in these other segments stain positively for aluminum.

The positive Morin staining in lumbar cord clearly demonstrates that post injection aluminum finds entry into this part of the nervous system. One possibility is that it does so by retrograde transport from muscles to motor neurons in particular segments. This seems unlikely given that our paradigm of injecting *subcutaneous* should not have targeted any particular spinal cord segment. Another possibility is that aluminum can enter the CNS in a systemic manner if it enters the circulatory system. Experiments in progress are designed to distinguish between these possibilities.

The presence of hyper-phosphorylated tau protein, one of the hallmarks of both Alzheimer's disease and ALS-PDC of Guam, in motor neurons in lumbar spinal cord clearly suggests that additional pathological processes associated with aluminum are occurring.

The behavioural outcomes in the second experiment reported here reinforce the pathological outcomes seen in the first studies. While the histological measurements from these studies are still pending, the extent of the behavioural deficits strongly suggests that we will observe widespread neuronal pathologies. The greater extent of the behavioural outcomes in this experiment may be related to the experimental paradigm that tripled the number of aluminum hydroxide injections.

Overall, the results reported here mirror previous work that has clearly demonstrated that aluminum, in both oral and injected forms, can be neurotoxic [15,16,32,33]. Potential toxic mechanisms of action for aluminum may include enhancement of inflammation (i.e., microgliosis) and the interference with cholinergic projections [34], reduced glucose utilization [33], defective phosphorylation-dephosphorylation reactions [35], altered rate of transmembrane diffusion and selective changes in saturable transport systems in the blood brain barrier (BBB [36], and oxidative damage on cellular processes by the inhibition of the glutathione redox cycle [37].

Given the above, it is not surprising that aluminum has been widely proposed as a factor in neurodegenerative diseases and has been found in association with degenerating neurons in specific CNS regions [38–41]. In animal studies, aluminum has been linked to the accumulation of tau protein and amyloid-beta protein and observed to induce neuronal apoptosis *in vivo* as well as *in vitro*<sup>30</sup>. Aluminum injected animals show severe anterograde degeneration of cholinergic terminals in cortex and hippocampus [42].

Aluminum in its adjuvant form can gain access to the CNS [42–44], however, oral administration of aluminum hydroxide gel does not appear to be neurotoxic in humans [45], although aluminum chloride is, in rats [46]. The route of exposure, and perhaps the form of aluminum, may be important factors that determine the potential for toxicity.

We speculate that the observed neurotoxic effects of aluminum hydroxide in the present study arise by both 'direct' and 'indirect' pathways, some of which are cited above. Direct toxicity refers to the physical presence (or close proximity) of aluminum and its potential for initiating cell death pathways. Accumulation of aluminum into the cytoplasm via cellular uptake mechanisms or diffusion could cause alterations in glutaminase and glutamine synthetase and easily alter the availability of the neurotransmitter glutamate [47]. Aluminum acting to induce abnormal tau protein accumulation could also increase neurofibrillary tangles and impair cellular transport mechanisms [48]. Outside the cell, aluminum could affect neurons by altering synapses. For example, aluminum has been shown to decrease the thickness of post-synaptic density, increase the width of the synaptic cleft, and increase the number of flat synapses [49]. Aluminum could also block voltage-activated calcium channels [50], augment the activity

of acetylcholinesterase [51], or interfere with synaptic transmission by merely accumulating in the synaptic cleft [52]. Aluminum can also induce apoptosis in astrocytes [53]. Since astrocytes are essential for maintaining neuronal health, any loss of astrocyte function could prove toxic to neurons. Indirect toxicity of aluminum could occur in various ways, including by activating various cytokines [54], releasing glutamate in an excitotoxic cascade, or by modifying various enzymatic pathways [55].

In addition to the above actions specifically on neural cells, aluminum might act indirectly by stimulating abnormal, generalized immune responses. This is, in fact, what adjuvants are placed in vaccines to do in the first place. Adjuvant neurotoxicity could thus be the result of an imbalanced immune response. Rook and Zumla [56] hypothesized that multiple vaccinations, stress, and the method of vaccination could lead to a shift in immune response [56,57]. Aluminum hydroxide has previously been shown to stimulate a Th2-cytokine response [9, 58].

While the current results and our previous study have demonstrated significant behavioural and neuropathological outcomes with aluminum hydroxide and some additionally significant outcomes due to a combination of adjuvants, it is important to recognize that these were achieved under *minimal* conditions. Table 1 summarizes aspects of human ALS and GWS symptoms compared with outcomes observed in aluminum-injected mice. The likelihood exists that a synergistic effect between adjuvants and other variables such as stress, multiple vaccinations, and exposure to other toxins likely occurs. A recent study examining some of these factors in combination showed that stress, vaccination, and pyridostigmine bromide (a carbamate anticholinesterase (AChE) inhibitor), may synergistically act on multiples stress-activated kinases in the brain to induce neurological impairments in GWS [59]. In addition, a genetic background in context to aluminum exposure may play a crucial role and may be an important area for future research.

The demonstration of neuropathological outcomes and behavioural deficits in aluminum hydroxide injected mice may provide some insight into the causes of not only GWS–ALS, but may open avenues of investigation into other neurological diseases.

## Acknowledgments

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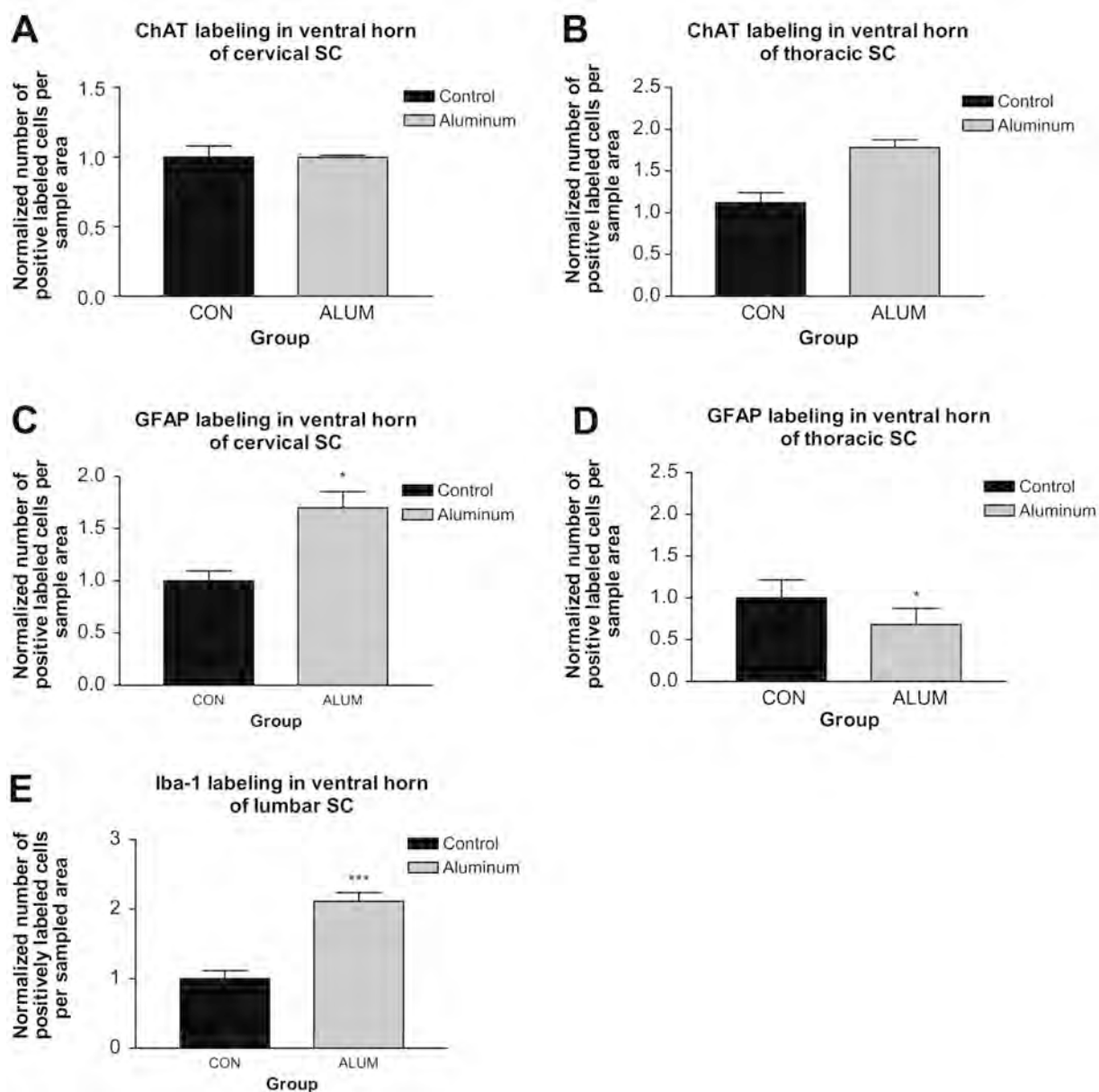


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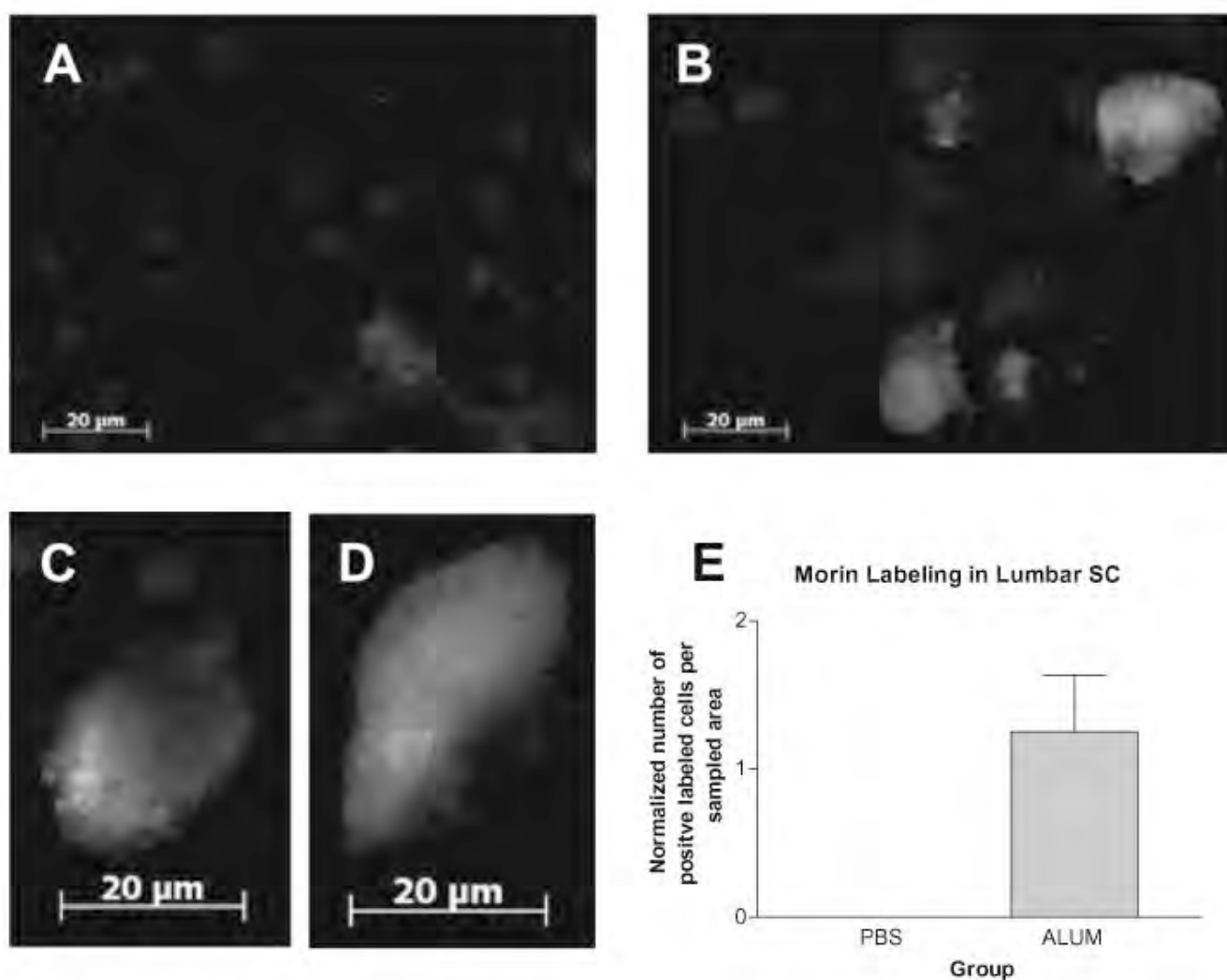
## Abbreviations

chE	Anticholinesterase
ALS–PDC	Amyotrophic lateral sclerosis- parkinsonism dementia complex
AVA	Anthrax vaccine adsorbed
BSA	Bovine serum albumin
GFAP	Glial fibrillary acidic protein
ChAT	Choline acetyltransferase
GWS	Gulf War Syndrome
NGS	normal goat serum
OCT	Optimum cutting temperature
PBST	Phosphate buffer saline – Tween 20
PFA	Paraformaldehyde

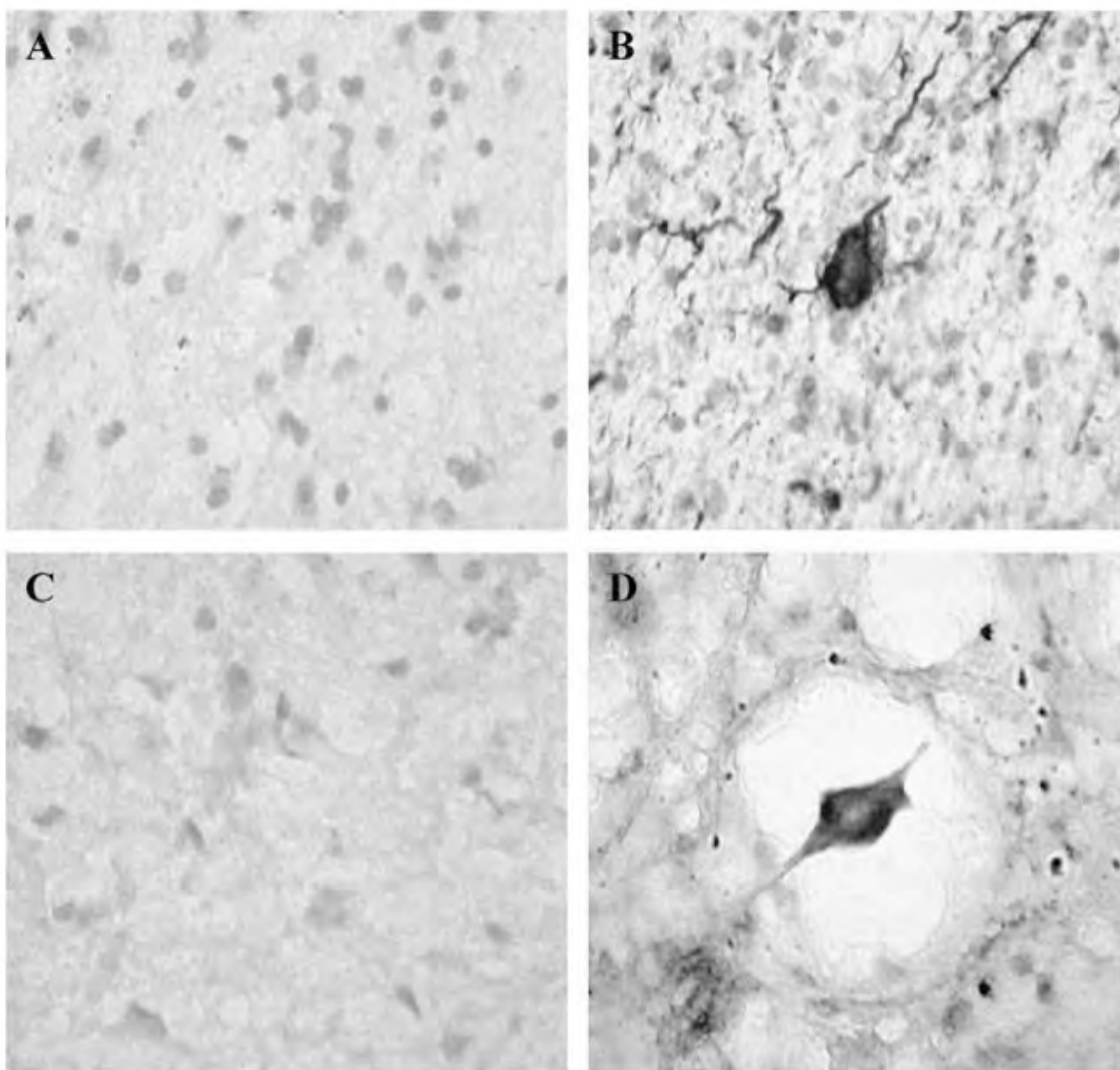
**Fig. 1.**

Impact of aluminum hydroxide on different levels of spinal cord (SC). (A and B) ChAT labeling in cervical and thoracic cords, respectively. (C and D) Normalized cell counts for GFAP labeling of reactive astrocytes in cervical and thoracic spinal cord, respectively. In cervical cord, the aluminum hydroxide treated groups showed higher levels of GFAP labeling with the aluminum alone group achieving statistical significance. (E) Iba-1 fluorescent labeling in the ventral horn of mouse lumbar cord showed that aluminum-injected mice had significantly increased numbers of activated microglia. Data are means  $\pm$  S.E.M. \*\*\* $p < 0.001$ , one-way ANOVA.

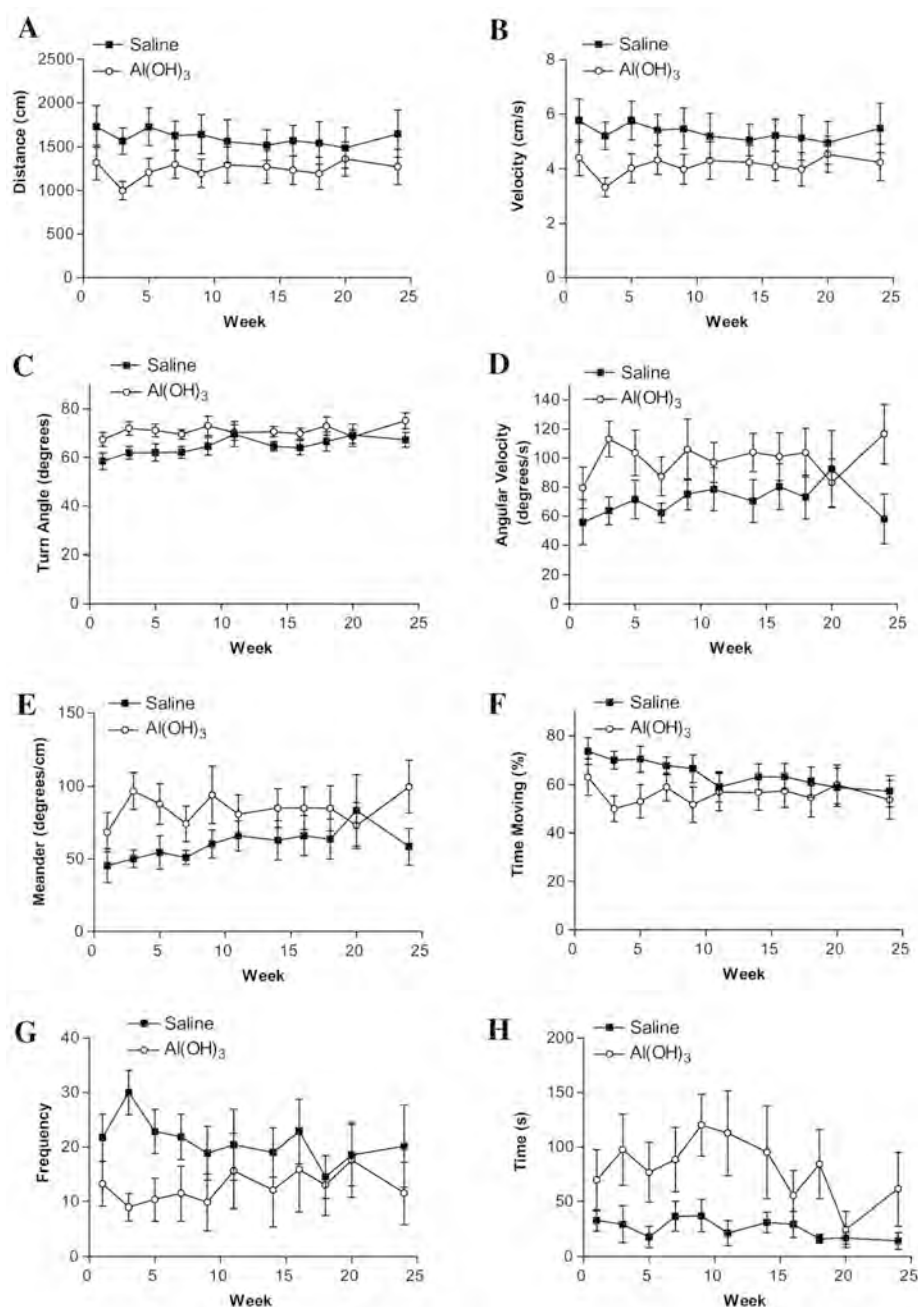




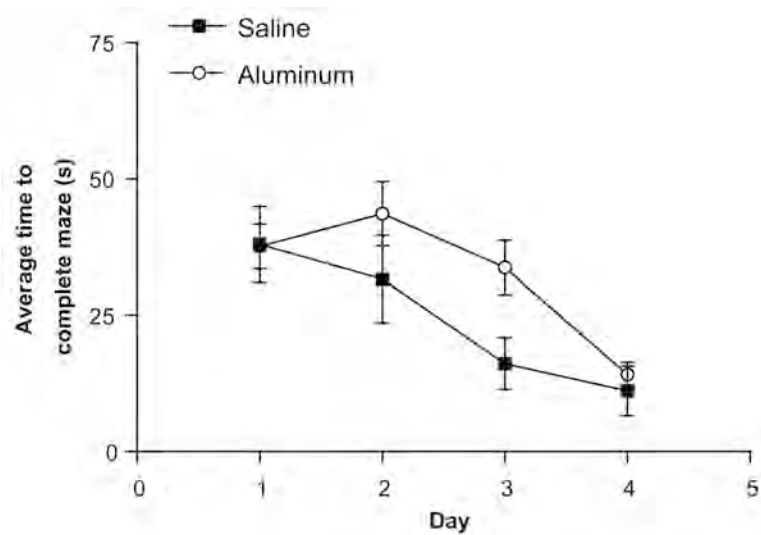
**Fig. 2.** Morin fluorescent labeling in ventral horn of mouse lumbar spinal cord. Sections from control (A) mice showed no Morin fluorescent labeling. Scale bar = 20 μm. (B) Morin-positive motor neurons in aluminum hydroxide treated mice. (C and D) Higher power of motor neurons in aluminum-injected mice showing show high levels of cytoplasmic Morin labeling. Scale bar = 20 μm. (E) Cell counts for Morin positive cells in the different treatment groups ( $n = 4$  mice/group, four sections each). Data are mean  $\pm$  S.E.M. One-way ANOVA analysis revealed a significance level of  $*p < 0.05$ .



**Fig. 3.** Hyper-phosphorylated tau immunostaining in the ventral horn of mouse lumbar spinal cord compared to Alzheimer's disease. (A) A section of human entorhinal cortex from a control patient. (B) Human entorhinal cortex section from a patient with Alzheimer's disease (sections kindly provided courtesy of Dr. P. McGeer). (C) Lumbar spinal cord sample from a saline injected mouse. (D) Equivalent section from an aluminum hydroxide injected mouse. All pictures are 100× magnification.

**Fig. 4.**

Open field movement analysis as an assessment of spontaneous activity and anxiety in control mice vs. mice injected six times with aluminum hydroxide. Aluminum hydroxide injected mice showed the following behavioural changes: (A) Shorter distances moved ( $***p < 0.0001$ ). (B) Slower movement ( $***p < 0.0001$ ). (C) Greater mean turn angle ( $***p < 0.0001$ ). (D) More rapid turning ( $***p < 0.0001$ ). (E) Greater meander ( $***p < 0.0001$ ). (F) Smaller percentage of time in overall movement ( $**p = 0.0030$ ). (G) Fewer entries into the centre of the open field ( $***p < 0.001$ ). Late entry into centre ( $***p < 0.0001$ ). (All measures, two-way ANOVA).



**Fig. 5.**

Water maze test as an evaluation of learning and memory. Mice injected 6× with aluminum hydroxide on average took significantly longer to complete the maze compared to saline injected mice (two-way ANOVA. \* $p = 0.0389$ ).

Table 1

Summary of human ALS and GWI symptoms compared with symptoms observed in aluminum-treated mice and rats. This table also outlines the similarities between human ALS and Gulf War illness.

Animal	Age	Dose	Injection type	Result	Reference
Female NIH mice	4 week	315–335 µg/kg	i.p.	Significantly elevated levels of Al in brain	Redhead et al., 1991
Male and female Long Evan rats	2 month	100 or 300 mg/kg/day	Oral	Significantly reduced learning ability and elevated levels of Al in brain	Bilkei-Gorzo, 1993
Male Swiss albino mice	Not stated	~20 µg/kg/day	Oral	Significantly elevated levels of Al in brain, kidney and liver.	Sahin et al., 1994
Pzh:SFIS mice	Not stated	1.0 mg every 2 weeks or 0.1 mg 5 days/week	i.p.	Significantly elevated levels of Al in liver and tibia (bone), but not in brain.	Fiejka et al., 1996

# Aluminum Vaccine Adjuvants: Are they Safe?

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**Abstract:** Aluminum is an experimentally demonstrated neurotoxin and the most commonly used vaccine adjuvant. Despite almost 90 years of widespread use of aluminum adjuvants, medical science's understanding about their mechanisms of action is still remarkably poor. There is also a concerning scarcity of data on toxicology and pharmacokinetics of these compounds. In spite of this, the notion that aluminum in vaccines is safe appears to be widely accepted. Experimental research, however, clearly shows that aluminum adjuvants have a potential to induce serious immunological disorders in humans. In particular, aluminum in adjuvant form carries a risk for autoimmunity, long-term brain inflammation and associated neurological complications and may thus have profound and widespread adverse health consequences. In our opinion, the possibility that vaccine benefits may have been overrated and the risk of potential adverse effects underestimated, has not been rigorously evaluated in the medical and scientific community. We hope that the present paper will provide a framework for a much needed and long overdue assessment of this highly contentious medical issue.

**Keywords:** Aluminum adjuvants, adjuvant safety, autoimmunity, autism, Gulf War Syndrome, multiple sclerosis, macrophagic myofasciitis, neurotoxicity, seizures, Th2 immune response, vaccines.

## INTRODUCTION

Aluminum is the most commonly used vaccine adjuvant and until recently the only one licensed for use in the U.S. [1-4]. In its absence, antigenic components of most vaccines (with the exception of live attenuated vaccines), fail to launch an adequate immune response [1, 5, 6]. Paradoxically, despite almost 90 years of widespread use of aluminum adjuvants [3] their precise mechanism of action remains poorly understood [1, 2]. Furthermore, a growing number of studies have linked the use of aluminum adjuvants to serious autoimmune outcomes in humans [5-8]. That concerns about aluminum adjuvant safety are indeed warranted is evident from the summary conclusions of the *Aluminum in Vaccines* workshop held in Puerto Rico in 2000 [2]. The written consensus amongst the participants of the workshop was listed under the rubric of "pervasive uncertainty", a term used to denote what remained unknown regarding potential aluminum toxicity from adjuvants. The specific areas of concern were: "1) toxicology and pharmacokinetics, specifically the processing of aluminum by infants and children, 2) mechanisms by which aluminum adjuvants interact with the immune system and 3) the necessity of adjuvants in booster doses." In the concluding paragraphs of the summary, the report nevertheless claimed that "the use of salts of aluminum as adjuvants in vaccines has proven to be safe and effective" [2]. In light of the items of "pervasive uncertainty", this statement remains questionable. Given that multiple aluminum-adjuvanted vaccines are often given to very young children (i.e., 2 to 6 months of age), in a single day at individual vaccination sessions [9, 10], concerns for potential impacts of total adjuvant-derived aluminum body burden may be significant [11, 12]. These issues warrant serious consideration since, to the best of our knowledge, no adequate studies have been conducted to assess the safety of simultaneous administration of different vaccines to young children. Another issue of concern is the lack of any toxicological evaluation about concomitant administration of aluminum with other known toxic compounds which are routine constituents of commercial vaccine preparations, e.g., formaldehyde, formalin, mercury, phenoxyethanol, phenol, sodium borate, polysorbate 80, glutaraldehyde [13, 14]. In spite of all this, aluminum adjuvants are generally regarded as safe [2, 13],

and some researchers have even recommended that no further research efforts should be spent on this topic despite "a lack of good-quality evidence"[15].

In the following paper we aim to provide an overview of what is currently known about aluminum adjuvants, their modes of action and mechanisms of potential toxicity. We first present well established evidence that implicates aluminum in a variety of neurological disorders. We then elaborate on the unresolved controversy about aluminum adjuvant safety.

## ALUMINUM TOXICITY IN ANIMALS AND HUMANS

Aluminum is a well demonstrated toxin in biological systems [16] whose more specific impacts on the nervous system have been widely documented (Table 1). As early as 1911, Dr. William Gies had summarized data from 7 years worth of experimental testing in humans and animals on the effects of oral consumption of aluminium salts, then used primarily in baking powders, food preservation, and dye manufacturing [17]. The outcome of these studies led Gies to conclude that: "the use in food of aluminum or any other aluminum compound is a dangerous practice." Gies' concerns have since been borne out by experimental studies showing that oral exposure to aluminum that is at levels "typically" consumed in an average "Western diet" over an extended period of time, produce strikingly similar outcomes in rodents to those induced by intracerebral injection of aluminum salts (Table 1) with the exception of seizures and fatalities [18, 19]. Animals intoxicated with dietary aluminum routinely show impaired performance in learning and memory tasks, impaired concentration, and behavioural changes including confusion and repetitive behaviours [18, 19]. Consistent with these observations, according to the most recent and elaborate toxicological report for aluminum prepared by the Agency for Toxic Substances and Disease Registry (ATSDR): "There is a rather extensive database on the oral toxicity of aluminum in animals. These studies clearly identify the nervous system as the most sensitive target of aluminum toxicity"[16].

In humans, aluminum toxicity has been solidly linked to dialysis-associated encephalopathy syndrome, also known as dialysis dementia (Table 1). This syndrome occurs in patients with renal failure subjected to chronic dialysis treatment and is caused by accumulation of intravenously administered aluminum from the dialysis fluid (which is derived from aluminum-treated tap water [20]). Dialysis dementia is associated with abnormally high levels of plasma and brain aluminum and is generally fatal within 3 to 7

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Table 1. Neurodevelopmental Toxicity of Aluminum Compounds in Various Species

Aluminum source/compound	Dose & duration	Route	Species	Neurodevelopmental adverse effects
Standard infant feeding solution	~20 µg/kg/day, >10 days	Intravenous (parenteral)	Human, premature infants	Reduced developmental attainment at the corrected post-term age of 18 months, as evidenced by significantly lower Bayley Mental Development Index (BMDI) scores (mean loss of one point on the BMDI/day of full intravenous feeding, after adjustment for potentially confounding factors) compared to infants fed with Al-depleted solutions [32]
Al-containing antacids	Chronic	Oral	Human infants	Craniosynostosis (premature ossification of the skull and obliteration of the sutures) [33]
Al-containing dialysis fluid (derived from Al-sulphate treated tap water)	1 ppm, chronic (2-5 years)	Intravenous	Human, kidney failure patients (15-61 years old at the start of the dialysis treatment)	Speech impairments (stuttering, dysarthria, dyspraxia, motor aphasia), movement disorders (twitches, tremors, myoclonic jerks, seizures, motor apraxia), cognitive impairments and behavioural changes (progressive dementia, paranoia, confusion, psychosis), death [21]
Al-sulphate (present as flocculant in potable water supplies, accidentally released in high amounts)	500-3000 x the acceptable limit under European Union legislation (0.200 mg/L), chronic (15 years)	Oral	Human adult (female, 44 years old)	Sporadic early-onset $\beta$ amyloid angiopathy (Alzheimer's-related disease), difficulty in finding words, progressive dementia, visual hallucinations headache, anxiety, cerebral ischaemia, death [34]
Various dietary	Chronic	Oral	Elderly human subjects	Impaired visuo-motor coordination, poor long-term memory, and increased sensitivity to flicker (correlated with high Al-serum levels [35])
Al-oxide fumes, occupational exposure	0.13-1.95 mg/m <sup>3</sup> , chronic	Inhalation	Human, adults (mean age 39 years)	Headache, emotional irritability, concentration difficulty, insomnia, mood lability [36]
Various: Al-chloride, Al-phosphate, Al-powder slurry	Single sub-lethal dose	Intracerebral injection	Cats, rabbits	Decline in memory, impaired learning responses, deterioration in psychomotor control, epileptic seizures and death, neurofibrillary degeneration (resembling Alzheimer's disease neurofibrillary tangles [37-42])
Al-hydroxide	2 injections, 2 weeks apart	Subcutaneous injection (behind the neck)	Mice, 3-month old	Motor neuron degeneration and apoptosis, motor function deficits, decrease in strength, cognitive deficits and decreased performance in learning tasks, decrements in spatial memory, activation of microglia [43, 44]
Al-containing food pellets	0.5-1.7 mg/kg/day (typical human), chronic (22-32 months)	Oral	Rats, 6-month old at the start of treatment	Cognitive deterioration and impaired performance in learning tasks, impaired concentration, behavioural changes including confusion and repetitive behaviour [45]
Al-lactate	500-1000 ppm, chronic (during gestation and lactation)	Oral	Mice dams	Hind limb paralysis, seizures and death (dams), lower neurobehavioral development and altered performance on a neurobehavioural test battery in pups (foot splay, forelimb and hind limb grip strengths [46])

months following the sudden overt manifestation of clinical symptoms in patients who had been on dialysis treatment for 3 to 7 years [21, 22] (unless treated with chelating agent such as desferrioxamine (DFO) or reverse osmosis to remove aluminum salts from the water used to prepare the dialysis fluid [20-23]). Symptoms appear suddenly and worsen either during or immediately after a dialysis session [21, 22, 24-26]. The first symptom to appear is a speech abnormality, then tremors, impaired psychomotor control, memory losses, impaired concentration, behavioural changes, epileptic seizures, coma and death [20-22, 24-26]. Although frequent ingestion of aluminum-containing medicines was also thought to be a contributing factor in dialysis dementia [26], it should be noted that there were no incidences of this syndrome prior to introduction of aluminum salts in water supplies [21, 27]. Furthermore, symptomatic patients rapidly improved when efforts were made to remove aluminum from the dialysis fluid, despite the fact they still ingested large amounts of

aluminum-containing phosphate binding gels [21]. In addition to dialysis dementia, a host of neurodegenerative complications and diseases such as Alzheimer's [11, 28], Parkinson's disease [29], amyotrophic lateral sclerosis (ALS) [29], multiple sclerosis [30], Gulf War Syndrome (GWS) [5, 6], autism [31], and epilepsy [12] may also be related to aluminum exposure. While it is likely that these diseases are of multifactorial etiologies, aluminum certainly has the potential to serve as a toxic co-factor.

#### ALUMINUM EXPOSURE FROM VACCINES: BODY BURDENS AND RISKS

During the course of the last 30 years, the number of officially scheduled vaccines deemed necessary for children in the U.S. has increased sharply, from 10 in the 1980s to 32 in the late 2000s, 18 of which contain aluminum adjuvants [11]. The issue of vaccine safety thus becomes even more pertinent given that, to the best of

our knowledge, no adequate clinical studies have been conducted to establish the safety of concomitant administration of two experimentally-established neurotoxins, aluminum and mercury, the latter in the form of ethyl mercury (thimerosal) in infants and children. Since these molecules negatively affect many of the same biochemical processes and enzymes implicated in the etiology of autism, the potential for a synergistic toxic action is plausible [31, 47]. Additionally, for the purpose of evaluating safety and efficacy, vaccine clinical trials often use an aluminium-containing placebo, either containing the same or greater amount of aluminum as the test vaccine [48-51]. Without exception, these trials report a comparable rate of adverse reactions between the placebo and the vaccine group (for example, 63.7% vs 65.3% of systemic events and 1.7% vs 1.8% of serious adverse events respectively [51]). According to the U.S. Food and Drug Administration (FDA), a placebo is “an inactive pill, liquid, or powder that has no treatment value” [52]. The well-established neurotoxic properties of aluminium (Table 1) therefore suggest that aluminum cannot constitute as a valid placebo.

In 1965, Klatzo *et al.* [38] demonstrated that aluminum phosphate, the primary constituent of Holt's adjuvant, induced degeneration and neurofibrillary tangle-like histological changes in neurons (a hallmark feature of Alzheimer's disease), when injected intracerebrally into rabbits. The aluminum-injected animals also suffered from convulsions [38]. While direct application of aluminum adjuvants to the central nervous system (CNS) is unquestionably neurotoxic [37, 38, 40, 42], little is known about aluminum transport into and out of the CNS, its toxicokinetics, and the impact on different neuronal subpopulations following subcutaneous or intramuscular injections. The reason for this is that under current regulatory policies, evaluation of pharmacokinetic properties is not required for vaccines [53]. This issue is of special concern in context to worldwide mass immunization practices involving children whose nervous systems are undergoing rapid development. Furthermore, an immature developing blood brain barrier (BBB) is more permeable to toxic substances than that of an

adult [16, 54]. In addition, there are critical periods in neurodevelopment that occur within first few years of postnatal life during which exposure to neurotoxic insults may induce CNS damage [16, 47, 55]. In that respect, it is worth noting that any potential CNS damage caused by aluminum in children may not be evident until a later stage of development [16].

Bishop *et al.* [32] have shown that, parenteral exposure to as little as 20 µg/kg bw of aluminum for >10 days may result in long-term detrimental outcomes in neurologic development in preterm infants. In 2004, the U.S. Food and Drug Administration (FDA) set a limit for aluminum from parenteral sources for individuals with impaired kidney function and premature neonates at no greater than 4 to 5 µg/kg bw/day, stating that levels above those have been associated with CNS and bone toxicity [56]. In addition, according to the FDA, tissue loading may occur at even lower levels of administration [56]. What the upper limit for “safe” aluminum exposure might be for healthy neonates is not known.

In spite of these above data, newborns, infants and children up to 6 months of age in the U.S. and other developed countries receive 14.7 to 49 times more than the FDA safety limits for aluminum from parenteral sources from vaccines through mandatory immunization programs (Table 2). Specifically, 2-month old children in U.K., U.S., Canada and Australia routinely receive as much as 220 to 245 µg/kg bw of aluminum per vaccination session (Table 2), a burden equivalent to 34 standard adult-dose injections of hepatitis B vaccine (Table 3). Similarly, newborns at birth receive 73.5 µg Al/kg bw/day from a single hepatitis B vaccine, which is a dose equivalent to 10 standard adult-dose injections of hepatitis B vaccine in a single day (Table 3). Whether such doses of aluminum are safe even for adults is not known. However, detrimental effects associated with multiple vaccinations over a short period of time in U.S. and other Coalition military personnel who developed GWS in an aftermath of only six anthrax vaccine inoculations [5, 6], may suggest that adults in some circumstances are also vulnerable to deleterious CNS effects of adjuvant-aluminum. Notably, these inoculations were not given in a

**Table 2.** Estimated total aluminum body burden (µg/kg bw/day) per vaccination session in various developed countries. Vaccine schedules were obtained from the following sources: U.K. (U.K. Department of Health [10]), U.S. (Centers for Disease Control and Prevention [9]), Canada (Public Health Agency of Canada [57]) and Australia (Australian Government Department of Health and Aging [58]). Aluminum content of vaccines was according to Offit and Jew [3]

	Birth	1 mo	2 mo	3 mo	4 mo	5 mo	6 mo
U.K.	73.5	62.5	245	184	193	0	0
U.S.	73.5	0	245	0	171.1	0	161.2
Canada	73.5	0	220	0	193	0	111.8
Australia	73.5	0	220	0	193	0	144.7

FDA safety limit for Al from parenteral sources: 5 µg/kg bw/day.

**Table 3.** Comparison of aluminum body burden from vaccines in children and adults. Note that the closest an adult can get to the aluminum body burden from vaccines that compares to that of a child is in special circumstances, such as Gulf War deployed military personnel. Each anthrax vaccine administered to Gulf War veterans contained 1200 µg Al/mL (600 µg Al/dose) [59]. Currently licensed hepatitis B vaccines Engerix-B and Recombivax contain 250 (pediatric) and 500 µg Al/dose (adult) [3]. Age-specific weights were sourced from Haddad and Krishnan [60]

	An infant receiving 1 HepB injection (250 µg/dose) at birth	A 2-month old receiving the full U.S. scheduled set of injections	An adult receiving 6 anthrax injections over 18 months	An adult receiving 73.5 µg/kg bw/visit from HepB at 500 µg/dose	An adult receiving 245 µg/kg bw/visit from HepB at 500 µg/dose
Total Al (µg)	250	1225	3600	5145	17,150
Bw (kg)	3.4	5	70	70	70
Total Al µg/kg bw/day	73.5	245	51.4	73.5	245
# of Al-adjuvanted HepB at 500 µg/dose	NA	NA	NA	10	34



single day but were spread out over several weeks and up to 18 months (Table 3).

In a recent review, Offit and Jew [3], in addressing concerns about potential aluminum adjuvant toxicity, cited as evidence an uncontrolled feeding study by Golub *et al.* [61], which used aluminum lactate as the form of treatment. The reviewers stated that: "No adverse reactions were observed when mice were fed quantities of aluminum as high as 62 mg/kg/day" [3], when in fact 20% of the mice showed significantly lower motor activity [61]. Moreover, Golub *et al.* [61] emphasized that: "The clear cut influence of dietary Al on motor activity suggests the value of further testing of Al fed animals in areas of sensory-motor competence as well as cognitive and social functioning". Also often unrecognized by researchers [3, 13] is the fact that different aluminum compounds may vary in their toxic potential or that the extent of toxicity of a particular compound depends on a specific route of administration, duration of exposure, and species studied. For example, while feeding aluminum hydroxide at 66.5, 133, and 266 mg Al/kg/day to mice does not appear to cause neurodevelopmental damage [62, 63], parenteral administration of aluminum chloride at 40 mg/kg/day causes maternal deaths in rats, as well as embryo lethality, growth retardation and fetal abnormalities [64]. The latter effects were also shown to occur at lower doses (20 mg/kg/day [64]). The authors of the former study that used higher doses of aluminum hydroxide concluded that this form of aluminum is very poorly absorbed and thus does not reach the fetus at levels which might pose a developmental hazard [63]. A rigorous survey of the primary literature further shows that evidence for pre, perinatal and postnatal aluminum neurotoxicity is well established [65-71], even at very low doses of aluminum. For example, Gonda *et al.* [72] have shown that parenteral exposure during gestation days 7 to 15 to as little as 2.5, 5 and 10 mg/kg/day of aluminum lactate results in diminished performance and lengthened latency in avoidance response in rat pups. The evidence for potential aluminum toxicity in early life is thus more firmly established than suggested by some researchers [3, 13, 15].

Finally, it should be fairly obvious that parenterally administered aluminum bears more relevance to vaccine exposure than dietary aluminum. In this context it is worth noting that unlike dietary aluminum of which only ~0.25 % is absorbed into systemic circulation [73], aluminum from vaccines may be absorbed at nearly 100% efficiency [74]. It is also important to note that ionic aluminum will not have the same toxicokinetic properties as aluminum bound to an antigen. While ionic aluminum may be excreted *via* the kidneys, the sizes of most antigen-aluminum complexes (24-83 kDa [59, 75, 76]), are higher than the molecular weight cut-off of the glomerulus (~18 kDa [12]), likely precluding efficient excretion of these compounds. Indeed, effective excretion would in fact obviate the basic reason that adjuvants are used at all. For all these reasons, vaccine-derived aluminum has a much greater potential to induce neurological damage than that obtained through diet, even in those with effective renal function. In addition, adjuvant-aluminum can gain access to the CNS as demonstrated by Redhead *et al.* [77], who showed that intraperitoneal injection of aluminum adsorbed vaccines in mice caused a transient rise in brain tissue aluminum levels peaking around the second and third day after injection.

#### ALUMINUM TOXICOKINETICS: DEVELOPING BRAIN, A SINK FOR ADJUVANT-ALUMINUM?

Experiments by Levy *et al.* [78] in which antibodies were raised against an immunogen prepared from aluminum and bovine serum albumin (BSA) suggested that aluminum on its own may act as an antigen. These results raise questions concerning the possibility that vaccination with aluminum adjuvants may increase an individual's susceptibility to subsequent exposure to aluminum. Given the

ubiquity of bioavailable aluminum compounds (food, water, cosmetics, pharmaceuticals [16]), such issues warrant further investigation. The existing data available on the pharmacokinetics of aluminum adjuvants suggest that these compounds may access systemic circulation and cross the blood brain barrier. Flarend *et al.* [79] estimated aluminum absorption in adult female rabbits following intramuscular injection of two forms of  $^{26}\text{Al}$  labeled adjuvants, aluminum hydroxide and aluminum phosphate. The results showed that both were rapidly absorbed, appearing in the blood as early as one hour after injection [79]. Blood levels of aluminum remained elevated for 28 days post-injection in both cases and subsequent tissue analysis revealed elevated levels of aluminum in kidney, spleen, liver, heart, lymph nodes and, notably, brain [79]. In Flarend *et al.*'s [79] study the level of aluminum in the brain was lower compared to the other organs, however the study by Yumoto *et al.* [80] indicated that such a pattern of tissue distribution may be age-dependent. Following a single subcutaneous injection of  $^{26}\text{Al}$  on gestation day 15, these investigators showed that 0.2% of the  $^{26}\text{Al}$  injected into a pregnant rat had been transplacentally transferred to the fetuses. Notably, the amount of the radiolabeled aluminum in the fetal brain was 30% higher than in the liver, while in the dams, brain aluminum levels were only 1% of the levels found in the liver [80]. The possibility that the fetal brain may act as a sink for aluminum may be of concern since under certain circumstances, vaccination of pregnant women with a number of aluminum-adjuvanted vaccines (tetanus, hepatitis A and B, meningococcal and pneumococcal is recommended [3, 81]) under the current U.S. immunization guidelines [82].

#### ADVERSE EFFECTS ASSOCIATED WITH ALUMINUM ADJUVANTS

A recently described syndrome termed macrophagic myofasciitis (MMF) has been specifically attributed to aluminum adjuvants in recipients of hepatitis A and B and tetanus toxoid (Td) vaccines [83]. MMF patients were found to suffer from diffuse arthromyalgias, chronic fatigue, muscle weakness and in some cases, multiple sclerosis [83]. Muscle biopsies show extensive infiltration by granular periodic acid-Schiff's reagent-positive macrophages and lymphocytes and inconspicuous muscle-fibre damage [2, 7, 83-85]. While most MMF patients appeared to have a normal white blood count, laboratory analysis showed evidence of increased inflammation and the presence of serum auto-antibodies. The former was indicated by significant increases in the levels of inflammatory cytokines interleukin (IL)-1 receptor antagonist and IL-6 [2]. Electron microscopy and microanalytical analysis showed that the appearance of MMF lesions was due to long-term persistence of aluminum adjuvants at the site of injections and concomitant ongoing local immune reactions [8, 83]. Aluminum was shown to persist at the site of injection from several months up to 8 years following vaccination [83, 85]. MMF lesions were subsequently also reproduced in rats by injection of aluminum adjuvants [86].

Aluminum adjuvants are exceptionally potent stimulators of the immune system and their specific action is to shift the immune response towards a Th2 profile. In that respect, Dr. Gherardi who first described MMF noted: "It is plausible that persistent systemic immune activation that fails to switch off represents the pathophysiologic basis of chronic fatigue syndrome associated with macrophagic myofasciitis, similarly to what happens in patients with post-infectious chronic fatigue and possibly idiopathic chronic fatigue syndrome" [8]. The symptoms of MMF are similar to those of GWS, a multisystem disorder which has been linked to multiple vaccinations administered over a short period of time (Table 3 [6, 8]). As with autism and MMF, GWS patients also show Th2 predominance and a significant risk factor in causing this syndrome may be aluminum hydroxide adjuvant from the anthrax vaccine.

Injections of aluminum hydroxide at levels comparable to those administered to Gulf War veterans, were shown to cause significant motor neuron degeneration as well as impairments in motor function and decrements in spatial memory capacity in young CD-1 male mice [43, 44].

Of even graver concern is that persistent Th2 stimulation, due to repeated administration of aluminum-adsorbed vaccines, may have profound long-term adverse effects on the developing immune system in children. A newborn infant has an undeveloped immune system which is limited in function [87] and requires a series of challenges to bring it to full capacity. Prior introduction of mandatory vaccines, these challenges were largely in the forms of relatively minor childhood diseases such as mumps and measles. Vaccinations targeted at stimulating antibody production by the humoral immune system (Th2) located in the bone marrow, bypass the cellular immune system (Th1) on mucosal surfaces (respiratory and gastrointestinal tract), leaving the latter unchallenged during the critical period of development. Since Th1 progenitors will not differentiate into Th1 cells in the absence of Th1-cytokines [88] (due chronic stimulation of the Th2 pathway), the end result of a prolonged Th2 shift may be permanently stunted cellular (Th1) immunity. Ironically, Th1 immunity is inherently far more efficient in clearing viral pathogens than Th2 immunity [6, 88, 89], which further raises a question about the general efficacy of aluminum-adsorbed vaccines in fighting viral infections. Notably, a similar mechanism by which acute, subacute or chronic stress selectively suppress cellular (Th1) immunity but boosts humoral (Th2) immunity, is thought to be responsible for the onset and/or course of many infectious, autoimmune/inflammatory, allergic and neoplastic diseases [89]. For example, research indicates that by inducing a Th2 shift, stress hormones may increase susceptibility to acute respiratory infections caused by flu viruses and enhance disease progression in human immunodeficiency virus (HIV)-positive individuals [89]. Furthermore, severe acute stress associated with high adrenaline output leads to histamine release from Th2 type immune cells (mast cells), which may either initiate new or exacerbate existing allergic reactions [89]. Finally, high histamine levels have been observed in various cancer tissues, suggesting that stress hormone dependent amplification of Th2 responses can increase the susceptibility to tumorigenesis [89]. Taken together, these observations potentially explain why naturally acquired immunity against common childhood diseases may protect against certain aggressive types of tumors in humans [90], asthma and other allergies [91, 92], as well as neurodegenerative disorders such as Parkinson's [93].

Although most autoimmune diseases are Th1-related, others such as lupus-like syndromes (Table 4), are mediated by Th2 cytokines IL-10 [89] and IL-4 [95]. It is thought that vaccine adjuvants may trigger autoimmunity through a bystander effect, by activating dormant autoreactive T-cells in predisposing individuals [96]. Notably, the repertoire of adverse reactions and syndromes associated with aluminum-adsorbed vaccines (Table 4), appears

to fit the spectrum of diseases stemming from immune dysfunction [5, 6]. In addition, fatalities have been reported among individuals who were vaccinated against with the anthrax vaccine. These included deaths from sudden cardiac arrest, myocardial infarction with polyarteritis nodosa, aplastic anemia, CNS lymphoma and suicide [59]. Since the anthrax vaccine contains a higher dose of aluminum than most other aluminium-adsorbed vaccines (0.6 mg/dose vs 0.5 mg/dose Engerix-B [59, 94]), combined with another potent adjuvant and Th2 stimulant, squalene [6], the potential for synergistic adverse actions by these two adjuvants in humans cannot be discounted.

Fatal outcomes have also been reported following administration of pediatric aluminum-adsorbed hexavalent vaccines, one of which (Hexavac) was subsequently withdrawn from use, apparently due to its poor effectiveness [97]. Zinka *et al.* [98] reported six cases of sudden infant death that occurred within 48 hours after vaccination with hexavalent vaccines. The post-mortem analysis of six children aged 4 to 17 months (five of whom were vaccinated with Hexavac and one with Infanrix Hexa), revealed abnormal pathologic findings particularly affecting the nervous system [98]. The overall pathological abnormalities included acute congestion, defective BBB, infiltration of the leptomeninges by macrophages and lymphocytes, perivascular lymphocytic infiltration, diffuse infiltration of the pons, mesencephalon and cortex by T-lymphocytes, microglia in the hippocampus and pons, and in one case, necrosis in the cerebellum [98]. Increased serum mast-cell tryptase and numbers of eosinophilic granulocytes were also found indicating that an anaphylactic reaction developed subsequent to vaccination [98]. As shown in Table 4, anaphylaxis appears to be a common side effect associated with aluminum-adsorbed vaccines. According to Zinka *et al.* [98], there was a 13-fold increase in infant death following introduction of hexavalent vaccines into immunization practice [97]. Although there is no conclusive proof that these deaths were directly caused by vaccination, the authors felt it was "important to inform vaccinating physicians and pediatricians as well as parents about such possibly fatal complications after application of hexavalent vaccines" [98]. Finally, the neuropathological findings by Zinka *et al.* [98] are consistent with neurotoxic properties of aluminum adjuvants. For example, as shown by our group as well others, aluminum is a BBB neurotoxin [54, 99] that has a propensity to activate brain microglia and increase the production of inflammatory cytokines thereby instigating and/or exacerbating inflammation and excitotoxicity in the brain [31, 43, 44, 100-104].

Permanent activation of brain inflammatory responses has long been recognized as a factor in etiology of many neurodegenerative diseases [105] including Alzheimer's disease [106, 107], autism [31, 108-110], multiple sclerosis [30] and dialysis dementia [111]. Notably, all of these diseases have been previously linked to aluminum exposure [12, 21, 28, 30, 31, 107, 111]. Aluminum potentiates inflammatory responses in the brain by multiple mechanisms, such as activation of microglia [31, 44, 100, 101, 107,

**Table 4. Engerix-B and BioThrax (Anthrax Vaccine) Common Post-Licensure Adverse Effects [59, 94]**

<b>Blood and Lymphatic System Disorders</b>	Idiopathic thrombocytopenia
<b>Immune System Disorders</b>	Anaphylaxis and/or other generalized hypersensitivity reactions, inflammatory arthritis/arthritis, fever, and dermatologic reactions such as erythema, systemic lupus erythematosus
<b>Nervous System Disorders</b>	Encephalitis, multiple sclerosis, Guillain-Barré syndrome, transverse myelitis, facial palsy, seizures, syncope
<b>Eye Disorders</b>	Visual disturbances
<b>Cardiac Disorders</b>	Cardiac arrhythmias
<b>Respiratory, Thoracic and Mediastinal Disorders</b>	Asthma
<b>Skin and Subcutaneous Tissue Disorders</b>	Angioedema, erythema
<b>Musculoskeletal and Connective Tissue Disorders</b>	Arthritis, myalgia, muscle weakness

112] and induction of pro-inflammatory gene expression [107]. Regarding the latter, aluminum at nanomolar to low micromolar concentrations augments specific neuroinflammatory and pro-apoptotic signalling cascades, strikingly similar to those observed in Alzheimer's disease brains [104], by driving expression from a subset of stress-inducible promoters in cultured human primary brain cells [113-115]. For example, out of 8 induced genes up-regulated in cultured human neurons by 100 nm aluminum, 7 showed expression patterns similar to those observed in Alzheimer's disease, including hypoxia inducible factor (HIF)-1 and nuclear factor (NF)- $\kappa$ B-responsive amyloid  $\beta$ -protein precursor (A $\beta$ PP), IL-1 $\beta$  precursor, NF- $\kappa$ B subunits, cytosolic phospholipase A<sub>2</sub> (cPLA<sub>2</sub>), cyclooxygenase (COX)-2 and DAXX, a regulatory protein known to induce apoptosis and repress transcription [114]. Both HIF-1 and NF- $\kappa$ B are up-regulated in Alzheimer's disease where they fuel the pro-inflammatory cycle which leads to further exacerbation of oxidative stress and inflammation, culminating in neuronal death [105, 116]. Taken together, these results underscore the potential of physiologically relevant levels of aluminum to drive genotoxic mechanisms characteristic of neurodegenerative disease processes [115].

## CONCLUSIONS

Aluminum in various forms can be toxic to the nervous system. The widespread presence in the human environment may underlie a number of CNS disorders. The continued use of aluminum adjuvants in various vaccines for children as well as the general public may be of significant concern. In particular, aluminum presented in this form carries a risk for autoimmunity, long-term brain inflammation and associated neurological complications and may thus have profound and widespread adverse health consequences. The widely accepted notion of aluminum adjuvant safety does not appear to be firmly established in the scientific literature and, as such, this absence may have lead to an erroneous conclusions regarding the significance of these compounds in the etiologies of many common neurological disorders. Furthermore, the continued use of aluminum-containing placebos in vaccine clinical trials may have lead to an underestimation of the true rate of adverse outcomes associated with aluminum-adjuvanted vaccines. In our opinion, a comprehensive evaluation of the overall impact of aluminum on human health is overdue. Such an evaluation should include studies designed to determine the short and long-term impacts of dietary aluminum as well as the potential impacts in different age groups of exposure to adjuvant aluminum alone and in combination with other potentially toxic vaccine constituents (e.g., formaldehyde, formalin, mercury, phenoxyethanol, phenol, sodium borate, polysorbate 80, glutaraldehyde). For the latter, until vaccine safety can be comprehensively demonstrated by controlled independent long-term studies that examine the impact on the nervous system in detail, many of those already vaccinated as well as those currently receiving injections may be at risk for health complications that exceed the potential benefits that vaccine prophylaxis may provide. The issue of aluminum adjuvanted vaccine safety is especially pertinent in light of the legislation which might mandate vaccination regimes for civilian populations (e.g., the Biodefense and Pandemic Vaccine and Drug Development Act of 2005). Whether the risk of protection from a dreaded disease outweighs the risk of toxicity from its presumed prophylactic agent is a question that demands far more rigorous scrutiny than has been provided to date.

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# Do aluminum vaccine adjuvants contribute to the rising prevalence of autism?

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## ABSTRACT

Autism spectrum disorders (ASD) are serious multisystem developmental disorders and an urgent global public health concern. Dysfunctional immunity and impaired brain function are core deficits in ASD. Aluminum (Al), the most commonly used vaccine adjuvant, is a demonstrated neurotoxin and a strong immune stimulator. Hence, adjuvant Al has the potential to induce neuroimmune disorders. When assessing adjuvant toxicity in children, two key points ought to be considered: (i) children should not be viewed as “small adults” as their unique physiology makes them much more vulnerable to toxic insults; and (ii) if exposure to Al from only few vaccines can lead to cognitive impairment and autoimmunity in adults, is it unreasonable to question whether the current pediatric schedules, often containing 18 Al adjuvanted vaccines, are safe for children? By applying Hill's criteria for establishing causality between exposure and outcome we investigated whether exposure to Al from vaccines could be contributing to the rise in ASD prevalence in the Western world. Our results show that: (i) children from countries with the highest ASD prevalence appear to have the highest exposure to Al from vaccines; (ii) the increase in exposure to Al adjuvants significantly correlates with the increase in ASD prevalence in the United States observed over the last two decades (Pearson  $r = 0.92$ ,  $p < 0.0001$ ); and (iii) a significant correlation exists between the amounts of Al administered to preschool children and the current prevalence of ASD in seven Western countries, particularly at 3–4 months of age (Pearson  $r = 0.89$ – $0.94$ ,  $p = 0.0018$ – $0.0248$ ). The application of the Hill's criteria to these data indicates that the correlation between Al in vaccines and ASD may be causal. Because children represent a fraction of the population most at risk for complications following exposure to Al, a more rigorous evaluation of Al adjuvant safety seems warranted.

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## 1. Introduction

During prenatal and early postnatal development the brain is extremely vulnerable to neurotoxic insults [1,2]. Not only are these highly sensitive periods of rapid brain development in general [3] but also, the blood brain barrier (BBB) is incomplete and thus more permeable to toxic substances during this time [2,4,5]. Further, immune challenges during early development, including those induced by vaccines, can lead to permanent detrimental alterations of nervous and immune system function [6–9]. Experimental evidence also shows that simultaneous administration of as little as two to three immune adjuvants, or repeated stimulation of the immune system by the same antigen, can overcome genetic resistance to autoimmunity in animals [10,11]. Moreover, in adult humans, a variety of conditions encompassed by the ‘Autoimmune/inflammatory syndrome induced by adjuvants’ (‘ASIA’) have been linked to exposure to aluminum (Al) vaccine adjuvants (Table 1).

In many Western countries, by the time children are 4–6 years old they will have received a total of 23–32 vaccines [12,13], many with Al adjuvants, through routine pediatric vaccine schedules [2,14]. According to the United States Food and Drug Administration (US FDA), safety assessments for vaccines have often not included appropriate toxicity studies because vaccines have not been viewed as inherently toxic [15]. However, if a few vaccines administered to adults can result in adverse outcomes, such as the ‘ASIA’ syndrome, should we *assume* without experimental evidence that the current pediatric schedules are safe for children?

Analysis of the relevant data shows that the number of vaccinations recommended prior to school entry increased from 10 in the late 1970s to 32 in 2010 (18 of which contain Al adjuvants) [16]. During this same period, the prevalence of autism spectrum disorders (ASD) in the US also increased by as much as 2000% [16]. While such observations have been of interest, the potential role of vaccines in the development of ASD remains controversial. ASD are characterized by marked impairments in social skills, verbal communication, behavior and cognitive dysfunction [17–19]. Although the etiology of 90% of ASD is still largely unknown [20,21], a growing body of scientific literature shows that neuroimmune abnormalities (i.e., abnormal cytokine profiles, neuroinflammation and presence of autoantibodies

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**Table 1**

Shared aspects between autoimmune/inflammatory diseases (including ASD) and immunostimulatory properties of Al vaccine adjuvants.

Condition			Al adjuvant	
Disease	Th shift	Inflammatory profile	Inflammatory profile	General immunostimulatory effects
Arthritis <sup>*,†</sup>	Excessive Th1 [129,155]	Increased IL-1, IL-6, IL-12, TNF- $\alpha$ , IFN- $\gamma$ , MIP-1 $\alpha$ and oxidative stress [129,134,155]	Increases cytokines (IL-1 $\alpha$ , IL-1 $\beta$ , IL-4, IL-5, IL-6, IL-18, TNF- $\alpha$ ), chemokines (IL-8, MCP-1, MIP-1 $\alpha$ , MIP-1 $\beta$ ), ROS, and nitric oxide (NO) [34,40,138,155,170,171]	Stimulates recruitment of monocytes, macrophages and granulocytes to the injection site Induces differentiation of monocytes to antigen presenting cells (APCs) Activates APCs
Autoimmune thyroid disease				
Inflammatory bowel disease (IBD)/Crohn's disease (CD)		Increased NLRP3 inflammasome complex signaling and NLRP3-dependent over-production of IL-1 $\beta$ , IL-6, IL-18, TNF- $\alpha$ and reactive oxygen species (ROS) in MS, EAE, Type 1 diabetes mellitus [164–166] and animal models of IBD [167]	Activates the NLRP3 inflammasome complex and NLRP3-dependent cytokines [33,34,172]	Promotes antigen uptake and processing by APCs and enhances antigen-specific T-cell responses Increases the expression of MHC class I and II and associated co-stimulatory molecules on peripheral blood monocytes
Type 1 diabetes mellitus <sup>*</sup>				
Multiple sclerosis (MS) <sup>*,†</sup> and experimental autoimmune encephalomyelitis (EAE)				
Systemic lupus erythematosus (SLE) <sup>*</sup>	Excessive Th2 [129,156]	Increased IL-10, IL-18, IL-6, IFN- $\gamma$ , TNF- $\alpha$ [129,156,168,169]		Activates the complement cascade
Macrophagic myofasciitis (MMF) and chronic fatigue syndrome (CFS) <sup>*,†</sup>	Excessive Th2 [53,157,158]	Increased IL-4, IL-6, B-cell hyperlymphocytosis, infiltration of large periodic acid-schiff (PAS)-positive macrophages, and CD8+ T lymphocytes in the absence of conspicuous muscle fibre damage [53,95,158]		Generally stimulates Th2 responses but can also induce a Th1 shift and activate cytotoxic T lymphocytes (CTLs) in the presence of other Th1 stimulators (i.e., lipopolysaccharide (LPS), CpG, recombinant influenza protein antigen [138,173–175]) Activates astrocytes and microglia [29,97,139]
Gulf War Syndrome (GWS) <sup>*,†</sup>	Mixed Th1/Th2 [159]	Increased IFN- $\gamma$ , IL-5, IL-6 [159]		
Autism spectrum disorders (ASD) <sup>*</sup>	Both Th1 and Th2 shifts have been reported [17,160–163]	Increased IL-1 $\beta$ , IL-4, IL-5, IL-6, TNF- $\alpha$ , IL-8, MCP-1, MIP-1 $\beta$ , MHC class II [17,160,162] Increased astrocyte and microglia reactivity [17,20]		

\* Linked to Al-adjuvanted vaccines [32,101,102,176,177].

† Specifically recognized as 'Autoimmune/inflammatory syndrome induced by adjuvants' ('ASIA') [32].

against brain proteins) occur in ASD patients and may contribute to the diversity of ASD phenotypes [17,20,22–26].

Al is an experimentally demonstrated neurotoxin whose ability to impact the human nervous system has been known for decades [16,27–29]. For example, exposure to as little as 20  $\mu\text{g}/\text{kg}$  bw of Al for period >10 days is sufficient to cause neurodevelopmental delays in preterm infants [28]. In addition, Al is a potent stimulator of the immune system, indeed this is the very reason why it is used as an adjuvant [14,30–34]. Given this, it remains surprising that in spite of over 80 years of use, the safety of Al adjuvants appears to rest largely on assumptions rather than experimental evidence. For example, nothing is known about the toxicology and pharmacokinetics of Al compounds in infants and children [35]. In addition, the mechanisms by which Al adjuvants interact with the immune system remain far from clear [34,35]. In this regard it is notable that many vaccine trials usually use an Al adjuvant containing "placebo" or another vaccine as the "control" group [36–38], rather than a saline control. This study design has not allowed a direct comparison of the efficacy and safety of the antigen alone versus the Al adjuvant. In spite of these gaps in our knowledge about Al adjuvants, the use of Al in vaccines is widely regarded as safe and effective [35,39,40].

Should it be of concern that so little is known about the potential deleterious impacts of Al adjuvants on the developing central nervous system (CNS) given that worldwide, preschool children are regularly exposed to significant amounts of Al from vaccines [2,14]? To address this question, we investigated pediatric vaccine schedules from various Western countries in order to gain a better understanding of potential Al exposure from vaccines in children. Our results, supported by the Hill's criteria for establishing causality between exposure and outcome [41], suggest that a causal relationship may exist between

the amount of Al administered to preschool children at various ages through vaccination and the rising prevalence of ASD.

## 2. Methods

### 2.1. Collection of ASD prevalence data

We analyzed the currently available data from the US Department of Education Annual Reports to Congress for ASD prevalence for the period from 1991 to 2008 [42–52] in the 6–21 year-old age cohort and correlated it with the estimated total Al exposure from pediatric vaccines (given to preschool children before the age of 6 years), sourced from the US Centers for Disease Control and Prevention (CDC [12]). In addition, we obtained the most recent available data for ASD prevalence and vaccination schedules from several other countries including the United Kingdom (UK), Australia, Canada, Sweden, Finland and Iceland (see below for source references). Using the latter data, we carried out a correlation analysis to investigate the potential association between ASD prevalence and estimated vaccine-derived Al exposures in preschool children at various ages. We also correlated ASD prevalence with the number of Al-adjuvanted vaccines given to preschool children according to the relevant vaccination schedules from each country.

### 2.2. Calculations of Al exposure from vaccines

For the purpose of correlating ASD prevalence to Al exposure, for each country studied, we calculated the cumulative amount of Al administered from all vaccines that children receive during the specified age period (i.e., the cumulative exposure to Al at 4 months of age



includes AI from vaccines given at 2, 3 and 4 months). This rationale for using cumulative amounts of adjuvant AI in our analysis is also supported by the following observation: AI has been shown to persist at the site of injection from several months up to 8–10 years following vaccination in patients suffering from macrophagic myofasciitis, an autoimmune disease linked to AI vaccine adjuvants [53]. The number and types of pediatric vaccines were sourced from the US CDC [12], UK Department of Health [13], Public Health Agency of Canada [54], Australian Government Department of Health and Aging [55], Swedish Institute for Infectious Disease Control [56], KTL (Finnish) National Public Health Institute [57] and Iceland's A Surveillance Community Network for Vaccine Preventable Infectious Diseases [58]. The AI content used was derived from an article by Offit and Jew [39] and manufacturer's product monographs (Table 2 [59–62]). Because the AI content varies between different brands of certain vaccines (Table 2), for each vaccination appointment, three possible exposures were calculated: (i) maximum, assuming exposure to vaccines with the highest AI content (i.e., 625 µg AI for DTaP from Infanrix and 225 µg AI for Hib from PedVax), (ii) mean, using the calculated mean AI-content values of different brands of DTaP and Hib (i.e., 375 µg for DTaP = (625 + 330 + 170)/3 and 112.5 µg for Hib = (0 + 225)/2); and (iii) minimum, assuming exposure to vaccines with the lowest AI content (i.e., 170 µg AI for DTaP from Tripedia and 0 µg AI for Hib from Hiberix). All three of these exposures were then correlated with the relevant ASD prevalence data. With regard to vaccine uptake in the US, we acknowledge that there are likely to be variations between individual states due to differences in adopting CDC's recommendations. However, since the ASD prevalence data pertain to the US population as a whole, rather than individual states, we felt that our overall evaluation with regard to US vaccine uptake was the most appropriate measure to use.

### 2.3. Exclusion/inclusion criteria

Certain vaccines were excluded from our calculations since the addition of these to childhood vaccination schedules occurred after the relevant ASD prevalence study periods. For example, in Australia, pneumococcal vaccine (PCV) was introduced in 2003 [63] and the ASD prevalence study conducted in 2005 provided data for 6–12 year-old children (1993–1999 birth cohort [64]); in Canada PCV and meningococcal serogroup C (MenC) were introduced in 2005 [65] and 2001 [66] respectively, and the ASD prevalence report was for 1987–1998 birth cohort [67]; in Sweden PCV was introduced in 2009 [68], ASD prevalence report was for 1977–1994 birth cohort [69]; in Finland, rotavirus vaccine was introduced in 2009 [70] and the ASD prevalence report was for 1979–1994 birth cohort [71]; in Iceland, meningococcal serogroup C (MenC) was introduced in 2002 [58] with ASD prevalence report for the 1984–1993 birth cohort [72]. ASD prevalence data for the US and UK were from Kogan et al. [73] and Baron-Cohen et al. [74], respectively. We included hepatitis B (HB) vaccine in our calculations for the UK vaccination schedule (at 0, 1 and 2 months [75]) since there was no rationale for excluding high risk groups from our analysis (as these groups have not been

specifically excluded from the UK ASD prevalence data [74]). We excluded HB vaccine from our calculations for Sweden and Finland since in these countries HB vaccination for high risk groups was introduced in the mid 1990s [76,77], after the relevant ASD prevalence study periods.

### 2.4. Statistical methods

The correlation analysis was carried out using GraphPad Prism statistical software to derive Pearson correlation coefficients (Pearson *r*; due to normal data distribution) between vaccine-derived AI exposures, AI-containing vaccine number and ASD prevalence. To control for type I errors due to multiple tests, we used permutation resampling-based multiplicity adjustment for *p*-values according to Westfall and Young [78] to determine whether the correlation between ASD prevalence in seven Western countries and AI exposure at various ages was statistically significant. Unlike the more popular Bonferroni-Holm method, Westfall and Young accounts for correlations between variables (e.g., age of exposure) and was hence a more appropriate choice. The Westfall and Young *p*-value adjustment was carried out in R software. The correlation was considered statistically significant at *p* < 0.05. In all of the data provided for AI vaccine exposure, AI is expressed either as total, or per kg of body weight. The latter was calculated by dividing total AI exposure with age-specific weight, sourced from Haddad and Krishnan [79].

### 2.5. Hill's criteria

The Hill's criteria for causation include: (1) the strength of the association (as measured by appropriate statistical tests), (2) the consistency of the observed association (i.e., the association has been repeatedly observed by different persons and/or in different places, circumstances and times), (3) the specificity of the association (established when a single putative cause produces a specific effect), (4) the temporal relationship of the association (exposure precedes the outcomes), (5) the biological gradient or dose-response curve (an increasing amount of exposure increases the risk), (6) biological plausibility (causation is biologically plausible and agrees with a currently accepted understanding of pathological processes of the disease in question), (7) the coherence with the current knowledge (data should be congruent with generally known facts of the natural history and biology of the disease), (8) experimental or semi-experimental evidence and (9) analogy with similar evidence (i.e., different toxins may result in similar disease outcomes because they adversely affect the same underlying processes linked to a specific disease) [41]. In neuropsychiatry, four of Hill's nine criteria are considered critical to assess causality: the strength of the association (criterion 1), the consistency of the observed association (criterion 2), the biologic rationale (criterion 6) and the temporal relationship of the association (criterion 4) [80]. Obviously, if evidence exists for the remaining criteria, conclusions about causality would be further strengthened. Note also that the specificity criterion (3) is not considered necessary in neuropsychiatry [80] given that many neuropsychiatric disorders have multiple causal factors. ASD for example, are partly determined by genetic susceptibility factors and hence fit this category [17,18,20,21].

## 3. Results

### 3.1. AI exposure from vaccines in adults and children based on body weight

Table 3 shows the estimated amounts of AI administered through vaccination to preschool children in the US. At 2 months of age, US infants receive the highest amount of AI per body weight from vaccines (172.5 µg/kg bw, mean exposure) compared to other ages. Table 4 shows AI exposure from vaccines per kg of body weight in children from seven Western countries: the UK, US, Canada, Australia, Sweden, Finland and Iceland. Note that children from countries with the highest ASD prevalence (i.e., UK, US, Australia and Canada) appear to have a higher exposure to AI from vaccines than do children from Scandinavian

**Table 2**  
AI-adjuvant content in licensed vaccines.

AI adjuvant	Vaccine	Trade name	Manufacturer	Amount (µg) per dose
Al hydroxide	DTaP	Infanrix	GlaxoSmithKline	625 [39]
	DTaP	Daptacel	Aventis Pasteur	330 [39]
	DTaP	Tripedia	Aventis Pasteur	170 [39]
	HA	Havrix	GlaxoSmithKline	250 [39]
	HB*	EngerixB	GlaxoSmithKline	250 [178]
	Hib	PedVax	Merck and Co	225 [39]
	Hib	Hiberix	GlaxoSmithKline	0 [62]
	Anthrax	Biothrax	Bioprot Corp	600 [60]
Al phosphate	PCV	Prevnar	Wyeth	125 [39]
	MenC	Meningitec	Wyeth	125 [59]
Al sulfate	HB*	Recombivax	Merck and Co	250 [61]

\* Pediatric dose = 250 µg, adult dose = 500 µg.



**Table 3**

Al administered from pediatric vaccines to children at different ages under the current US vaccination schedule [12] assuming mean exposure. Ages are expressed in months (mo).

Vaccine	Birth	2 mo	4 mo	6 mo	15 mo	24 mo	72 mo
HB	250	250		250			
DTaP*		375	375	375	375		375
Hib†		112.5	112.5	112.5	112.5		
PCV		125	125	125	125		
HA					250	250	
Total Al (μg)	250	862.5	612.5	862.5	862.5	250	375
Total Al (μg/kg bw)	73.5	172.5	107.5	113.5	78.4	19.8	19.3

\* Mean value from three different brands of DTaP (Infanrix, Daptacel, Tripedia, see Table 2).

† Mean value from two different brands of Hib (PedVax and Hiberix, see Table 2).

countries where autism prevalence is lower. Table 5 shows a comparison between vaccine-derived Al exposures in adults and children. Due to their lower body weight, children attain a much higher Al exposure per kg of body weight than adults (73.5–172.5 μg/kg bw versus 7.1 μg/kg bw).

### 3.2. Correlation between ASD prevalence and vaccine-derived Al exposures in the US

Al exposure from vaccines in the US vaccination schedule from 1991 to 2008 shows a highly significant positive linear correlation with ASD prevalence at all three levels of exposure (Pearson  $r = 0.92$ ,  $p < 0.0001$ ), with 95% CI = 0.79–0.97 (Fig. 1; Table 6). In addition, we show in Table 7 that the number of Al-adjuvanted vaccines in the yearly vaccination schedules from 1991 to 2008 also yields a highly significant positive correlation with ASD prevalence (Pearson  $r = 0.90$ ,  $p < 0.0001$ ) with 95% CI = 0.76–0.96.

### 3.3. Correlation between ASD prevalence in the US, UK, Canada, Australia, Sweden, Finland and Iceland and Al exposure from pediatric vaccines

In Table 8 we show that the estimated cumulative vaccine-derived Al exposure yields a significant positive correlation with the current prevalence of ASD in seven Western countries at all three levels of exposure at 3–4 months of age. (Pearson  $r = 0.89$ – $0.94$ ,  $p = 0.0018$ – $0.0248$ ). ASD prevalence in these countries also significantly correlates with the number of Al-adjuvanted vaccines given at 3–18 months of age (Pearson  $r = 0.89$ – $0.94$ ,  $p = 0.0018$ – $0.0368$ ; Table 8).

**Table 4**

Estimated total Al exposure from vaccines (μg/kg bw) per vaccination schedule in various Western countries at different ages. Minimum to maximum range of exposure is given where applicable (where DTaP and Hib are scheduled). Age is expressed in months (mo).

	ASD prevalence/10,000	Birth	1 mo	2 mo	3 mo	4 mo	5 mo	6 mo
UK	157 [74]	73.5	62.5	109–245	55.7–184	73.7–193	0	0
US	110 [73]	73.5	0	109–245	0	51.8–171.1	0	71.7–161.2
Canada	65 [67]	73.5	0	84–220	0	73.7–193	0	22.4–111.8
Australia	62.5 [64]	73.5	0	84–220	0	73.7–193	0	55.3–144.7
Sweden	53.4 [69]	0	0	0	32.1–160.4	0	25.4–126.9	0
Iceland	12.4 [72]	0	0	0	32.1–160.4	0	25.4–126.9	0
Finland	12.2 [71]	0	0	0	32.1–160.4	0	25.4–126.9	0

**Table 5**

Comparison of Al exposure from vaccines in children and adults. An infant's vaccine-derived Al exposure of 73.5 μg Al/kg bw is equivalent to that from 10 HB vaccines given in a single day to a 70 kg adult ((73.5 μg Al/kg bw x 70 kg)/(HB dose (500 μg Al)) = 5147/500 = 10.3). The vaccine-derived Al exposure in a 2 month old receiving 172.5 μg Al/kg bw is equivalent to that from 24 HB vaccines given in a single day to a 70 kg adult ((172.5 μg Al/kg bw x 70 kg)/(HB vaccine dose (500 μg Al)) = 12075/500 = 24.2).

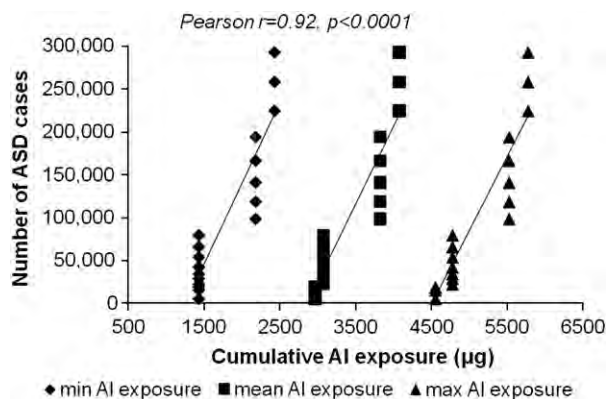
	An adult receiving a single HB vaccine (adult dose)	An infant receiving a single HB vaccine at birth (pediatric dose)	A 2 month old receiving the recommended set of injections (mean exposure)
Al (μg)	500	250	862.5
Bw (kg)	70	3.4	5
Total Al μg/kg bw	7.1	73.5	172.5

## 4. Discussion

### 4.1. Summary and implications of main findings

To the best of our knowledge, these results are the first to show that Al, a highly neurotoxic metal and the most commonly used vaccine adjuvant, may be a significant contributing factor to the rising prevalence of ASD in the Western world. In particular, we show here that the correlation between ASD prevalence and Al adjuvant exposure appears to be the highest at 3–4 months of age (Pearson  $r = 0.89$ – $0.94$ ,  $p = 0.0018$ – $0.0248$ ; Table 8). We also show that children from countries with the highest ASD prevalence appear to have a much higher exposure to Al from vaccines, particularly at 2 months of age (Table 4). In this respect, we note that several prominent milestones of brain development in humans coincide with these periods. These include the onset of synaptogenesis (birth), maximal growth velocity of the hippocampus (2–3 postnatal months) [3] and the onset of amygdala maturation (8 weeks postnatal age) [81]. In addition, the period between 2 and 4 months is also one of major developmental transition in many biobehavioural systems, including sleep, temperature regulation, respiration and brain wave patterns [82,83], all of which are regulated by the neuroendocrine network [84,85]. Many of these aspects of brain function are known to be impaired in autism (i.e., sleeping and brain wave patterns [86–88]).

According to the FDA, vaccines represent a special category of drugs as they are generally given to healthy individuals [15]. Further according to the FDA, “this places significant emphasis on their [vaccine] safety” [15]. While the FDA does set an upper limit for Al in vaccines at no more than 850 μg/dose [89], it is important to note that this amount was selected empirically from data showing that Al in such amounts enhanced the antigenicity of the vaccine, rather than from existing safety



**Fig. 1.** Correlation between the number of children with ASD (6–21 years of age) and the estimated cumulative Al exposure (μg) from pediatric vaccines in the period from 1991 to 2008 (US data).

**Table 6**

Statistical analysis summary. Correlation between the number of children with ASD (6–21 years of age) and the estimated AI exposure ( $\mu\text{g}$ ) from pediatric vaccines in the period from 1991 to 2008 (US data). Significant change is indicated by the asterisk (\*).

	ASD prevalence and estimated yearly cumulative vaccine-derived AI exposures		
	Minimum	Mean	Maximum
Pearson r	0.92	0.92	0.92
95% CI	0.79–0.97	0.80–0.97	0.80 to 0.97
P value (two-tailed)	<0.0001	<0.0001	<0.0001
P value summary	*	*	*
Is the corr. significant? (p<0.05)	Yes	Yes	Yes
R <sup>2</sup>	0.84	0.85	0.85

data or from the basis of toxicological considerations [89]. However, in preventative vaccination where a vaccine is administered to healthy individuals, a compromise in efficacy for additional margins of safety should not necessarily be viewed as an unreasonable expectation [30]. It is also of note that the FDA requires limits on AI in parenteral feeding solutions and requires warning labels about potential AI hazards, while setting no safety limits or issuing warnings for AI in vaccines [90].

The lack of an established safety margin for AI in vaccines may be concerning for numerous reasons: (i) AI is highly neurotoxic and can impair prenatal and postnatal brain development in humans and experimental animals [28,91]; (ii) a pilot study showed higher than normal AI levels in the hair, blood and/or urine of autistic children (according to the authors, the correlation between the severity of signs and symptoms and the behavioral pattern found in many patients appeared to be compatible with metabolism disturbances provoked by AI overload [92]); (iii) children are regularly exposed to much higher levels of AI adjuvants than adults (Table 5 [14]); (iv) practically nothing is known about the pharmacokinetics and toxicodynamics of AI adjuvants in children [35] and paradoxically, evaluation of pharmaco- and toxicokinetics is not required for vaccine licensing purposes [93]; (v) in adult humans, AI vaccine adjuvants have been linked to serious neurological impairments, chronic fatigue and autoimmunity (Table 1) [31,32,94–96]; (vi) injection of AI adjuvants at levels comparable to those that are administered to humans have been shown to cause motor neuron death, impairments in motor function and decrements in spatial memory capacity in young mice [29,97]; and (vii) intraperitoneal injection of AI adsorbed vaccines in 4-week old mice was followed by a transient peak in brain AI levels on the second and third days after injection [98]. The latter experiment demonstrated that even a fully developed BBB does not impede AI access to the brain tissue. Altogether, the above observations raise plausible concerns about the overall safety of the use of AI adjuvants in childhood vaccines.

An additional concern is that for certain AI-adjuvanted vaccines the risks/benefit ratio appears to preclude widespread use. The HB vaccine, the only vaccine recommended to newborn babies, is one such example, since: (i) the HB virus is primarily transmitted through sexual contact with an infected person or by injections with contaminated material and, hence, poses no risk to infants unless the mother is a carrier [99];

**Table 7**

Statistical analysis summary. Correlation between the number of children with ASD (6–21 years of age) and the number of AI-adjuvanted vaccines in the yearly vaccination schedule in the period from 1991 to 2008 (US data). Significant change is indicated by the asterisk (\*).

	ASD prevalence and yearly number of AI-adjuvanted vaccines
Pearson r	0.90
95% CI	0.76–0.96
P value (two-tailed)	<0.0001
P value summary	*
Is the corr. significant? (p<0.05)	Yes
R <sup>2</sup>	0.82

**Table 8**

Pearson correlation summary according to age of vaccine exposure for ASD prevalence data in seven Western countries. Ages are expressed in months (mo). The adjusted p-values were derived using the resampling-based multiplicity adjustment according to Westfall and Young [78]. Note that for each country studied, the AI exposure is from all vaccines that children receive during the specified age period (i.e., the cumulative exposure to AI at 4 months of age includes AI from vaccines given at 2, 3 and 4 months). Significant change is indicated by the asterisk (\*).

Age	ASD prevalence in the US, UK, Canada, Australia, Sweden, Finland and Iceland in correlation with			
	Minimum AI exposure	Mean AI exposure	Maximum AI exposure	# AI-adjuvanted vaccines
2 months				
Pearson r	0.89	0.86	0.83	0.86
95% CI	0.40–0.98	0.29–0.98	0.21–0.97	0.30–0.98
p (unadjusted)	0.0077*	0.014*	0.0199*	0.0131*
p (adjusted)	0.0346*	0.0682	0.1283	0.0594
R <sup>2</sup>	0.79	0.73	0.69	0.74
3 months				
Pearson r	0.94	0.94	0.92	0.94
95% CI	0.63–0.99	0.62–0.99	0.55–0.99	0.50–0.99
p (unadjusted)	0.0017*	0.0019*	0.0032*	0.0014*
p (adjusted)	0.0018*	0.0018*	0.0038*	0.0018*
R <sup>2</sup>	0.88	0.88	0.85	0.89
4 months				
Pearson r	0.89	0.90	0.90	0.93
95% CI	0.43–0.98	0.45–0.99	0.46–0.99	0.60–0.99
p (unadjusted)	0.0067*	0.0059*	0.0055*	0.0022*
p (adjusted)	0.0248*	0.020*	0.0168*	0.0038*
R <sup>2</sup>	0.80	0.81	0.81	0.87
6 months				
Pearson r	0.85	0.83	0.82	0.90
95% CI	0.26–0.98	0.21–0.97	0.17–0.9	0.44–0.98
p (unadjusted)	0.0160*	0.0206*	0.0248*	0.0064*
p (adjusted)	0.0895	0.1333	0.157	0.0248*
R <sup>2</sup>	0.72	0.69	0.67	0.80
18 months				
Pearson r	0.82	0.80	0.77	0.89
95% CI	0.18–0.97	0.13–0.97	0.05–0.96	0.40–0.98
p (unadjusted)	0.0227*	0.0297*	0.0408*	0.0079*
p (adjusted)	0.1467	0.1871	0.3133	0.0368*
R <sup>2</sup>	0.68	0.64	0.60	0.79
72 months				
Pearson r	0.78	0.76	0.74	0.86
95% CI	0.055–0.97	0.03–0.96	–0.02–0.96	0.29–0.98
p (unadjusted)	0.0402*	0.0456*	0.0550	0.0138*
p (adjusted)	0.3087	0.353	0.4128	0.0682
R <sup>2</sup>	0.60	0.58	0.55	0.73

(ii) the incidence of the HB infection in Western countries is extremely low (0.9–2.7 per 100,000) and some of these countries indeed only vaccinate high-risk groups [100]; (iii) a striking decline in the incidence of HB virus infections in these countries occurred during the second half of the 1980s, but only a minor part of this decline was due to HB vaccination since rather limited vaccination programs have been introduced in most Western countries at that time [99]; and (iv) epidemiological studies implicate HB vaccination as a risk factor for ASD. For example, in the US, males aged 0–9 years who received a complete triple series of HB vaccine were found to be significantly more susceptible to developmental disabilities [101], while those aged 3–17 years who received HB vaccination during the first month of life had a 3-fold greater risk of ASD than unvaccinated males [102]. Finally, in newborn primates, a single dose of the HB vaccine is sufficient to cause neurodevelopmental delays in acquisition of neonatal reflexes essential for survival [7]. Although the HB vaccines are adjuvanted with AI (Table 2), both the primate and the epidemiological studies mentioned above only draw attention to thimerosal (ethyl mercury vaccine preservative). This point was also noted by Dorea and Marques in their analysis of infant exposure to AI from vaccines and breast milk during the first 6 months of life [2]. These authors also noted that in general, mercury toxicity is well recognized and has been more studied and better understood than AI toxicity

[2]. Altogether, these observations suggest that, in spite of its well documented neurotoxic effects, Al is not perceived as a potential hazard in vaccines.

#### 4.2. Dietary versus injectable Al: what is the difference?

Given the bioavailability of Al through food sources, a common assertion in relation to Al in vaccines is that children obtain much more Al from diet. From this perspective, Al from vaccination does not represent a toxicological risk factor [39,103]. However, this notion contradicts basic toxicological principles. For instance, it should be obvious that the route of exposure which bypasses the protective barriers of the gastrointestinal tract and/or the skin will likely require a lower dose to produce a toxic outcome [14,16]. In the case of Al, only ~0.25% of dietary Al is absorbed into systemic circulation [104]. In contrast, Al hydroxide (the most common adjuvant form) injected intramuscularly may be absorbed at nearly 100% efficiency over time [105]. In addition, although the half-life of enterally or parenterally absorbed Al from the body is short (approximately 24 h), the same cannot be assumed for adjuvant-Al because the sizes of most antigen-Al complexes (24 to 83 kDa [60,106,107]) are higher than the molecular weight cut-off of the glomerulus of the kidney (~18 kDa [108]) which would preclude efficient excretion of Al adjuvants. In fact, a longer elimination period is one of the major properties of effective vaccine adjuvants, including those using Al salts [2,14]. Additionally, the tightness of bonding between the Al adjuvant and the antigen is considered a desired feature that can be used to predict the immunogenicity of vaccines [109]. Experiments in adult rabbits demonstrate that even in an antigen-free form, Al-hydroxide, the most commonly used Al adjuvant (Table 2) is poorly excreted. The cumulative amount of Al-hydroxide in the urine of adult rabbits as long as 28 days post intramuscular injection was less than 6% as measured by accelerator mass spectrometry [110]. Al-phosphate was more efficiently excreted (22%) [110]. Finally, it is important to recognize that neonates have anatomical and functional differences crucial for toxicokinetics and toxicodynamics of neurotoxic metals (e.g., an immature renal system and an incomplete BBB), which would further compromise their ability to eliminate Al adjuvants [2,4,5].

#### 4.3. Study results in relation to Hill's criteria: is there a causal relationship between Al vaccine adjuvants and the prevalence of ASD?

The positive correlation between Al exposure from vaccines and prevalence of ASD does not necessarily imply causation. However, if the correlation is strong (criterion 1), consistent (criterion 2) and if there is a biologically plausible mechanism by which it can be explained (criterion 6), as well as an appropriate temporal relationship between the proposed cause and the outcome (criterion 4), then the satisfaction of these criteria supports the notion that the two events may indeed be causally related. Our results satisfy not only all four of these criteria applicable for establishing causation in neuropsychiatry [80], but also four others. These additional criteria are: (5) biological gradient, (7) coherence with the current knowledge, (8) experimental or semi-experimental evidence and (9) the analogy with similar evidence (Table 9). These are discussed below as they are extremely relevant for the ways in which Al might induce ASD.

Thus, in total, the results of our study satisfy eight out of nine of Hill's criteria for causation [41]. The only criterion that our current study fails to satisfy is the "specificity" criterion which is actually not applicable to ASD given that the latter is recognized as a multifactorial disease [20,21,111]. Overall, an analysis of our results indicates that the adjuvant effect of Al in vaccines may be a significant etiological factor in the increasing prevalence of ASD in some Western countries.

#### 4.4. Al-adjuvants and the immature brain and immune system

There is a growing body of data that supports a significant role for immune system-related molecules in the etiology of a variety of neurological disorders, including autism [25,111–115]. In addition, some 15 years ago, Cohen and Shoenfeld made the important observation that, "It seems that vaccines have a predilection to affect the nervous system" [116]. With regard to this statement, as well as the ensuing discussion, four key observations ought to be considered. First, there are critical periods in brain development during which even subtle immune challenges (including those induced by vaccinations) can lead to permanent detrimental alterations of brain and immune function [7,9,117,118]. Second, preschool children in developed countries are regularly exposed to significant amounts of Al adjuvants through vaccination programs (250–862.5 µg; Table 3). Such high exposures to adjuvant-Al which are repeated over relatively short intervals during these critical periods of brain development (i.e., first 2 years post-natal) constitute a significant neurotoxicological as well as an immunological challenge to neonates and young children [2,14]. Third, despite a prevalent view that peripheral immune responses do not affect brain function, overwhelming research suggests that neuro-immune cross-talk may be the norm rather than the exception [25,84,119–128]. Indeed, it is now clearly established that this bi-directional neuro-immune cross-talk plays crucial roles in immunoregulation and brain function [84,128–135]. In turn, perturbations of the neuro-immune axis have been demonstrated in many diseases encompassed in the 'ASIA' syndrome (Table 1) and are thought to be driven by a hyperactive/unrestrained immune response [130,135]. Fourth, the very same components of the neuro-immune regulatory system that are known to play key roles in proper brain development and immune function (i.e., interleukin (IL)-1, IL-6, major histocompatibility complex (MHC) class I, complement cascade [25,84,119–129,133,135]), are heavily targeted by Al adjuvants (Table 1). The latter experimental evidence suggests that Al adjuvants have all the necessary biochemical properties needed to induce neurological and immune disorders. In this regard, it is interesting to note that autism is a multisystem disorder characterized by dysfunctional immunity and impaired CNS function [17,20,22].

Although vaccines are credited for decreasing the risk of neurodevelopmental complications arising from natural infections in early childhood, the problem is that in many ways the immune challenge from vaccinations may be much greater in magnitude than that arising from a natural infection. The main reason for this is that early-life immune responses (before 6 months of age) are weaker and of shorter duration than those that are elicited in immunologically mature hosts [136,137]. Hence, in order to provoke and sustain an adequate B-cell immune response in a neonate, strong immune adjuvants and repeated closely spaced booster doses are needed [137]. Furthermore, in the absence of Al, most antigenic compounds fail to launch an adequate immune response [31,40,138], suggesting that a large part of the immunostimulatory effects of vaccines may be driven by the Al-adjuvant itself. While it is generally accepted that potency and toxicity of immune adjuvants must be adequately balanced so that the necessary immune stimulation is achieved with minimal side effects, in practical terms, such a balance is very difficult to achieve. This is because the same adjuvant-mediated mechanisms which drive the immunostimulatory effects of vaccines have the capacity to provoke a variety of adverse reactions (Table 1). The potential hazards of vaccination with Al adjuvants thus not only arise from the possibility that a single vaccine may change the pre-programmed immune milieu in a neonate and thus compromise neural development, but also that multiple Al-adjuvanted vaccinations are administered simultaneously. Multiple exposure magnifies the inflammatory response and while this is essential for linking the innate and adaptive immune responses, it may also be responsible for the immunotoxic effects of Al adjuvants (Table 1).

**Table 9**

Study results in relation to Hill's criteria applicable for establishing causality between exposure and outcome.

Hill's criterion	Does the current study satisfy the criterion?	Comment
Strength (1)	Yes	The association is highly statistically significant (Tables 6–8).
Consistency (2)	Yes	The positive and statistically significant correlation between vaccine-derived Al exposures (as well as the overall uptake of Al-adjuvanted vaccines), and ASD prevalence is consistently observed in different populations (Table 8). While ours is, to the best of our knowledge, the first study to investigate the possible association between Al vaccine adjuvants and ASD, at least three more studies have found a positive association between the prevalence of autism (and developmental disabilities) and vaccination uptake in early childhood, a result consistent with our findings [101,102,179]. In addition, a recent study showed that autistic children have higher than normal levels of Al in the body (hair, blood and/or urine) [92]. In contrast, neither copper, lead nor mercury were elevated beyond normal levels in these children [92].
Specificity (3)	No	Not applicable to diseases such as ASD with possible multifactorial etiologies [79].
Biological rationale (4)	Yes	Al is a neurotoxin and a strong immune stimulator, hence, Al has the necessary biochemical properties to induce neuroimmune disorders such as ASD. The immunostimulatory properties of Al adjuvants are numerous and affect both innate and adaptive immune responses (see Table 1). Chronic hyperactivation of immune responses by repeated short-interval administration of Al-adjuvants could: (i) disrupt the delicate balance of immune mediators which is crucial for proper brain development and function (i.e., members of the MHC, complement, pro-inflammatory cytokines TNF- $\alpha$ , IL-1 $\beta$ and IL-6 [25,119–127,141,142]); (ii) promote activation of neuroglia and brain inflammation [29,97,139]; and (iii) promote aberrant immune responses [31,32,157], all of which are known pathophysiological features of ASD [17,20,23,111,147].
Temporal relationship (5)	Yes	Up until and during the early 1980s, the prevalence of ASD was relatively low (<5 in 10,000 children [180,181]). Currently, 1 in 91 children in the US is diagnosed with ASD (110 per 10,000 [73]). In the United Kingdom, current reported ASD prevalence is 1 in 64 children (157 per 10,000 [74]). The increase in the number of vaccines given to children precedes the “autism epidemic” (i.e., from 10 in the late 70s to 32 in 2010 (18 of which contain Al adjuvants) [16]. Note also that the dramatic increase in the prevalence of ASD observed over the last three decades in the US and the UK (2000–3000%) cannot be convincingly explained by genetic factors alone nor by changes in diagnostic criteria. Concerning the latter, in many ways such criteria have become more restrictive [182]. Moreover, in a recent analysis comparing the prevalence of autism with that of other disabilities among successive birth cohorts of US school-aged children, Newschaffer et al. [180] clearly show that autism prevalence has been increasing with time, as evidenced by higher prevalences among younger birth cohorts.
Biological gradient (6)	Yes	The higher the Al exposure from vaccines, the higher the prevalence of ASD (Fig. 1; Table 4).
Coherence (7)	Yes	The same pro-inflammatory mediators that are induced by Al adjuvants were shown to be elevated in the blood, cerebrospinal fluid (CSF) and post-mortem brain tissue of ASD patients (see Table 1). Increase in pro-inflammatory mediators in autistic brains was also found concurrent with widespread activation of astro- and microglia and increased immunoreactivity to MHC class II [17], all of which can also be activated by Al-adjuvants (Table 1).
Experimental/semi-experimental evidence (8)	Yes	Al can impair prenatal and postnatal brain development in humans and experimental animals [28,91]. Other well-documented symptoms of Al intoxication in humans that are relevant to ASD include loss of speech skills, cognitive and behavioral impairments, increased incidence of seizures, increased inflammation and microgliosis in the brain, impairment of synaptic plasticity, synaptic loss and myelin sheath damage [16,29,91,94,183–186].
Analogy (9)	Yes	Peripheral stimulation of the immune system during critical periods of brain development can lead to ASD-related outcomes [9,118,187–189].

#### 4.5. Al adjuvants and brain inflammation

Repeated injections of 1 mg/kg of Al nanoparticles to adult Sprague–Dawley rats is sufficient to produce significant inflammatory effects in the rat brain [139]. Comparable amounts of Al are administered to 2, 6 and 15 month old infants according to the US vaccination schedule (Table 3). Moreover, as we have demonstrated previously, only two subcutaneous injections of Al adjuvants (relevant to adult human exposure) in young male mice, spaced two weeks apart, were sufficient to cause dramatic activation of microglia and astrocytes that persisted up to 6 months post-injection. This outcome was accompanied by motor neuron death, impairments in motor function and decrements in spatial memory capacity [29,97]. What then might be the effects of repeated, closely spaced administration of Al adjuvanted vaccines (i.e., every 2–4 months from birth up until 12 months of age) in immature human infants? One possibility is that such treatment would increase the risk of chronic brain inflammation. In this regard, it is worth noting that neuroinflammatory mechanisms appear to play an important role in the pathophysiology of autism [17,20].

It is well established that peripheral immune insults can directly stimulate the synthesis of pro-inflammatory cytokines (i.e., IL-1 $\beta$ , IL-6 and tumor necrosis factor (TNF)- $\alpha$ ) within the brain [84,140], acting to promote inflammation even in the absence of a direct CNS infection. Moreover, the same pro-inflammatory mediators that are normally induced by Al adjuvants have been shown to be elevated in the blood, cerebrospinal fluid (CSF) and brain tissues of ASD patients (Table 1). The aberrant neuroinflammatory cytokine profile in autistic

brains was found concurrently with widespread microglial and astrocyte activation. In particular, microgliosis in autism coincided with increased immunoreactivity to MHC class II markers [17]. Microglia, astrocytes, as well as members of the MHC and the complement cascade are crucial regulators of synaptic connectivity, function and plasticity and play key roles in establishing and modulating neuronal circuitry in the developing CNS [25,112,119–126,141,142]. Notably, abnormal brain connectivity is well recognized as one of the hallmarks of autism [143,144]. Cerebellar Purkinje cells, which are significantly reduced in autism, are a site of prominent MHC class I expression. One hypothesis currently under investigation is that specifically timed changes in neuronal MHC class I expression could contribute to autism [143].

Given that Al adjuvants activate both MHC class I and II, components of the complement cascade, increase pro-inflammatory cytokines IL-1 $\beta$ , IL-6 and TNF- $\alpha$ , as well as activate microglia and astrocytes in the brain (Table 1), it is possible that they may also interfere with synaptic pruning and developmental activity-dependent synaptic remodeling/plasticity. At present, there is experimental evidence that Al can impair synaptic plasticity *in vivo* [91,145,146], which can be reversed by vasopressin treatment of Al-exposed rats [146].

#### 4.6. Al adjuvants as promoters of autoimmune/inflammatory reactions in the brain

Experimental evidence clearly shows that simultaneous administration of as little as two to three immune adjuvants can overcome genetic resistance to autoimmunity in animals [10]. While currently there is no



direct evidence that Al can induce autoimmunity, it is important to recognize that it certainly has a biochemical potential to do so.

Autoimmune manifestations, particularly those affecting the CNS, are prevalent in autistic individuals and do not appear to be limited to only a few nervous system antigens. For example, Vojdani et al. [147] demonstrated elevated levels of immunoglobulins (Ig)G, IgM and IgA against nine different neuron-specific antigens in ASD children. Such widespread manifestation of autoimmunity may have arisen from an alteration in the BBB which would then have enabled access of immunocompetent cells to many different central nervous system antigens [147].

Al is known to disrupt the BBB and can increase its permeability by increasing the rate of trans-membrane diffusion and by selectively altering saturable transport systems [5,148,149]. Even in an adjuvant form, Al can enter the brain [98]. Furthermore, much like mercury, Al may induce autoimmunity through the so-called “bystander” effect [150]. Finally, Al's ability to upregulate chemo-attractants such as monocyte chemoattractant protein (MCP)-1, monocyte inflammatory protein (MIP)-1 $\alpha$  and MIP-1 $\beta$  [40], could promote the active recruitment of immunocompetent cells into the brain, leading to inflammation and/or autoimmunity. Consistent with this interpretation, post-mortem analysis of six children aged 4–17 months who died within 48 h of exposure to Al-adjuvanted hexavalent vaccines revealed abnormal pathologic findings in the nervous system, including a defective BBB, infiltration of the leptomeninges by macrophages and lymphocytes, perivascular lymphocytic infiltration, diffuse infiltration of the pons, mesencephalon and cortex by T-lymphocytes and increased microglia in the hippocampus and pons [151]. The neuropathological observations made by Zinka et al. [151] are consistent with the well established immunostimulatory and neurotoxicological properties of Al vaccine adjuvants.

## 5. Conclusions and future directions

By satisfying eight of the Hill's criteria for establishing causality applicable to our study (Table 9), we show that Al-adjuvanted vaccines may be a significant etiological factor in the rising prevalence of ASD in the Western world. We also show that children from countries with the highest ASD prevalence appear to have a much higher exposure to Al from vaccines, particularly at 2 months of age. In addition, the correlation between ASD prevalence and Al adjuvant exposure appears to be the highest at 3–4 months of age. Of note, these periods (i.e., first 4 post-natal months) coincide with several critical stages of human brain development and biobehavioural transitions that are known to be impaired in autism (i.e., onset of synaptogenesis, maximal growth velocity of the hippocampus [3], onset of amygdala maturation [81] and development of brain-wave and sleeping patterns [82,83]).

Clearly, we cannot draw definite conclusions regarding the link between Al adjuvants and autism based on an ecological study such as the present one and hence the validity of our results remains to be confirmed. A case control study with detailed examination of vaccination records and Al body burden measurements (i.e., hair, urine, blood) in autistic and a control group of children would be one step toward this goal. Nonetheless, given that the scientific evidence appears to indicate that vaccine safety is not as firmly established as often believed, it would seem ill advised to exclude pediatric vaccinations as a possible cause of adverse long-term neurodevelopmental outcomes, including those associated with autism.

We have thus provided a hypothesis which we hope will encourage future research into this area in order to resolve the issue of whether or not vaccines might be responsible in some part for the growing prevalence of autism in the developed world. Such future research should consider the following: (i) the postnatal period represents a very sensitive phase in development during which the physiology of the nervous as well as the immune system can be influenced and sometimes permanently changed [8,9,118,119,152–154]; (ii) Al is a

neurotoxin and a strong immune adjuvant (Table 1), hence Al has all the necessary biochemical properties to induce neurological and immune disorders; and (iii) autism is a multisystem disorder characterized by dysfunctional immunity and impaired brain function [17,20,22]. Because the current safety data for Al exposure in infants and children is unsatisfactory and because this demographic represents those who may be most at risk for complications following vaccination, a more rigorous evaluation of Al adjuvant safety than what has been provided to date seems warranted.

## 6. Competing interests

CAS is a founder and shareholder of Neurodyn Corporation, Inc. The company investigates early state adult neurological disease mechanisms and biomarkers. This work and any views expressed within it are solely those of the authors and not of any affiliated bodies or organizations. CAS and LT are in favor of a more rigorous evidence based medicine approach to vaccine safety.

### Abbreviations

ASD	autism spectrum disorders
Al	aluminum
APC	antigen presenting cells
BBB	blood brain barrier
CDC	Centers for Disease Control and Prevention
CNS	central nervous system
CFS	chronic fatigue syndrome
CTL	cytotoxic T cell
DTaP	Diphtheria, Tetanus, acellular Pertussis
EAE	experimental autoimmune encephalomyelitis
FDA	Food and Drug Administration
GFAP	glial fibrillary acidic protein
GWS	Gulf War syndrome
HA	Hepatitis A
HB	Hepatitis B
Hib	Haemophilus influenza type b
IDEA	The Individuals with Disabilities Education Act
Ig	Immunoglobulin
IL	interleukin
LPS	lipopolysaccharide
MCP	monocyte chemoattractant protein
MenC	Meningococcal serogroup C
MHC	major histocompatibility complex
MIP	monocyte inflammatory protein
MMF	Macrophagic myofasciitis
MS	multiple sclerosis
NLRP3	nucleotide-binding domain, leucine-rich, repeat containing family, Pyrin-domain containing 3
NO	nitric oxide
PCV	Pneumococcal
ROS	reactive oxygen species
TNF- $\alpha$	tumor necrosis factor

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## EDITORIAL

### The Biochemistry/Toxicity of Aluminum

We live in what one author of this *Hot Topic* issue has correctly labeled “the Age of Aluminum” [1]. Aluminum, the third most abundant element in the Earth’s crust and the most abundant metal, is one of the most remarkable elements in the periodic table. Compounds made with aluminum are strong, durable, light and corrosion resistant. Aluminum is also an excellent conductor of electricity.

For these reasons, aluminum currently finds its way into virtually every aspect of our daily lives. Industrially, aluminum is used in cans and cookware, aluminum foil, housing materials, components of electrical devices, airplanes, boats, cars and numerous hardware items of all descriptions. Aluminum is found in drinking water, as a food additive in typical Western diets, cosmetics, pharmaceutical products and because of such ubiquity, it is increasingly found in our bodies [2, 3].

None of this would necessarily be a problem if aluminum was inert in biological systems. However, in spite of a widely held belief that this is true, it is demonstrably not the case. Aluminum is highly reactive with oxygen and carbon, two of the most abundant organic elements, yet appears to have no intrinsic nor beneficial role in organic chemistry of any biota on the planet [1]. Instead, evidence clearly shows that aluminum is toxic to plants, animals and humans.

For example, aluminum intoxication frequently impairs learning, memory, concentration and behaviour in both animals and humans. The latter is typically reflected in confusion, anxiety, repetitive behaviours and sleep disturbances. Notably, all of these symptoms typical of an aluminum overload are also typical to two most common neurological disorders of the Western world, one neurodegenerative and the other one neurodevelopmental: Alzheimer’s disease and autism. Moreover, there is now sufficient experimental evidence implicating elevated levels of aluminum in both of these disease conditions [2, 4, 6].

In this Hot Topic issue of Current Inorganic Chemistry we have brought together some of the world’s experts on the biochemistry of aluminum to consider the potential impacts of aluminum compounds on human health. The issue starts with a discussion of aluminum’s exposome (Exley) and then proceeds to explore how aluminum can impact biological systems through some of its modern compounds, specifically fluoroaluminates (Strunecka *et al.*). In the central part of the issue, Walton challenges the long-term notion that aluminum’s role in Alzheimer’s disease rests on a myth. Focusing on inflammation, the fourth contribution highlights the ways in which aluminum compounds might promote the onset and progression of neurological diseases in general (Bondy). Blaylock further expands on this concept and shows how not only inflammation, but rather, the interaction between inflammatory mediators and excitotoxins is crucial for the way by which aluminum exerts its toxic actions throughout the central nervous system (CNS). Finally, Yokel demonstrates the many molecular mechanisms by which aluminum might reach the CNS. Importantly, this final evidence clearly negates past notions that aluminum’s accumulation in Alzheimer’s is an artifact of passive uptake by dysfunctional neurons.

If the focus of this series of articles seems to the reader to be heavily weighted toward the nervous system, then this perception is correct. Aluminum does many things in biological systems, none of them beneficial. But perhaps the most deleterious actions are on CNS structures and function where aluminum impacts seem to be the most egregious at the two ends of the age spectrum: early postnatal life and old age. While in the first case aluminum exposure could precipitate adverse neurodevelopmental outcomes associated with autism [6], in the second case it could lead to one of the most devastating neurodegenerative diseases known to man [4].

The potential for aluminum to do harm can hardly be disputed. The means of remediation from aluminum intoxication are limited at present while the risk of exposure is increasing. It would thus appear that the practical considerations of warnings given by William Gies are now 100 years overdue, “*These studies have convinced me that the use in food of aluminum or any other aluminum compound is a dangerous practice*” [7]. Perhaps the key to real progress against the rapidly increasing neurological disease burden is to eliminate unnecessary human exposure to aluminum.

This issue, we hope, will trigger the long delayed and much needed debate about aluminum in the biosphere and its impact on human health. Finally, we thank all the authors who keenly accepted our invitation to contribute to this issue, as well as the reviewers who invested their valuable time to ensure the high scientific quality of all contributions.

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## SPECIAL ARTICLE

# Mechanisms of aluminum adjuvant toxicity and autoimmunity in pediatric populations

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Immune challenges during early development, including those vaccine-induced, can lead to permanent detrimental alterations of the brain and immune function. Experimental evidence also shows that simultaneous administration of as little as two to three immune adjuvants can overcome genetic resistance to autoimmunity. In some developed countries, by the time children are 4 to 6 years old, they will have received a total of 126 antigenic compounds along with high amounts of aluminum (Al) adjuvants through routine vaccinations. According to the US Food and Drug Administration, safety assessments for vaccines have often not included appropriate toxicity studies because vaccines have not been viewed as inherently toxic. Taken together, these observations raise plausible concerns about the overall safety of current childhood vaccination programs. When assessing adjuvant toxicity in children, several key points ought to be considered: (i) infants and children should not be viewed as “small adults” with regard to toxicological risk as their unique physiology makes them much more vulnerable to toxic insults; (ii) in adult humans Al vaccine adjuvants have been linked to a variety of serious autoimmune and inflammatory conditions (i.e., “ASIA”), yet children are regularly exposed to much higher amounts of Al from vaccines than adults; (iii) it is often assumed that peripheral immune responses do not affect brain function. However, it is now clearly established that there is a bidirectional neuro-immune cross-talk that plays crucial roles in immunoregulation as well as brain function. In turn, perturbations of the neuro-immune axis have been demonstrated in many autoimmune diseases encompassed in “ASIA” and are thought to be driven by a hyperactive immune response; and (iv) the same components of the neuro-immune axis that play key roles in brain development and immune function are heavily targeted by Al adjuvants. In summary, research evidence shows that increasing concerns about current vaccination practices may indeed be warranted. Because children may be most at risk of vaccine-induced complications, a rigorous evaluation of the vaccine-related adverse health impacts in the pediatric population is urgently needed. *Lupus* (2012) **21**, 223–230.

**Key word:** adjuvants; aluminum; autoimmunity; immunotoxicity; inflammation; neurotoxicity; vaccine safety

## Introduction

Aluminum (Al) is highly neurotoxic and has been shown to impair both prenatal and postnatal brain development in humans and experimental animals.<sup>1–2</sup> In addition to its neurotoxic properties, Al is a potent stimulator of the immune system, which

is the very reason why it is used as an adjuvant.<sup>3–8</sup> Given this, it is somewhat surprising to find that in spite of over 80 years of use, the safety of Al adjuvants continues to rest on assumptions rather than scientific evidence. For example, nothing is known about the toxicology and pharmacokinetics of Al adjuvants in infants and children.<sup>9</sup> On the other hand, in adult humans long-term persistence of Al vaccine adjuvants can lead to cognitive dysfunction and autoimmunity.<sup>6,10</sup> Yet, in spite of these observations children continue regularly to be exposed to much higher levels of Al adjuvants than adults, via routine childhood vaccination programmes.<sup>3,11</sup>

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An additional concern to using a neurotoxic substance such as Al as an adjuvant in pediatric vaccine formulations is the fact that infants and young children should not be considered simply as “small adults” when it comes to toxicological risk. In spite of this, a review of the literature to date relating to Al-toxicology indicates that the vast majority of previous research and testing has been dedicated to Al exposure in adults.<sup>12</sup> If a few vaccines administered to adults can result in adverse outcomes associated with the “ASIA” syndrome, is it reasonable to assume in the absence of experimental evidence that the current pediatric schedules, often exceeding 30 vaccinations in the first 4 to 6 postnatal years,<sup>3,13</sup> are safe for children? The purpose of this review is to address the mechanisms of Al adjuvant toxicity with special reference to the developing neuro-immune system and the “ASIA” syndrome in order to shed light on this unresolved and hotly debated question.

### **Al adjuvants: a toxicological risk to a developing child?**

Some 15 years ago, Cohen and Shoenfeld made an important observation: “It seems that vaccines have a predilection to affect the nervous system.”<sup>14</sup> Furthermore, according to Israeli and co-workers, alongside their supportive role in vaccine-induced immune responses, vaccine adjuvants were found to inflict, by themselves, illnesses of an autoimmune nature.<sup>5</sup> With regard to these statements, as well as the ensuing discussion, five key observations ought to be considered. First, there are critical periods in brain development during which even subtle immune challenges (including those induced by vaccinations) can lead to permanent detrimental alterations of brain and immune function.<sup>15–17</sup> Indeed, a single Al-adjuvanted hepatitis B vaccine administered to newborn primates within 24 h of birth is sufficient to cause neurodevelopmental delays in acquisition of neonatal reflexes essential for survival.<sup>17</sup> Second, through multiple vaccinations preschool children are regularly exposed to significant amounts of Al adjuvants.<sup>3,18</sup> Such high exposures to Al repeated over relatively short intervals during critical neurodevelopmental periods constitute a significant neuro-immunotoxicological challenge to neonates and young children.<sup>18</sup> Third, despite the prevalent view that peripheral immune responses do not affect brain function, overwhelming research evidence clearly points to the contrary. Namely, it is

now firmly established that there is a bidirectional neuro-immune cross-talk which plays crucial roles in immunoregulation, brain function, and maintenance of general homeostasis.<sup>19,20</sup> In turn, perturbations of the neuro-immune axis have been demonstrated in a variety of autoimmune/inflammatory diseases encompassed in the “ASIA” syndrome.<sup>21–24</sup> Fourth, the very same components of the neuro-immune regulatory system that demonstrably play key roles in both brain development and immune function (e.g., immune cytokines),<sup>19–20,25</sup> are heavily targeted by Al adjuvants (Table 1). Fifth, experimental evidence demonstrates that a strong adjuvant effect can overcome genetic resistance to autoimmunity.<sup>26</sup>

Thus, the possibility needs to be considered that repeated immune system stimulation with multiple vaccines during critical periods of brain development could result in adverse neurodevelopmental outcomes and or/autoimmunity.<sup>18</sup>

### **Mechanisms of immune stimulation by Al adjuvants: what are the risks?**

The success of Al as a vaccine adjuvant is due to its potent and multifactorial stimulatory effects on the immune system (Table 1). In fact, with the exception of attenuated viruses, in the absence of Al most antigenic compounds fail to launch an adequate immune response,<sup>5,27–28</sup> suggesting that a significant part of the immunostimulatory effects of vaccines may be driven by the Al-adjuvant itself. While the potency and toxicity of Al-adjuvants should be adequately balanced so that the necessary immune stimulation is achieved with minimal side effects, such balance is difficult to achieve in practice. This is because the same mechanisms that drive the immunostimulatory effects of adjuvants have the capacity to provoke a variety of adverse reactions, including those associated with the “ASIA” syndrome (Table 1).

There are additional problems with using a neurotoxic substance such as Al as an immune stimulator in pediatric vaccinations. First, during prenatal and early postnatal development the brain is extremely vulnerable to neurotoxic insults. Not only are these highly sensitive periods of rapid brain development but also, the blood–brain barrier is incomplete and thus more permeable to toxic substances during this time.<sup>11,12,29</sup> Additionally, the immature renal system of neonates significantly compromises their ability to eliminate environmental toxicants.<sup>11,12</sup> For all these reasons, children are

at much greater risk of adverse reactions from Al adjuvants than adults.

Although vaccines are often credited for decreasing the risk of neurodevelopmental complications arising from natural infections in early childhood, it should be noted that immune stimulation induced by vaccinations may be much greater in magnitude than that resulting from natural infections. The main reason for this is that early-life immune responses (before 6 months of age) are weaker and of shorter duration than those elicited in immunologically mature hosts.<sup>30,31</sup> Thus, to provoke and sustain an adequate B-cell immune response in neonates, strong immune adjuvants such as Al, as well as repeated closely spaced booster doses are needed.<sup>31</sup> In contrast, during the course of natural infections, children are in most cases exposed to one pathogenic agent (or immune stimulant) at a time (i.e., measles only as opposed to measles,

mumps, and rubella all at once). This allows for a more subtle priming of the immature immune system, as well as brain recovery from the potential neuro-immune challenge.

The inability of an immature immune system to mount a robust immune response to certain antigens stems in part from an inherent anti-inflammatory phenotype of neonatal splenic macrophages which fail to produce sufficient amounts of pro-inflammatory cytokines (such as interleukin (IL)-1 and IL-6, both of which are induced by Al adjuvants; Table 1). These cytokines are needed for adequate stimulation of antibody-producing B-cells.<sup>32</sup> This attenuation of pro-inflammatory cytokine production by neonatal macrophages may be an important developmental program of the neonate, rather than a defect because the anti-inflammatory phenotype may be beneficial to the neonate at a time when

**Table 1** Shared aspects between autoimmune/inflammatory conditions and immunostimulatory properties of Al vaccine adjuvants

Disease	Condition		Al adjuvant	
	Th shift	Inflammatory profile	Inflammatory profile	General immunostimulatory effects
Arthritis*†	Excessive Th1 <sup>20</sup>	Increased IL-1, IL-6, IL-12, TNF- $\alpha$ , IFN- $\gamma$ , MIP-1 $\alpha$ and oxidative stress <sup>20,55,63</sup>	Increases cytokines (IL-1 $\alpha$ , IL-1 $\beta$ , IL-4, IL-5, IL-6, IL-18, TNF- $\alpha$ ), chemokines (IL-8, MCP-1, MIP-1 $\alpha$ , MIP-1 $\beta$ ), ROS, and nitric oxide (NO) <sup>8,27,28,41,42,55</sup>	Stimulates recruitment of monocytes, macrophages and granulocytes to the injection site
Autoimmune thyroid disease	Excessive Th1 <sup>20</sup>			
Inflammatory bowel disease (IBD)/ Crohn's disease (CD)	Excessive Th1 <sup>55</sup>	Increased NLRP3 inflammasome complex signalling and NLRP3-dependent overproduction of IL-1 $\beta$ , IL-6, IL-18, TNF- $\alpha$ and reactive oxygen species (ROS) in MS, EAE, Type 1 diabetes mellitus <sup>64-66</sup> and animal models of IBD <sup>67</sup>	Activates the NLRP3 inflammasome complex and NLRP3-dependent cytokines <sup>7,8</sup>	Induces differentiation of monocytes to antigen presenting cells (APCs)
Type 1 diabetes mellitus*	Excessive Th1 <sup>20</sup>			Activates APCs
Multiple sclerosis (MS)*† and experimental autoimmune encephalomyelitis (EAE)	Excessive Th1 <sup>20</sup>			Promotes antigen uptake and processing by APCs and enhances antigen-specific T-cell responses
Systemic lupus erythematosus (SLE)*	Excessive Th2 <sup>20,56</sup>	Increased IL-10, IL-18, IL-6, IFN- $\gamma$ , TNF- $\alpha$ <sup>20,56,68</sup>		Increases the expression of MHC class I and II and associated co-stimulatory molecules on peripheral blood monocytes
Macrophagic myofasciitis (MMF) and chronic fatigue syndrome (CFS)*†	Excessive Th2 <sup>57-59</sup>	Increased IL-4, IL-6, B-cell hyperlymphocytosis, infiltration of large periodic acid-schiff (PAS)-positive macrophages, and CD8 <sup>+</sup> T lymphocytes in the absence of conspicuous muscle fibre damage <sup>57,59,69</sup>		Activates the complement cascade
Gulf War Syndrome (GWS)*†	Mixed Th1/Th2 <sup>60</sup>	Increased IFN- $\gamma$ , IL-5, IL-6 <sup>60</sup>		Generally stimulates Th2 responses but can also induce a Th1 shift and activate cytotoxic T lymphocytes (CTLs) in the presence of other Th1 stimulators (i.e., lipopolysaccharide (LPS), CpG, recombinant influenza protein antigen) <sup>27,73-75</sup>
Autism spectrum disorders (ASD)*	Both Th1 and Th2 shifts have been reported <sup>61, 62</sup>	Increased IL-1 $\beta$ , IL-4, IL-5, IL-6, TNF- $\alpha$ , IL-8, MCP-1, MIP-1 $\beta$ , MHC class II <sup>61,70,71</sup>		Activates astrocytes and microglia <sup>76</sup>
		Increased astrocyte and microglia reactivity <sup>70, 72</sup>		

\*linked to Al-adjuvanted vaccines.<sup>6,35,38,77-79</sup>

†specifically recognized as 'Autoimmune/inflammatory syndrome induced by adjuvants' ('ASIA').<sup>6</sup>

tissue development is taking place at a rapid pace.<sup>32</sup>

The risks from current childhood vaccination schedules are thus twofold. First, a single vaccine may disrupt the delicate balance of immune mediators required for normal brain development and thus compromise neurodevelopmental programs. Second, such multiple vaccinations are routinely administered simultaneously (Table 2), thus magnifying the inflammatory response which, although being essential for linking the innate and adaptive immune responses, is also responsible for adjuvant's immunotoxic effects.<sup>4</sup> The repetitive taxing of the immune system by high doses of Al adjuvants may also cause a state of immune hyperactivity, a known risk for autoimmune diseases.<sup>6,33,34</sup>

Consistent with all of the above, in an epidemiological study examining the impact of hepatitis B vaccination in male children, Gallagher and Goodman<sup>35</sup> showed that those receiving a single vaccine during the first month of life had a

threefold greater risk of neurodevelopmental disorders compared with those vaccinated later or not vaccinated. Further evidence from case reports validates the highly contentious hypothesis that multiple vaccinations may precipitate developmental regression, at least in susceptible individuals.<sup>36</sup> Finally, routine vaccination in children has been associated with a variety of autoimmune conditions, including transverse myelitis,<sup>37</sup> insulin-dependent diabetes mellitus (IDDM),<sup>38</sup> multiple sclerosis (MS)<sup>39</sup> and anti-N-methyl-D-aspartate receptor (NMDA) receptor encephalitis.<sup>40</sup>

## Al vaccine adjuvants and autoimmunity

A major difficulty in understanding how the Al-adjuvant effect could account for the vast heterogeneity of autoimmune manifestations described in the "ASIA" and related syndromes, relates to the fact that most of these conditions are driven by an overactive Th1 immune response (Table 1). Although Al

**Table 2** Summary of vaccine ingredients according to the current US vaccination schedule<sup>80</sup>

	Birth	2 m	4 m	6 m	12 m	18 m	24 m	4–6 y	
Vaccine (#antigen)	EngerixB (1)	Infanrix-IPV (5) Comvax (3) Prevnar (14)	Infanrix-IPV (5) Pedvax (2) Prevnar (14)	EngerixB (1) Infanrix-IPV (5) Prevnar (14)	Hiberix (2) Prevnar (14)	Daptacel 5	–	Daptacel (5)	
Total # antigens	1	22	21	20	16	5	–	5	90
Viral attenuated vaccine (#attenuated viruses)	–	Infanrix-IPV (3) Rotarix (1)	Infanrix-IPV (3) Rotarix (1)	Infanrix-IPV (3)	Imovax Polio (3) MMR-II (3) Varivax (1) Havrix (1) Fluviral (3)	Havrix (1)	Fluviral (3)	Imovax Polio (3) MMR-II (3) Varivax (1) Fluviral (3)	
Total # attenuated viruses	0	4	4	3	11	1	3	10	36

Vaccine ingredients were sourced directly from the manufacturer's monographs. EngerixB, HBsAg adsorbed on 250 µg Al hydroxide; Infanrix-IPV, diphtheria toxoid, tetanus toxoid, pertussis toxoid, FHA, pertactin, inactivated polioviruses Type 1 (Mahoney), Type 2 (MEF1) and Type 3 (Saukett), Al hydroxide; Comvax, Hib capsular polysaccharide PRP conjugated to OMPC of *Neisseria meningitidis* serogroup B, HBsAg, Al hydroxyphosphate sulphate; Prevnar, *Streptococcus pneumoniae* serotypes 1, 3, 4, 5, 6A, 6B, 7F, 9V, 14, 18C, 19A, 19F, 23F saccharides, diphtheria CRM<sub>197</sub> carrier protein, Al phosphate; Rotarix, live attenuated RIX4414 strain of human rotavirus of the G1P[8] type; Pedvax, 7.5 µg of Hib PRP, *N. meningitidis* OMPC, Al hydroxyphosphate sulphate; Hiberix, Hib capsular polysaccharide PRP conjugated to tetanus toxoid; Imovax Polio, inactivated polioviruses Type 1 (Mahoney), Type 2 (MEF1) and 32 Type 3 (Saukett); MMR-II, measles virus, Enders' Edmonston strain (live, attenuated), mumps virus, Jeryl Lynn® (B level) strain (live, attenuated), rubella virus, Wistar RA 27/3 strain (live, attenuated); Varivax, varicella virus, Oka/Merck strain (live, attenuated); Havrix, inactivated hepatitis A virus (HM175 strain), Al hydroxide; Fluviral, inactivated influenza strains A/California/7/2009 (H1N1)-like strain, A/Perth/16/2009 (H3N2)-like strain, B/Brisbane/60/2008-like strain; Daptacel, pertussis toxoid, FHA, pertactin, fimbriae types 2 and 3, diphtheria toxoid, tetanus toxoid, Al adjuvant. Abbreviations: HBsAg, hepatitis B surface antigen; IPV, inactivated poliomyelitis vaccine; Hib, *Haemophilus influenzae* type b; PRP, polyribosylribitol phosphate; OMPC, outer membrane protein complex; FHA, filamentous hemagglutinin.



adjuvants have been historically known as potent and specific stimulators of Th2 immunity and presumably could not activate cytotoxic T cells (CTL),<sup>41,42</sup> current evidence suggests that the classical Al-induced Th2 responses can be shifted towards Th1 polarization in the presence of other Th1-inducing compounds such as lipopolysaccharide (LPS) or recombinant influenza protein antigen (Table 1). Routine contamination of vaccine formulations with residual compounds from the production process, including LPS and various peptidoglycans,<sup>4</sup> could thus account for different adjuvant properties of individual batches. Furthermore, it is also possible for Al adjuvants to trigger autoimmunity through a bystander effect by activating dormant autoreactive T cells in certain individuals.<sup>43,44</sup>

It is of interest to note that a typical vaccine formulation contains all the necessary components for the induction of an autoimmune disease. For example, vaccines contain antigens that may share mimetic epitopes with self-antigens ("molecular mimicry") and immune adjuvants for the upregulation of immune cytokines, which in turn are able to trigger polyclonal activation of autoreactive T cells.<sup>4,44</sup> Consistent with these observations, the immunotoxic effects of vaccine adjuvants are generally recognized to be a consequence of hyperstimulation of immunological responses and are known to be mediated by pro-inflammatory cytokines.<sup>4</sup>

It is perhaps not surprising then to find that simultaneous administration of as little as two to three immune adjuvants, or repeated stimulation of the immune system by the same antigen, can overcome genetic resistance to autoimmunity.<sup>26,45</sup> These facts are often overlooked in the design of routine vaccination schedules. For example, as shown in Table 2, according to the US vaccination schedule currently recommended for preschool children, 2-month-old infants receive a total of 22 viral/bacterial antigens and 4 attenuated viruses along with high amounts of Al adjuvants. Such a potent immune challenge is then more or less repeated at 4, 6, and 12 months of age (Table 2). Hence, by the time children are 4 to 6 years of age, they will have received a total of 126 antigenic compounds (90 viral/bacterial antigens, 36 attenuated viruses) following the current US vaccination guidelines.

### Vaccine safety: how reassuring is the evidence?

In spite of the widespread agreement that vaccines are largely safe and serious adverse complications are extremely rare, a close scrutiny of the scientific

literature does not support this view. For example, to date, the clinical trials that could adequately address vaccine safety issues have not been conducted (i.e., comparing health outcomes in vaccinated versus non-vaccinated children). The lack of such controlled trials may be because historically, vaccines have not been viewed as inherently toxic by regulatory agencies (as documented in the 2002 publication by the US Food and Drug Administration).<sup>46</sup>

Although the temporal association between vaccinations and serious adverse reactions (ADRs) is clear, causality is rarely established.<sup>47</sup> Thus, it is often concluded that, (i) the majority of serious ADRs that do occur are coincidental<sup>48</sup> and (ii) true serious ADRs following vaccinations (i.e., permanent disability and death) are extremely rare.<sup>49</sup> However, the lack of evidence of causality between serious ADRs and vaccinations may simply be due to methodological inadequacy of vaccine trials (Table 3). In addition, the fact that a large number of vaccine safety trials use an Al adjuvant-containing placebo or another Al-containing vaccine as a "control"<sup>50</sup> precludes correct calculations of vaccine-related ADRs. In addition, historically, vaccine trials have routinely excluded vulnerable individuals with a variety of pre-existing conditions (i.e., premature birth, personal or immediate family history of developmental delay, or neurologic disorders including convulsive disorders of any origin, hypersensitivity to vaccine constituents including Al, etc.).<sup>51–53</sup> Because of such selection bias, the occurrence of serious ADRs resulting from vaccinations may be considerably underestimated. All this should be of concern given that the conditions named above are precisely those which are under current immunization guidelines considered as "false-contraindications" to vaccinations.<sup>54</sup> For all these reasons, the true health risks from vaccinations remain unknown.

### Conclusions and future goals

Infants and young children should not be viewed as "small adults." Their unique physiology makes them much more vulnerable to noxious environmental insults in comparison with the adult population. In spite of this, children are routinely exposed to much higher levels of Al vaccine adjuvants than adults, even though adequate safety data on these compounds are lacking. That Al vaccine adjuvants can induce significant autoimmune conditions in humans can hardly be disputed,



**Table 3** Sample of vaccine safety study designs

Study	Methods	Comments
Miller et al. <sup>81</sup>	For safety assessment, children were observed for 7 days post- vaccination for local reactions such as erythema, swelling, or tenderness at site of injection, or fever	The follow-up of study participants was too short and hence detected only the most immediate minor adverse reactions
GlaxoSmithKline <sup>82</sup>	Study subjects were monitored for only 4 days post-hepatitis B vaccination	As above. Given that hepatitis B is the only vaccine mandated to newborn babies and to prevent a disease to which an infant is extremely unlikely to be exposed (i.e., hepatitis virus is transmissible through sexual contact or injection with contaminated material), <sup>83</sup> a more rigorous safety assessment would appear to have been warranted
Verstraeten et al. <sup>84</sup>	Authors state that the safety study on new ASO-4 adjuvanted vaccines (including the human papilloma virus [HPV] vaccine) was not set up primarily to study autoimmune disorders	If the purpose of the study was to assess ADRs of autoimmune etiology, as the title itself clearly states, <sup>84</sup> then the study should have been designed to detect these. An increasing number of reports of previously unrecognized severe autoimmune conditions in HPV vaccine recipients have emerged in recent years <sup>78,79,85,86</sup>
Phillips et al. <sup>87</sup>	In exploring the potential association between Gulf War syndrome and anthrax vaccination, potential subjects were excluded if they reported bad reactions to immunizations or injections	It should be obvious that subjects who reported adverse reactions to immunizations should have been included in the study

although still debatable is how common such side effects are. However, the existing data (or lack thereof) raise questions on whether the current vaccines aimed at pediatric populations can be accepted as having adequate safety profiles. Because infants and children represent those who may be most at risk for complications following vaccination, a more rigorous evaluation of potential vaccine-related adverse health impacts in pediatric populations than what has been provided to date is urgently needed.

## Conflict of interest

CAS is a founder and shareholder of Neurodyn Corporation, Inc. The company investigates early-state adult neurological disease mechanisms and biomarkers. This work and any views expressed within it are solely those of the authors and not of any affiliated bodies or organizations.

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# Administration of aluminium to neonatal mice in vaccine-relevant amounts is associated with adverse long term neurological outcomes



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## ABSTRACT

Our previous ecological studies of autism spectrum disorder (ASD) has demonstrated a correlation between increasing ASD rates and aluminium (Al) adjuvants in common use in paediatric vaccines in several Western countries. The correlation between ASD rate and Al adjuvant amounts appears to be dose-dependent and satisfies 8 of 9 Hill criteria for causality. We have now sought to provide an animal model to explore potential behavioural phenotypes and central nervous system (CNS) alterations using s.c. injections of Al hydroxide in early postnatal CD-1 mice of both sexes. Injections of a “high” and “low” Al adjuvant levels were designed to correlate to either the U.S. or Scandinavian paediatric vaccine schedules vs. control saline-injected mice. Both male and female mice in the “high Al” group showed significant weight gains following treatment up to sacrifice at 6 months of age. Male mice in the “high Al” group showed significant changes in light–dark box tests and in various measures of behaviour in an open field. Female mice showed significant changes in the light–dark box at both doses, but no significant changes in open field behaviours. These current data implicate Al injected in early postnatal life in some CNS alterations that may be relevant for a better understanding of the aetiology of ASD.

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## 1. Introduction

Aluminium (Al) is the most abundant metal and third most common element in the Earth's crust [1]. Normally chemically bound to other elements, Al is not typically bioavailable and indeed seems to play no role in any known biochemistry of plants, animals or humans. In the last 150 years, however, Al through human activities has become much more prevalent in the human environment. Notably, Al is widely used in industrial and material applications, is widely found in processed foods, is contained in various medicinal compounds, and can be used as a flocculant in water treatment. Because of such ubiquity, it is increasingly found in our bodies [2–5]. Overall, we now live in what has been termed “The Aluminium Age” [6].

For all of its positive properties as a material, Al is also demonstrably toxic to biological systems [1], an observation that has been in the scientific literature for at least a century [7]. Although Al may deleteriously impact various organ systems, some of its worst impacts may be on the nervous system (for a review, see [2]). Some of the toxic actions of Al on the nervous system include: disruption of synaptic activity, misfolding of crucial proteins, promotion of oxidant stress, and increased permeability of the blood–brain barrier [2,8], to mention only

a few of the more egregious impacts. In particular, Al has been implicated in Alzheimer's disease [2,4,9,10] and animal models of the disease clearly demonstrate Al-induced cognitive deficits and pathologies [11–13]. Al vaccine adjuvants, in use since the mid 1920s [14], have been shown to produce Lou Gehrig's-like motor phenotypes in mice and motor neuron degeneration [15,16]. The neurotoxic effects of Al adjuvants have been discussed in previous publications by our group [17–19] and by others [20–23]. Additionally, Al in vaccines has been linked to the induction of autoimmune diseases [24–27].

Recently, we compared the amount of Al in various national paediatric vaccine schedules with increasing rates of autism spectrum disorder (ASD) and found a significant correlation that appeared to be dose-dependent [28]. These ecological data satisfied 8 or 9 so-called Hill criteria for causality [29]. Similar conclusions about a potential role of Al adjuvants in ASD have been discussed by other investigators [30,31].

The above results led us to attempt to create an animal model of ASD based on early life administration of Al adjuvants by injection. The current manuscript describes the behavioural outcomes of this study. A future publication will address central nervous system (CNS) alterations.

## 2. Materials and methods

### 2.1. Aluminium adjuvant

Alhydrogel®, an aluminium hydroxide (Al(OH)<sub>3</sub>) gel suspension, was used as a source of aluminium hydroxide. Alhydrogel is manufactured by

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**Table 1**

Approximate amounts of Al from paediatric vaccines administered to preschool children at different ages under the 2010 U.S. vaccination schedule (adapted from [28]) are shown. In the dotted portion of the table is the approximate mouse equivalent administered to CD-1 mice under the “high” and “low” Al schedules during three postnatal weeks (according to the timetable shown in Table 2).

Vaccine	Birth	2 m	4 m	6 m	15 m	2 yr	6 yr
Hepatitis B	250	250		250			
Diphtheria-pertussis-tetanus*		375	375	375	375		375
Haemophilus influenza type b <sup>‡</sup>		112.5	112.5	112.5	112.5		
Pneumococcal		125	125	125	125		
Hepatitis A					250	250	
Total Al (µg)	250	862.5	612.5	862.5	862.5	250	375
Total Al (µg/kg bw)	73.5	172.5	107.5	113.5	78.4	19.8	19.3
Total Al (µg/kg bw) injected into neonatal CD-1 mouse (“high Al” group)	–	170	150	110	80	20	20
Total Al (µg/kg bw) injected into neonatal CD-1 mouse (“low Al” group)	–	–	90	80	50		20

\*Mean value from three different brands of DTaP (Infanrix, Daptacel, Tripedia).

<sup>‡</sup>Mean value from two different brands of Hib (PedVax and Hiberix).

Superfos Biosector a/s (Denmark) and was purchased from SIGMA Canada. This formulation of the gel is presumed to be similar to that used in proprietary commercial vaccines, which may, however, differ in some chemical properties.

## 2.2. Dosage and administration

An example of the U.S. vaccination schedule is shown in Table 1 for reference. Previously, we estimated the amounts of Al per kg of body weight that children in Western countries receive according to their respective countries' immunization schedules [28]. We found that children from countries with the highest ASD prevalence (i.e., U.S., Canada) appeared to have a much higher exposure to Al from vaccines than those from countries where the ASD prevalence is lower (i.e., Scandinavian countries). Moreover, according to their respective immunization guidelines, children in Scandinavia receive fewer vaccines in general and these later in life than children in North America [28].

Based on these schedules, we sought to mimic the U.S. and the Scandinavian vaccination schedules as closely as practically possible in our mouse model (Table 2). For this purpose, CD-1 mouse pups were divided in three groups (“high Al” U.S. schedule), “low Al” (Scandinavian schedule) and saline control, each consisting of 14 animals, both males and females (n = 7–10 males; n = 4–7 females). The dosages of Al adjuvant administered to mice were approximately equivalent (µg/kg) to those administered to children in the U.S. and Scandinavian countries (Table 1). Note that while the groupings reflect individual litters, the size of the mothers, litters and pups pre-treatment did not differ significantly.

Mice were weaned at approximately 5–6 weeks of age when they reached sexual maturity (equivalent to a post-puberty in humans, i.e. 12–15 years) and hence the first three weeks in mice approximately corresponds to a human equivalent of 0–6 years of age. (This is, of

course, an approximation based largely on life span and various aspects of early postnatal neural development may differ significantly between humans and mice). Since most paediatric vaccinations are given to children before the age of 6 years (Table 1), we spread out the schedule of injections in mice over their first three postnatal weeks (Table 2).

The “high Al” schedule received six injection of Al hydroxide (at 170, 150, 110, 80, 20 and 20 µg/kg body weight respectively), for a total of 550 µg/kg body weight. The “low Al” schedule received approximately half of that amount or 240 µg/kg body weight (Table 2), spread out over four injections (at 90, 80, 50 and 20 µg/kg body weight respectively). Although most paediatric vaccines are given intramuscularly (*i.m.*), the treated mice were injected subcutaneously (*s.c.*) into the loose skin-behind the neck (the “scruff”) to minimize discomfort and for the ease of injection. Mice up to 12 days postnatal were injected with a micro-needle while older mice were injected with a standard 30 G needle. The total injection volume for each animal was 15 µl of either Al hydroxide in saline or saline alone.

## 2.3. Animals and breeding

Male and female CD-1 breeders were obtained from Charles River (Wilmington, MA). All animals were housed at the Jack Bell Research Centre Animal Care Facility in Vancouver, BC, Canada. Females and males were housed separately (apart from breeding purposes) at no more than five animals per cage and at an ambient temperature of 22 °C and a 12/12 h light cycle. All mice were fed Purina mouse chow and water ad libitum.

For the purposes of breeding, 3 female and 3 male mice of 16 weeks of age were housed together (total of four cages of breeders). Following impregnation, males were removed from the breeder's cage and housed separately and the females were monitored for the parturition date, which was taken as postnatal day (PND) 0. After birth at PND2, the pups from the four litters were distributed so that each litter consisted of 14 pups. Mice from the fourth litter were used for other purposes. Note that because not all females gave birth on the same day (i.e., two females delivered the pups on the same day, the third female on the following day and the fourth female another day later), injections were started at PND2 (Table 2).

All mice were weaned at PND35 (five postnatal weeks) and were kept housed at 3–5 animals per cage until the end of the experiment. Mice were weighed every two days until they were 10 weeks of age and from then on they were weighed once a week. At 4 months of age (16 weeks), the mice were exposed to an open field environment and given the light/dark box test. These two tests were repeated once every two weeks over a period of two months.

Following the completion of behavioural testing the mice were sacrificed by perfusion with saline followed by 4% paraformaldehyde, and the spinal cord and brain tissues collected for immunohistochemistry (IHC). The IHC analysis is ongoing and the final results will be reported separately.

All experimental procedures on animals were approved by the University of British Columbia's (UBC) Animal Care Committee (protocol #A11-0042) and were in compliance with the Canadian Council on Animal Care regulations and guidelines.

**Table 2**

Schedule of injections with Al hydroxide in treated mice. The approximate mouse equivalent administered to CD-1 mice under the “high” and “low” Al schedules during the first three postnatal weeks were as follows: “high Al” (170, 150, 110, 80, 20 and 20 µg/kg body weight), “low Al” (90, 80, 50 and 20 µg/kg body weight).

Treatment group	Mouse age (days postnatal)																	Total AI injected (µg/kg bw)
	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17		
High AI (U.S.)		x		x	x				x		x					x	550	
Low AI (SCA)			x		x				x							x	240	
Control (saline)	x		x					x		x					x		0	

## 2.4. Behavioural tests

### 2.4.1. Light-dark box

A light/dark box was used to evaluate anxiety and exploratory behaviour [32]. This test was performed in a standard two-compartment chamber. The dark box insert was made of black perspex designed to cover one third of the area of the activity chamber (45 cm × 30 cm × 21 cm) with a 7 cm × 7 cm hole placed in the middle of the wall at floor level. Time spent in and latency to enter light (171 lx) and dark zones (0 lx) as well as the number of full body transitions between the light and dark compartments were automatically scored by the EthoVision system (Noldus Information Technology, Seattle, WA) employing a video camera and a tracking software (Noldus EthoVision® 3.1). A mouse began the test in the dark compartment and its behaviour was recorded over a period of 5 min, after which it was returned to the home cage. The light/dark box was then cleaned with a solution of 70% ethanol and permitted to dry between tests.

### 2.4.2. Open field

The open-field test was used to evaluate locomotor activity and exploratory behaviours [32,33]. Mice were placed in the centre of the arena and were allowed to explore the open field (41 cm in diameter and 30 cm high) for the following 5 min under moderately light conditions (96 lx), while their activity was measured automatically using the EthoVision automated tracking system. The movement of the mice was measured with a camera mounted above the open field. Measurements included total distance moved, velocity, total time spent moving (measures of locomotor activity) and rearing frequency (measure of exploratory behaviour).

## 2.5. Statistical analysis

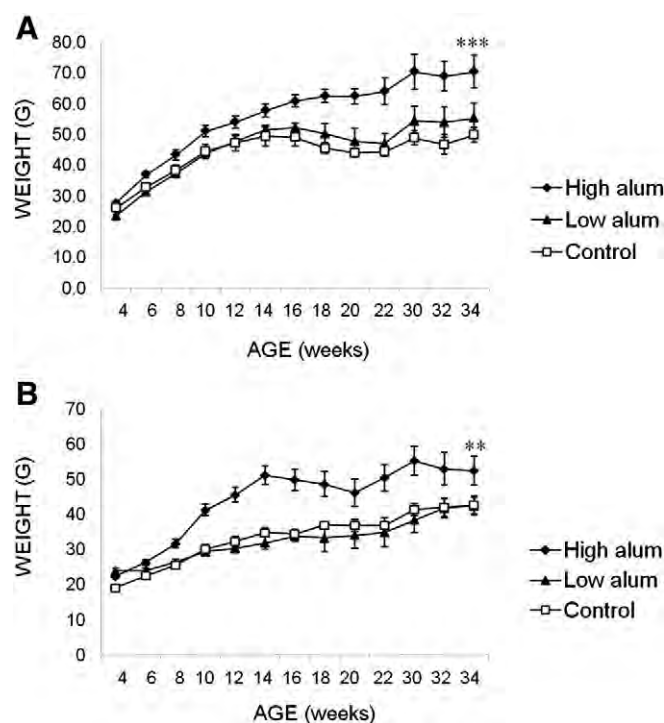
Values for each mouse on the individual tasks were used to calculate mean ± S.E.M. for each group. The means were compared using two-way and repeated measures analysis of variance (ANOVA) using GraphPad Prism statistical software (San Diego, CA). Probability (*p*) levels less than 0.05 were considered significant.

## 3. Results

### 3.1. Overall mouse development

No significant mortality and no overt morbidity were observed in the groups of pups injected with either Al or saline control. There were however two cases of mortality recorded during the experimental period. One was a case of bilateral pyelonephritis with subsequent septicaemia in the group of male mice who received the “high Al” injection schedule. According to the necropsy report by the Animal Care Facility, the pyelonephritis may have been caused by bacterial infections (i.e., *E. coli* and/or *Klebsiella*). Such events may occur spontaneously in a mouse colony and given that the other mice belonging to the same experimental group remained unaffected, it is most likely that this particular case was indeed spontaneous and not directly related to the treatment. The second case of morbidity occurred in the female saline control group where one mouse was found dehydrated and euthanized according to the veterinarian's suggestion. Both of these cases occurred in the post-weaning period. However, the latter occurred during the period of behavioural testing (when the mouse was 22 weeks old). Hence we were unable to perform the repeated measures ANOVA using the behavioural data recorded during the fourth (and final) time point of testing for female mice.

The general development of mice was monitored by systematic recording of their weights from week 1 till the time of sacrifice (week 34). All mice started off at the same weight and increased their weight at a similar rate for the first 8–10 weeks. Marked differences became apparent at weeks 16 and 10 for males and females, respectively

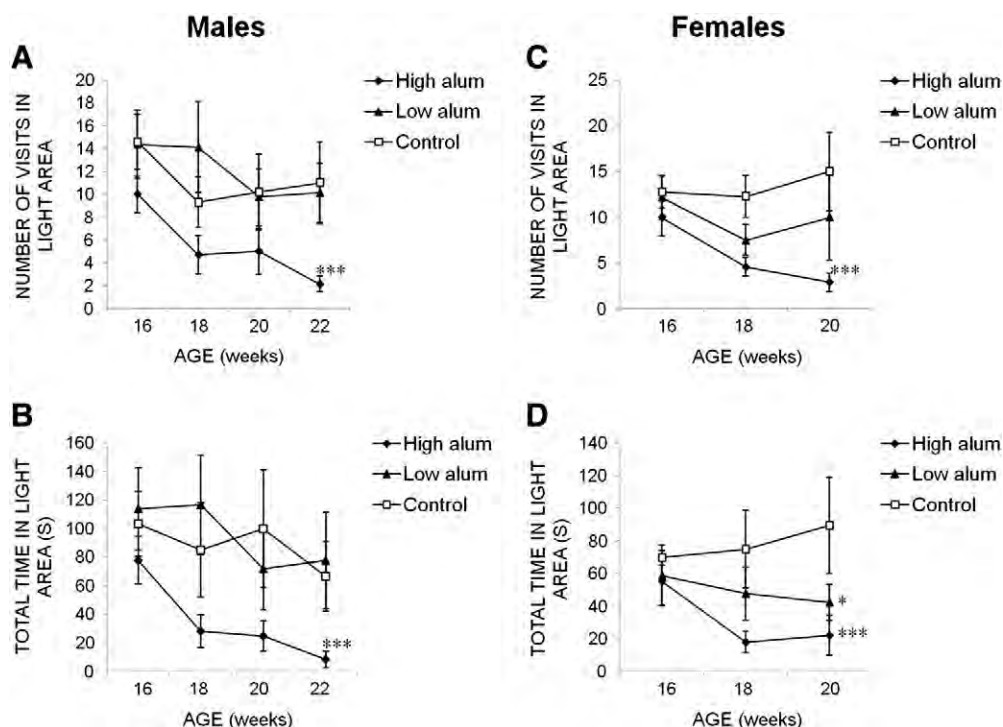


**Fig. 1.** The effects of Al adjuvant injections on body weight in young male (A) and female (B) CD-1 mice. Data are mean ± S.E.M. (animals per group, *n* = 7–10 males; *n* = 4–7 females). Mice were weighed once a week post-weaning. Both male and female mice injected with high Al showed a highly significant increase in weight compared to control mice (\*\**p* = 0.0005 males; \*\**p* = 0.001 females).

(Fig. 1). In particular, between weeks 4 and 16, the control male mice that were injected with saline increased their weight by 88% while the males in the “high Al” group increased their weight by 119%. Between week 4 and the end of the experimental period (week 34), males on “high Al” had a total of 154% increase in their body weight. In contrast, the weight of the control male mice remained relatively stable between weeks 16 and 34, showing only an additional 3.5% increase. Although the effect of “high Al” adjuvant exposure on body weight wasn’t as dramatic in females as it was in males (i.e., between weeks 4 and 34 the females in the “high Al” group showed a total increase of 134% compared to the 123% increase observed in the saline controls), overall it was still highly significant (Fig. 1). Overall, male and female mice in the “high Al” group showed a highly significant increase in weight compared to control mice (*p* = 0.0005 males; *p* = 0.001 females). Moreover, this increase was sustained till the week of sacrifice. In contrast, mice in the “low Al” group did not significantly differ in weight from the control mice.

### 3.2. Light/dark box test

The results of the light/dark box test showed that Al injections in the neonatal period significantly increased anxiety-like behaviours and reduced exploratory activities in mice when they were tested as adults approximately 4 months later (Fig. 2). These adverse behavioural outcomes were long-lasting and persisted throughout the two month period of testing. In particular, mice of both sexes injected according to the “high Al” schedule showed a highly significant increase in anxiety (*p* = 0.0001 males; *p* < 0.0001 females) and a highly significant reduction in exploratory activities (*p* < 0.0001 males; *p* < 0.0001 females) compared to saline controls. Females however were more severely affected, showing significant increase in anxiety even at “low Al” exposure (*p* < 0.034).



**Fig. 2.** The effects of Al adjuvant injections on indices of anxiety and exploratory behaviour in the light/dark box test in young CD-1 mice. Data are mean  $\pm$  S.E.M. ( $n = 7$ –10 males;  $n = 4$ –7 females). Mice were tested at 14 weeks of age for a total of four tests, once every two weeks. Male (A) and female (C) mice injected according to the “high Al” schedule visited the light area less frequently than control mice (indicative of reduced exploratory behaviour; \*\*\* $p = 0.0001$  males; \*\*\* $p < 0.0001$  females). Male (B) and female (D) mice receiving the “high Al” schedule spent less time in the light area than controls (indicative of increased anxiety; \*\*\* $p < 0.0001$  males; \*\*\* $p < 0.0001$  females). Females (D) but not males (B) under the “low Al” schedule were also significantly affected in the measure of anxiety compared to controls (\* $p < 0.034$ ). Note that we were unable to perform the repeated measures ANOVA using the behavioural data recorded during the fourth time point of testing for the female mice due to one unexpected case of morbidity in the control female group which occurred within this period (22 weeks of age).

### 3.3. Open field test

The results of the open field test in Fig. 3 show that the “high Al” adjuvant injections significantly reduced the locomotor activity in male but not female mice. In particular, the young male CD-1 mice exposed to high doses of Al adjuvant travelled shorter distances ( $p < 0.0001$ ), spent significantly less time moving ( $p < 0.0001$ ) and moved more slowly ( $p < 0.0001$ ) than the control animals. These mice also showed reduced rearing frequency in the “high Al” male group compared to controls ( $p < 0.0004$ ). Overall, the adverse effects of high Al adjuvant exposure on locomotor activities in male mice were long-lasting and persisted throughout the two month period of testing. We note that the observed decrease in locomotor activity was unlikely to be weight-related as both female and male mice injected according to the “high Al” schedule showed a comparable significant increase in body weight (Fig. 1) yet the locomotor activity was only significantly impaired in the male group (Fig. 3).

## 4. Discussion

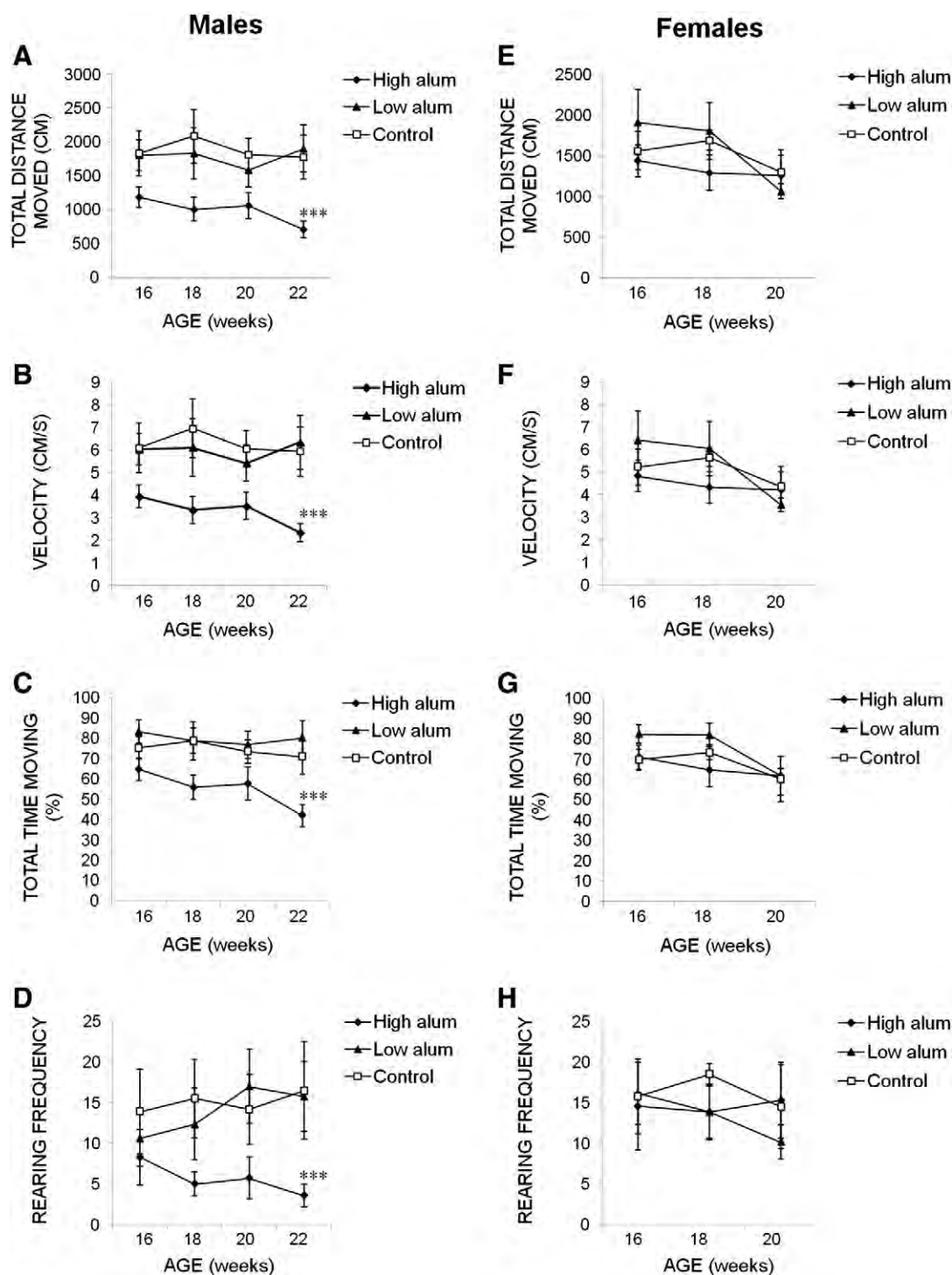
The present results demonstrate, to our knowledge for the first time, long-term alteration of behavioural responses in mice as a result of Al treatment by injection early in postnatal life. The administration of Al was meant to mimic the exposure of human infants to the standard paediatric schedules of various Western countries which we have previously linked to changing rates of ASD in these same countries [28].

In our experiment, mice of both sexes injected under the “high Al” schedule showed a highly significant increase in anxiety ( $p = 0.0005$  males;  $p = 0.0001$  females) and a marked reduction in exploratory behaviour ( $p = 0.013$  males;  $p = 0.0001$  females) compared to controls. Females however were more severely affected, showing a significant increase in anxiety even at “low Al” ( $p = 0.034$ ). In addition,

males but not females receiving “high Al” were significantly more lethargic and less active than control males or those on the “low Al” schedule ( $p < 0.0001$ ). Finally, both males and females in the “high Al” group showed a highly significant and sustained increase in body weight ( $p = 0.0005$  males;  $p = 0.001$  females). We did not perform tests of various forms of learning and memory in the current experiments, although such tests would clearly be advantageous to do in the next series of experiments. In addition, it will be worthwhile to examine social interactions, vocalizations, and other features which are known to be impacted in ASD. Nonetheless, our current results while clearly preliminary, show that administration of Al in vaccine-relevant exposures in neonatal mice is associated with long-term adverse neurological and metabolic outcomes.

The various behavioural outcomes noted, and the differences between male and female mice treated with Al point to sex difference in sensitivity to neurotoxic/neurodisruptive actions of Al. For example, while locomotor activity seemed to be disrupted in males treated with “high Al”, in females under same treatment no impairments were observed (Fig. 3). Of note, Olczak et al. [34] while investigating the neurotoxic potential of Thimerosal (ethyl mercury vaccine preservative) in vaccine relevant exposures in young adult Wistar rats reported similar outcomes in locomotor activity. Namely, male rats were more sensitive to Thimerosal disruption in the locomotor parameters measured in the open field. Of note, anxiety parameters were altered in both sexes even at the lowest doses of Thimerosal [35]. These results may reflect differential chronic neurotoxicity to mercury vs. Al, or may instead highlight species differences. The former is likely since the adverse effects of Thimerosal on anxiety parameters in rats were already highly significant at the dose of 12  $\mu\text{g}/\text{kg}$  of body weight administered in four injections (for a total of 48  $\mu\text{g}/\text{kg}$ ) [34]. On the other hand, the lowest dose of Al resulting in increased anxiety in female but not in male mice in our hands was 240  $\mu\text{g}/\text{kg}$  (spread out over four injections; Table 2).





**Fig. 3.** The effects of Al adjuvant injections on locomotor activity in the open field test in young CD-1 mice. Data are mean  $\pm$  S.E.M. ( $n = 7$ – $10$  males;  $n = 4$ – $7$  females). Mice were tested at 14 weeks of age for a total of four tests, once every two weeks. Male but not female mice injected with high Al showed highly significant reductions in the following indices of locomotor activity: (A) shorter distances moved ( $***p < 0.0001$ ); (B) slower movement ( $***p < 0.0001$ ); (C) smaller percentage of time in overall movement ( $***p < 0.0001$ ); (D) decreased rearing frequency ( $***p < 0.0004$ ). As with the light/dark box, we were unable to perform the repeated measures ANOVA using the behavioural data recorded during the fourth time point of testing for the female mice due to one unexpected case of morbidity in the control female group which occurred within this period of testing as cited in Fig. 2.

The adverse neurobehavioural alterations are presumed to reflect underlying alterations in CNS structure and/or function. In particular, changes in weight in the treated mice above the normal levels achieved by control mice may reflect alterations in the hypothalamus. Similarly, the other function tests may suggest alterations in so-called emotion regions of the brain, particularly the amygdala. All of these outcomes at the behavioural level remain to be confirmed at a cellular level. In

this regard, various assays for neuronal and glial cell numbers, apoptosis, stress markers, neuroinflammation, and autoimmune labelling for various regions of the CNS are in progress and will be reported at a later date.

An alternative explanation to the highly significant and sustained increase in body weight in both male and female mice (Fig. 1) may be related to the activation of the NLRP3 inflammasome pathway (and its downstream mediators caspase-1 and IL-1 $\beta$ ), which is the principal

immunostimulatory pathway through which Al adjuvants operate [36,37]. Unfortunately, activation of the NLRP3 inflammasome is also critically involved in the development of several autoimmune and inflammatory diseases, including type 2 diabetes, CNS demyelinating diseases, colitis, and atherosclerosis [38–42]. In particular, the way in which NLRP3 activation triggers type 2 diabetes is through interference with insulin signalling and promotion of insulin resistance. For example, using NLRP3 knockout mice, Wen et al. [41] demonstrated that the absence of inflammasome components leads to a better maintenance of glucose homeostasis and higher insulin sensitivity. Consistent with this, in other animal studies, blocking caspase-1 activity resulted in decreased weight gain, decreased inflammation, and improved insulin sensitivity [43]. Studies in human have further confirmed the positive association between abnormal inflammasome activation, the resultant IL-1 $\beta$  expression and obesity [44]. In summary, the above observations re-emphasize the fact that there is a very fine balance between the efficacy of vaccine adjuvants and their potential toxicity [23,24,27,28,45–47], precisely because the same mechanisms that drive the immunostimulatory effect of Al (i.e., activation of the NLRP3 inflammasome [36,37]), have the capacity to provoke a variety of autoimmune and/or inflammatory adverse reactions. Coupled with this, the neurotoxic potential of Al indicates that this element has all the necessary biochemical properties to induce neuroimmune disorders, including those of the autism spectrum.

Autism and related disorders of the autism spectrum (i.e., Asperger syndrome, pervasive developmental disorder not otherwise specified, and Rett syndrome) are neurodevelopmental disorders characterized by dysfunctional immune function and various degrees of impairments in social skills, speech and cognition [48,49]. By some estimates, in North America there has been a sharp increase in the prevalence of autism by as much as 2000% since the early 1990s [28]. A countervailing viewpoint is that autism has not changed in its yearly incidence over the last 20 years and that any apparent increases are due to (a) new and broader diagnostic criteria, (b) physicians more adept at diagnosing the condition [50] and/or (c) enhanced awareness by parents and paediatricians leading to a tendency to characterize unrelated conditions as ASD, (d) an increase in the general population, and (e) genetic factors. Of these, we note that (a) diagnostic criteria have not changed yearly although ASD has increased yearly [51]; (b–c) the evidence to support these assertions appears to rests on assumptions rather than solid data; (d) the increase in the population of the US since 1992 is closer to 35%, not 2000%; (e) the occurrence of a massive shift in the genetics of the general population in a time span of only a few decades is highly unlikely.

Indeed, the most conclusive data clearly show that autism prevalence has been increasing with time as shown by higher prevalence among younger groups [52,53]. However, despite considerable research efforts aimed at unravelling the possible causes of the “autism epidemic”, thus far no satisfactory answer has emerged from the research literature. Nonetheless, the fact that ASD rates have indeed been rapidly increasing over the last two decades strongly points to environmental components as possible triggering factors. In particular, early life immune insults (both peri- and post-natal) by various xenobiotics are now strongly implicated in the pathogenesis of disorders of the autism spectrum [54]. Notably, extensive research data has underscored the tight connection between development of the immune system and that of the CNS, thus substantiating the notion that disruption of critical events in immune development may play a role in neurobehavioural disorders including those of the autism spectrum [54–56]. Indeed, early-life immune challenges have been shown to produce long-lasting, highly abnormal cognitive and behavioural responses, including increased fear and anxiety, impaired social interactions, deficits in object recognition memory and sensorimotor gating deficits [34,57–61]. These symptoms are typical of ASD and results from the heightened vulnerability of the developing immune system to disruption by immuno-modulating environmental pollutants [54].

Inflammatory processes and immune dysfunction associated with autism [49,54,62] can result following exposure to many toxic metals including lead and mercury [54,63,64]. However, one of the most common metals to which children are exposed regularly throughout the world is Al from vaccines [17,28,30,31]. This is especially true following the removal of mercury from most vaccines used in the developed world [64]. As mentioned, in our previous research we observed a positive and statistically significant correlation between Al adjuvant exposures (as well as the overall uptake of Al-adjuvanted vaccines), and ASD prevalence [28]. While ours was, to the best of our knowledge, the first study to investigate the possible association between Al vaccine adjuvants and ASD, at least three other studies have found a positive association between the prevalence of autism (and developmental disabilities) and vaccination uptake in early childhood, a result consistent with our findings [65,66]. In addition, Seneff et al. [30] recently reported results from their analyses of the VAERS database which strongly suggest that the Al in vaccines is toxic to vulnerable children and is likely implicated in autism.

Furthermore, Melendez et al. [31] have recently confirmed that Al is a likely environmental risk factor for the development of ASD and behavioural impairments. Specifically, they showed that some metals such as chromium, arsenic and particularly Al were elevated in the blood of autistic children ( $n = 38$ ) when compared to reference values of a normal child. In their study the authors identified two important data regarding exposure to toxic metals. Notably, in 80% of cases the autistic children have used controlled drugs and 90% of them have taken all vaccines. In addition, 70% of mothers took vaccines and 80% of them ate canned food and fish during pregnancy. Hence the results by Melendez et al. [31] suggest that cumulative exposure to Al from dietary and pharmaceutical sources (i.e., Al-containing drugs and vaccines) in early periods of developmental vulnerability (both pre- and postnatal) contributes to the development of ASD. Their findings are thus consistent with our hypothesis that Al is another environmental agent that can now be added to the list of xenobiotics associated with developmental immunotoxicity (as defined by Dietert and Dietert [54]) and thus an important and yet underappreciated risk factors in ASD.

There is little dispute regarding the neurotoxicity of Al. However, it is currently viewed by the pharmaceutical industry and the regulatory authorities that the relatively low concentrations at which Al is used in vaccines do not represent a health risk [67,68] and that “the benefits of using vaccines containing Al adjuvant outweigh any *theoretical* concerns” [69] [emphasis added]. Contrary to these *assertions* however is experimental data from both human and animal studies which has consistently demonstrated the inherent ability of Al adjuvants to inflict neuroimmuno-inflammatory conditions [15,16,20–22,26,27,70–74].

A further common assertion made about Al is that children obtain much more of this element from their diets than from routine paediatric vaccinations and hence the small amount in most vaccines does not represent a significant risk factor for ASD [68]. However, this assertion contradicts basic toxicological principles because injected Al bypasses the protective barriers of the gastrointestinal tract and thus will likely require a lower dose to produce a toxic outcome. In fact, unlike dietary Al which is poorly absorbed (only 0.25% of total ingested Al) and normally clears rapidly from the body [75], Al used in vaccines may be completely absorbed over time [76]. Additionally, the tightness of bonding between the Al adjuvant and the antigen is considered a desired feature as it enhances the immunogenicity of vaccines [77]. However, this feature represents an additional problem for effective clearance of Al from the body as the sizes of most Al-adsorbed antigen complexes are higher than the molecular weight cut-off of the glomerulus [28]. Indeed, long-term persistence of Al (up to 8–10 years) following administration of Al-adjuvanted vaccines has been demonstrated in adult humans and in particular, is strongly associated with deterioration of cognitive skills and chronic fatigue syndrome [47,73,78,79]. Finally, the data by Melendez et al. [31] indicate that even dietary exposure to Al cannot be considered as innocuous in certain circumstances,

especially in the context of an overall Al burden to which a child might be exposed. In other words, an individual susceptibility to an adverse reaction from Al may be dependent upon the combination of a previous sensitization to Al, for example, via childhood vaccination or maternal exposure to Al during pregnancy (either from food or vaccines), and an ongoing Al overload [80]. While the body may cope robustly with a mild exposure to Al, the coping mechanisms will be suddenly and dramatically overwhelmed by increasing and continuous exposures.

It is further worth noting that both the drug regulators and the pharmaceutical industry appeared to have ignored thus far the fact that the potential toxicity of Al will not only be influenced by its bio-persistence but also, by its bio-distribution (i.e., whether the bioactive Al adjuvant nanoparticles remain localized at injection sites or scatter and accumulate in distant organs and tissues). In particular, the micron/submicron-sized aggregates of nano-sized particles of Al adjuvants were initially assumed to remain extracellular until their complete solubilisation in interstitial fluids [81]. We now know however that quite the reverse is true and that following injection, antigen-presenting cells (APCs) avidly take up Al particles [82], and, in so doing, become long-lived cells [83] thus impeding Al solubilisation [73]. Thus a proportion of Al nanoparticles escapes the injected muscle, mainly within immune cells, travels to regional draining lymph nodes, then exits the lymphatic system to reach the bloodstream eventually gaining access to distant organs including the brain. Notably, the Trojan horse-like mechanism by which Al loaded in macrophages enters the brain, results in its slow accumulation due to lack of recirculation and is plausibly responsible for the cognitive deficits associated with administration of Al-containing vaccines in adult humans [20,21]. Based on animal experiments, the bioaccumulation of Al in the brain occurs at a very low rate in normal conditions thus potentially explaining good overall tolerance of Al despite its strong neurotoxic potential. However, according to Khan et al. [84], continuously increasing doses of this poorly biodegradable adjuvant may become insidiously unsafe, especially in cases of repetitive closely-spaced vaccinations and immature/altered blood–brain barrier. In this context, the latest research by Lujan et al. [27] who described a severe neurodegenerative syndrome in commercial sheep, linked to the repetitive inoculation of Al-containing vaccines, is noteworthy. In particular, the “sheep adjuvant syndrome” mimics in many aspects human neurological diseases linked to adjuvanted vaccines [85–88]. Moreover, the “sheep syndrome” which was first identified following mass-vaccination campaigns against bluetongue, was successfully reproduced under experimental conditions following administration of Al-containing vaccines [27]. Notably, the adverse chronic phase of this syndrome affects 50–70% of flocks and up to 100% of animals within a flock. It is characterized by severe neurobehavioural outcomes (restlessness, compulsive wool biting, generalized weakness, muscle tremors, loss of response to stimuli, ataxia, tetraplegia, stupor, coma and death), inflammatory lesions in the brain and the presence of Al in CNS tissues. The latter findings thus confirm the ones by Khan et al. [84] who demonstrated the ability of Al adjuvants to penetrate the blood–brain barrier in mice, and further show that the resulting presence of Al in the brain can trigger severe neurological damage with devastating consequences.

One possibility for the observed dramatic neurobehavioural alterations in our mouse model may be due to the choice of the route of administration (s.c., rather than *i.m.*, due to the very young age of mice at the start of the experiment when the animals lacked abundant muscle tissue). According to Khan et al. [84] the s.c. route appears to be more effective in delivering Al nanoparticles into the brain. However, even the *i.m.* injection of Al resulted in the appearance of Al deposits in distant organs (including spleen and brain) where they were still detected one year after injection (note that most childhood vaccines are given *i.m.*). In particular, the *i.m.* injected Al nanoparticles linearly accumulated in the brain up to the six-month endpoint. Notably, the apparently irreversible accumulation of the nanomaterials after *i.m.* injection was unique to the brain tissue which lacks conventional

lymphatic pathways and may hence retain immune cells [84]. In other words, the lack of recirculation will favour the bio-accumulation of Al in the brain regardless of the route of administration. Hence, as Khan et al. [84] pointed out, the hazard related to Al lies in repetitive administration of continuously increasing doses of this adjuvant to vulnerable populations such as young infants, due to its poor biodegradability and its tendency to accumulate in the CNS.

## 5. Conclusions

Al salts are the most widely used adjuvants today and have been since the 1920s [14]. The fact that they can trigger pathological immunological responses and a cascade of unwanted health effects has been relatively under-appreciated to date [16–27,30,45,72,73,80,84,89]. Nevertheless, it is clear that the problem with vaccine-derived Al is three-fold: it can persist in the body, it can trigger pathological immunological responses and it can make its way into the CNS where it can drive further deleterious immuno-inflammatory and excitotoxic processes [15,16,27,70,72,73,78–80]. This paper reports only preliminary data on the adverse neurodevelopmental effects of early Al exposure in paediatric vaccine-relevant doses in an animal model and hence does not provide conclusive evidence on the hypothesized causative role of Al in autism. However, our current results are consistent with the existing evidence on the toxicology and pharmacokinetics of Al adjuvants which altogether strongly implicate these compounds as contributors to the rising prevalence of neurobehavioural disorders in children. Given that autism has devastating consequences in a life of a child, and that currently in the developed world over 1% of children suffer from some form of ASD [28], it would seem wise to make efforts towards reducing infant exposure to Al from vaccines.

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# Aluminum and the human diet revisited

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**C**oncerns about aluminum (Al) exposure in the human diet have persisted for one century. We suggest that continued research would benefit from better reporting of environmental factors that are known to influence Al accumulation in plant organs that are consumed, focusing on subsets of the general public that exhibit the highest risk for neuropathological responses, increased evaluation of commercial processing procedures that may concentrate Al or other toxic substances, and designing studies with low dose, chronic exposure rather than solely on further study of acute, brief exposure.

## Introduction

Traditional medicines derived from botanical products have been used in regions such as Africa, Asia, and India for many years.<sup>1,2</sup> The use of herbal materials in medicines, food supplements, and teas has increased substantially in Western countries in recent years, leading to concerns about safety assessment of these products.<sup>3-6</sup> Noni (*Morinda citrifolia*) is a popular species for formulating home, regional, and international products for human consumption, and this led us to evaluate the potential for excessive Aluminum (Al) exposure via ingestion of noni leaf products.<sup>7</sup> We reported that Al accumulation in leaves could pose a health risk in some situations, such as harvest of old leaves from trees growing in volcanic soils.

Aluminum is the most abundant metal and one of the most common elements in the Earth's crust. As a metal, it is light,

strong, durable, resistant to corrosion, and a good conductor of electricity. Over the past 200 years, refined Al has found its way into a variety of industrial and materials applications.<sup>8</sup> From the early 1800s Al became increasingly bioavailable due to its extraction from bauxite and other compounds. It is now found in numerous products such as industrial applications, in various processed foods as an anti-caking agent, in water treatment as a flocculant, and in a variety of medicinal products, antiperspirants, and cosmetics. In regard to medicinals, it is commonly used as an adjuvant in vaccines.

## Aluminum not Inert

Geologically, Al is normally bound into molecular complexes, usually those involving silicates. For this reason, Al appears to have no known role in any normal biochemistry on Earth. Some researchers have gone further to suggest that Al has been "selected out" of evolution due to its lack of bioavailability.<sup>9</sup> Aluminum is routinely toxic where it does occur in living systems.<sup>10-13</sup> However, growth of pathological cells such as some cancers may be enhanced by Al.<sup>14</sup>

As an element, Al is extremely avid and binds to then interferes with many molecules essential for life, notably carbon, oxygen, sulfur, and phosphorus among others. It also binds to fluoride, forming highly toxic aluminofluoride compounds.<sup>15</sup> Concerns over the potential for Al toxicity in human food and drink products have persisted for a century.<sup>16</sup> Aluminum toxicity was later discounted as an etiological factor in Alzheimer disease as the amounts available

**Keywords:** adjuvant, autoimmunity, neurodegeneration, neurotoxins, traditional medicine

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from food, water, or aluminum cookware were usually found to be low. A rather large body of evidence by various investigators now shows that chronic exposure to Al can be highly neurotoxic and may indeed have a link to Alzheimer disease.<sup>17-19</sup>

## Neurological Disorders

Cognitive decline and central nervous system (CNS) pathologies that resemble those of Alzheimer are induced by Al in older rats.<sup>20</sup> Soil and water sources of Al were implicated in the ALS-parkinsonism dementia complex on Guam.<sup>21</sup> Additionally, the acute effects of higher doses of Al-induced dialysis associated encephalopathy in humans are well documented.<sup>22</sup>

The route of administration of Al plays a key role in the type of neurotoxicity exhibited. While most dietary Al is removed by the kidneys, those lacking mature or patent kidney function such as pediatric and geriatric subjects may be more likely to accumulate Al in different organs, including the CNS. Injected Al from Al adjuvants in vaccines have a very different fate and appear to be picked up from the draining lymph nodes by circulating macrophages and transported into the CNS.<sup>23</sup> Motor neuron loss following Al hydroxide injections in mice and sheep<sup>24-26</sup> and macrophagic myofasciitis in humans involving cognitive dysfunction in humans has been reported.<sup>27</sup> Al adjuvants have also been linked to a series of autoimmune disorders in humans.<sup>28</sup>

Developmental neurological disorders such as autism spectrum disorder (ASD) also have a potential Al link through the accumulative weight of pediatric vaccines, many of which contain Al as adjuvants.<sup>29</sup> Indeed, there is a highly significant correlation between ASD rates and cumulative Al adjuvant amount,<sup>30</sup> a correlation that satisfies eight of nine Hill criteria for causality. Similar outcomes are found in newborn mice injected with Al adjuvants.<sup>31</sup> A recent review also links Al to ASD.<sup>32</sup>

## Areas of Concern

To view Al in biological systems as either inert or without toxic consequence

is to ignore a rapidly growing body of evidence to the contrary. Inter-disciplinary teams may offer the most efficient means of advancing our understanding of risks of Al exposure in the human diet and from other sources. The following are issues to guide ongoing research.

- Aluminum availability to plants is governed by soil pH, and in accumulator plant species by age of organ. Our study revealed that young noni leaves from trees growing in alkaline soils posed minimal calculated risk, but old leaves from trees growing in acidic soils posed the greatest risk.<sup>7</sup> During recent years, substantial advances have been made in understanding the mechanisms of Al toxicity in plants and approaches to assess the potentially toxic Al species in environmental samples.<sup>33</sup> Yet soil traits, harvested organ age, and other arguably mandatory experimental details are omitted from research articles on traditional knowledge and folk medicines. These omissions do not acknowledge the current status of knowledge and disallow adequate comparisons among studies.

- Consumption of unprocessed herbal products or home concoctions carries relatively minimal risk of excessive Al exposure. However, superfruits, nutritional therapeutics, nutraceuticals, and functional foods are among the arsenal of innovative marketing strategies to reach consumers who demand what they believe to be healthy food options.<sup>34-36</sup> Some commercial procedures concentrate herbal products, then boast about the supposed added benefits of the concentrated product. These processing and concentrating steps may take a raw herbal product that carries minimal risk and turn it into an internationally marketed product that carries greater risk.

- Risks of Al toxicity are elevated for some easily defined subsets of the general public. For example, infants and small children carry greater risk because intake limits are based on body weight. Individuals with kidney immaturity or abnormalities because the normal pathway for excreting aluminum from the body is via urine and feces.<sup>37</sup> Continued research on pediatric, geriatric, and other high risk groups rather than the general

population may increase the efficiency of research.

- Numerous investigations have revealed that the chronic component of exposure to Al is what leads to neurotoxicity.<sup>8,17</sup> The body burden of Al is spread among various tissues, but incremental doses of small amounts of Al over a lifetime favor brain tissues as the site of bioaccumulation. This form of exposure reproduces neuropathological traits of Alzheimer disease. Long-term studies on chronic exposure to Al should be the thrust of dietary research.

- Al compounds are numerous, and the biological responses may be highly specific to the form of Al to which the body is exposed. Studies should be specific to the form of aluminum administered.

A high percentage of the world's population uses alternative self-medication and herbal treatments for prophylactic purposes without being aware of the possible toxic components and toxin synergies that may impact their health. When these herbal treatments inadvertently contain Al or other known toxicants, environmental conditions, post-harvest treatment in processing, and age, general health, and genetic traits of the consumer are factors that may increase risk of developing neurological disorders. These examples illuminate why the public should be better informed on potential health risks associated with using herbal products in self-medication.<sup>38</sup>

## Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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# Aluminum in the central nervous system (CNS): toxicity in humans and animals, vaccine adjuvants, and autoimmunity

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**Abstract** We have examined the neurotoxicity of aluminum in humans and animals under various conditions, following different routes of administration, and provide an overview of the various associated disease states. The literature demonstrates clearly negative impacts of aluminum on the nervous system across the age span. In adults, aluminum exposure can lead to apparently age-related neurological deficits resembling Alzheimer's and has been linked to this disease and to the Guamanian variant, ALS-PDC. Similar outcomes have been found in animal models. In addition, injection of aluminum adjuvants in an attempt to model Gulf War syndrome and associated neurological deficits leads to an ALS phenotype in young male mice. In young children, a highly significant correlation exists between the number of pediatric aluminum-adjuvanted vaccines administered and the rate of autism spectrum disorders. Many of the features of aluminum-induced neurotoxicity may arise, in part, from autoimmune reactions, as part of the ASIA syndrome.

**Keywords** Autism · ALS · Alzheimer's · Neurodegeneration · Immune response

## Introduction

We live in what one leading researcher on the chemistry of aluminum has called “the Aluminum Age” [1]. Aluminum, the third most abundant element in the Earth's crust and the most abundant metal, is one of the most remarkable elements in the periodic table. Objects made with aluminum are strong, durable, light and corrosion resistant. Aluminum is an excellent conductor of electricity. For these

reasons, aluminum currently finds its way into virtually every aspect of our daily lives. Aluminum is used in cans and cookware, aluminum foil, housing materials, components of electrical devices, airplanes, boats, cars and numerous hardware items of all descriptions [2].

With aluminum geologically bound up in various molecular complexes, it is only in the last century that has become available for human use and, importantly, become bioavailable [2, 3]. In terms of bioavailability, aluminum is now found in drinking water due to its action as a flocculant, is a common additive in various processed foods, is added to cosmetics of many types, and, increasingly, shows up pharmaceutical products (Table 1). Notably, in regard to the latter, various aluminum salts are used as vaccine adjuvants. As a result of all of this, aluminum in the human environment is increasingly found in our bodies (Fig. 1) [4–7].

Aluminum is extremely reactive with carbon and oxygen, two of the leading elements of life on Earth. For this reason, the widespread use of bioavailable aluminum may have immense and far reaching implications for the health of humans and animals. In fact, much evidence shows that

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**Table 1** Estimates of daily and weekly intakes of aluminum in humans (Adapted from 9)

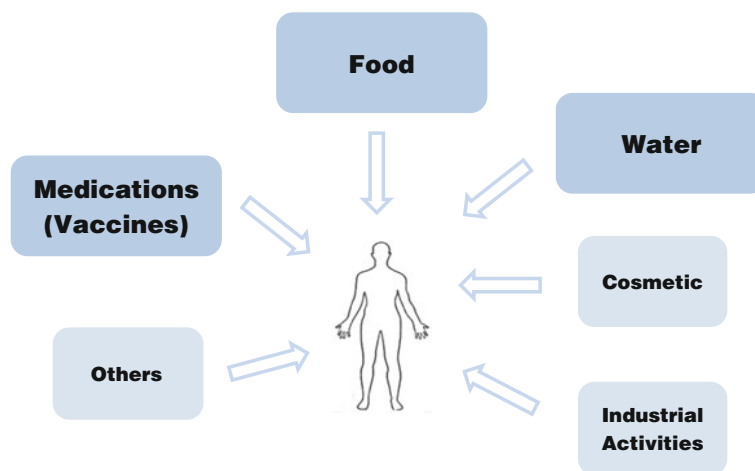
Major sources of Al exposure in humans	Daily Al intake (mg/day)	Weekly Al intake (mg/day)	÷ PTWI * (1 mg/kg body weight; for an average 70 kg human, PTWI = 70 mg)	Amount delivered daily into systemic circulation (at 0.25 % absorption rate*)
Natural food	1–10	7–70	0.1–1	2.5–25 µg
Food with Al additives	1–20 (individual intake can exceed 100)	7–140 (700)	0.1–2 [10]	2.5–50 µg (250 µg)
Water	0.08–0.224	0.56–1.56	0.008–0.02	0.2–0.56 µg
Pharmaceuticals (antacids, buffered analgesics, anti-ulceratives, anti-diarrheal drugs)	126–5000	882–35,000	12.6–500	315–12,500 µg
Vaccines (HepB, Hib, Td, DTP)	0.51–4.56	NA	NA	510–4560 µg**
Cosmetics, skin-care products and antiperspirants***	70	490	NA	8.4 µg (at 0.012 % absorption rate)
Cooking utensils and food packaging	0–2	0–14	0–0.2	0–5 µg

\* PTWI (provisional tolerable weekly intake) is based on orally ingested Al; generally, only 0.1–0.4 % of Al is absorbed from the gastrointestinal tract; however, Al may form complexes with citrate, fluoride, carbohydrates, phosphates and dietary acids (malic, oxalic, tartaric, succinic, aspartic and glutamic), which may increase its gastrointestinal absorption (0.5–5 %). Co-exposure with acidic beverages (lemon juice, tomato juice, coffee) also increases Al absorption as well as conditions of  $\text{Ca}^{2+}$ ,  $\text{Mg}^{2+}$ ,  $\text{Cu}^{2+}$  and  $\text{Zn}^{2+}$  deficiency

\*\* A single dose of vaccine delivers the equivalent of 204–1284 mg orally ingested Al (0.51–4.56 mg), all of which is absorbed into systemic circulation

\*\*\* The risk of antiperspirants is both from dermal exposure and inhalation of aerosols. Inhaled Al is absorbed from the nasal epithelia into olfactory nerves and distributed directly into the brain

**Fig. 1** Aluminum in the human environment. The schematic shows some of the key sources of bioavailable aluminum that are suspected, or demonstrated, to negatively impact human health



aluminum seems to be toxic to all forms of life on Earth, and where it appears in terrestrial biochemistry, it is invariably deleterious [1].

The notion that aluminum is toxic is hardly novel: Dr. William Gies, with 7 years of experimental testing in humans and animals on the effects of oral consumption of aluminum salts use in baking powders and food preservatives, had this to say in 1911:

These studies have convinced me that the use in food of aluminum or any other aluminum compound is a dangerous practice. That the aluminum ion is very toxic is well known. That aluminized food yields

soluble aluminum compounds to gastric juice (and stomach contents) has been demonstrated. That such soluble aluminum is in part absorbed and carried to all parts of the body by the blood can no longer be doubted. That the organism can ‘tolerate’ such treatment without suffering harmful consequences has not been shown. It is believed that the facts in this paper will give emphasis to my conviction that aluminum should be excluded from food. [8].

One hundred and one years after Gies’ prophetic concerns, the notion of aluminum toxicity, in particular in relation to a spectrum of neurological diseases such as Alzheimer’s,

ALS and autism spectrum disorders (ASD), requires a reevaluation based on the science of the last century. A now abundant literature shows that exposure of humans and animals to aluminum from various sources can have deleterious consequences on the developing and adult nervous systems (summarized in part in ref. [9]). These impacts may depend in large part on various factors, for example, the form(s) of aluminum, the route of administration, and the concentration and duration of exposure. Included in this latter category is the issue of dietary versus injected aluminum, the latter a key component of many current vaccines. In addition, the final impact of aluminum will likely depend on a number of biological variables including age, gender and the potential and largely yet unidentified genetic susceptibility factors enhancing aluminum toxicity.

The current review will briefly highlight the studies which have demonstrated aluminum toxicity in the nervous system in humans and in animal model systems, discuss the potential CNS neurotoxic role of aluminum vaccine adjuvants, and finish with a consideration of the potential negative contribution of aluminum to autoimmune reactions in disease.

### **Aluminum and its harmful biochemical interactions with animals and humans**

As noted above, aluminum is abundant but has not typically come into direct contact with humans until relatively recently [10]. This situation changed dramatically during the last half of the nineteenth century when aluminum salts began to be used routinely in the dyeing of fabrics and in food preservation [2, 9, 11, 12]. Aluminum now routinely shows up in infant formula (where it may represent a contaminant or a deliberate additive in the production process [13], in cheese, bakery products, ready-made cake mixes, soft-drinks, etc., as well as in less processed products such as coffee and tea [9, 14]). It may also enter the body through the use of aluminum cookware and packaging [11]. Aluminum also shows up in various cosmetics, as an antiperspirant in many commercial deodorants, and in a variety of medicinal formulations [2, 5, 9, 15]. Antacids also often contain high levels of aluminum hydroxide [2, 16].

Much of the aluminum that enters the human body comes through food. A smaller amount enters through the skin, such as in antiperspirants. Both of these routes would put aluminum into the circulatory system relatively quickly, and most of this aluminum is typically rapidly removed by the kidneys [9]. The exceptions for such excretion are those who lack patent kidney function, infants until age one [17–19] and the elderly [18, 19]. It is these three groups that are most susceptible to aluminum accumulation in the body.

### **Vaccines and aluminum**

Aluminum is added to vaccines to help the vaccine work more effectively [20], but unlike dietary aluminum which will usually clear rapidly from the body, aluminum used in vaccines and injected is designed to provide a long-lasting cellular exposure [18, 19]. Thus, the problem with vaccine-derived aluminum is really twofold: It drives the immune response even in the absence of a viral or bacterial threat and it can make its way into the central nervous system.

The origin of aluminum salts in vaccines has a curious, and largely unknown, history: In the early part of the twentieth century, vaccine researchers frustrated by low antibody titers in experimental vaccines added various compounds in the hope of making the vaccines more effective. In 1926, Glenney et al. [21] first experimented using aluminum salts as “helpers,” hence the term adjuvant. Aluminum worked so well at increasing antibody titers that it became the primary vaccine adjuvant in use, a circumstance which has continued to the present day. Unfortunately, the potential for aluminum to be harmful to various organ systems, including the central nervous system, does not appear to have been rigorously tested [19].

Safety concerns for aluminum in vaccines are twofold: First, the very real toxicity of aluminum compounds to be discussed below. The second is the more general issue of the type of immune response elicited, in particular if the aluminum adjuvant induces either allergic or abnormal autoimmune responses. Such responses are now considered by some investigators to play a role in Guillain–Barre disease, multiple sclerosis and Gulf War syndrome (see [22] for references).

### **Aluminum and neurological disease**

#### **ALS**

Amyotrophic lateral sclerosis (ALS) is a progressive disease of still unknown origin that targets the motor neurons in the brain and spinal cord. Typically, at end-stage disease, both sets of motor neurons have undergone degeneration with resulting loss of motor function. Death typically occurs by respiratory failure. The typical age for the onset of ALS starts is mid-50 s to 70 s, and the survival time after diagnosis ranges from 3 to 5 years. Many ALS victims show a significant loss of cognitive function as well at the latter stages of the disease.

About 90 % of all ALS cases (sporadic ALS) arise from unknown factors, while 10 % are “familial” with a variety of genes involved, notably mutations in the genes coding for the protein superoxide dismutase (SOD). Of the 90 % of sporadic cases, a current view is that environmental

toxins, alone or in synergy with still unknown “susceptibility” genes, are to blame. What these toxins might be remains controversial [23].

Some of the strongest evidence for an environmental toxin causing ALS has come from studies of the two confirmed clusters of ALS: ALS–parkinsonism dementia complex (ALS–PDC) in Guam and the Western Pacific and the ALS associated with Gulf War syndrome (GWS). In regard to the first, neurologists on Guam after World War II noted an extremely high incidence of what appeared to be almost classical ALS among the indigenous Chamorro population. A second disorder, PDC, described a form of parkinsonism with an associated dementia. Approximately 10 % of all patients in Guam developed both the ALS and PDC disorders, usually with the ALS features appearing first [24].

The cause of the disorder in Guam was eventually narrowed down to various putative environmental toxins, including toxins from the seed of the cycad palm which the Chamorro people once frequently ate, and abnormally high aluminum in the soil and water in southern Guam [25]. These data remain controversial but clearly point to a potential link between aluminum and ALS.

GWS (or illness) represents a spectrum of disorders primarily in military personnel in service during the Persian Gulf War (1990–1991). This set of disorders is now considered to fall into a broader category of autoimmune disorders termed “autoimmune syndrome induced by adjuvants” or ASIA 20, 26, 27. GWS is characterized by symptoms such as fatigue, muscle and joint pains, emotional disorders, posttraumatic stress reactions, headaches, and memory loss [28, 29]. Syndrome 1 includes excess fatigue and concentration and memory problems, anxiety, depression, and sleep disorders. Syndrome 2 includes blurred vision, concentration and memory problems, irregular heartbeat, loss of balance and dizziness, speech difficulties, sudden loss of strength, and tremors and shaking. Syndrome 3 includes generalized muscle aches, joint aches, numbness in the hands and feet, and swelling in the joints and in the extremities. Syndrome 2 is particularly of interest for the neurological disease community since four of the seven symptoms are consistent with early phases of ALS (loss of balance and dizziness, slurred speech, sudden loss of strength and muscle weakness, especially the arms and legs, and tremors and shaking).

The suggestion that ALS might be part of GWS became clear in 2003. First, the numbers of ALS cases in military personnel were three times higher in GWS patients than in the general population. Secondly, GWS/ALS victims tended to be younger than those with classical ALS, specifically 20–30 s instead of the normal North American onset age of 50–70 s. The age shift was consistent with a pattern familiar from the variety of forms of ALS–PDC on Guam:

As incidence levels increased, the age of onset tended to decrease.

Studies of Gulf War ALS and GWS in general have suggested a variety of putative environmental factors as causal or contributing (exposure to depleted uranium [30, 31], nerve gas [32, 33], organophosphates [34, 35], vaccines [36], heavy metals [37] and bacterial infections [38, 39]). Some genetic susceptibility factors have also been considered and could work in concert with the various toxic substances listed above [23].

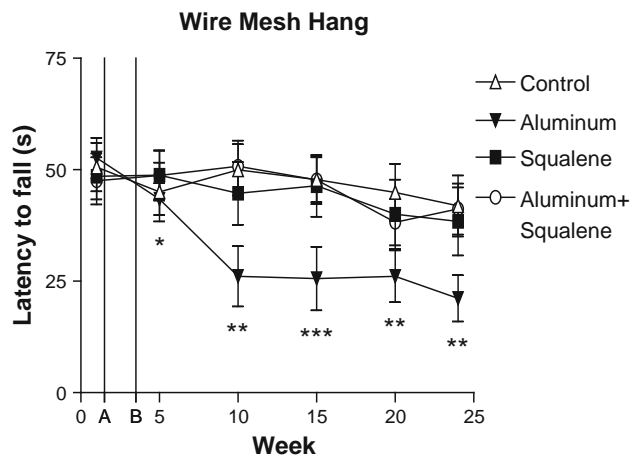
In recent years, increased scrutiny has focused on vaccines, in particular the anthrax vaccine which contained aluminum as an adjuvant [40]. Soldiers from the United Kingdom who also received the anthrax vaccine with aluminum showed increased psychological distress and chronic fatigue compared with those who did not get the vaccine [41]. French soldiers participating in the war did not receive the anthrax vaccine but did show some GWI-related disorders (respiratory, neurocognitive, psychological and musculoskeletal), but no ALS symptoms were reported [42]. As above, many of the features of the disease place it firmly within the ASIA family of disorders.

To explore the ALS component among GWS patients, we injected aluminum hydroxide compared to a more novel vaccine adjuvant, squalene, into young, male colony mice. We compared the outcomes in these animals to those that received both adjuvants and to those that had only saline injections [43, 44]. We tested the mice with various motor and cognitive behavioral tests over a period of 6 months. The mice injected with aluminum hydroxide showed a 50 % decrease in muscular strength and endurance compared with control mice (Fig. 2). Aluminum-injected mice also showed a 138 % increase in anxiety levels, and mice injected with aluminum and squalene had significant late-stage long-term memory loss. A second study confirmed a clear loss of spatial memory capabilities in aluminum-injected mice [44] (Fig. 3).

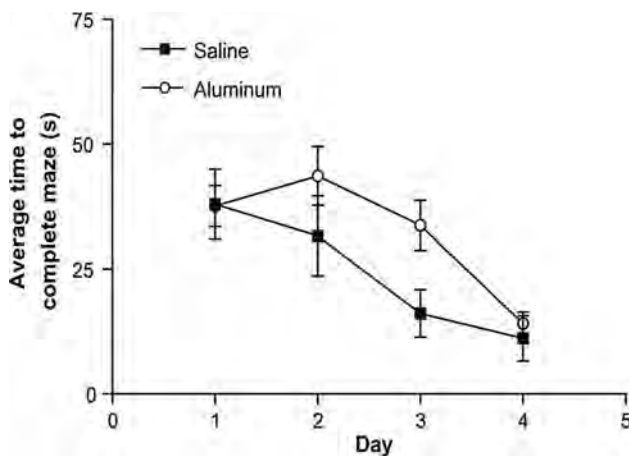
Mice injected with aluminum hydroxide showed a significant increase in cell death in the spinal cord and motor cortex (Figs. 4, 5), primarily affecting the motor neurons as well as neuroinflammation in the spinal cord and motor cortex as evidenced by increases in activated reactive astrocytes (Fig. 6) and microglia (data not shown).

These studies demonstrated that severe behavioral motor deficits and the loss of motor neurons throughout the nervous system resulted when an aluminum vaccine adjuvant was applied to an animal model. The effects closely resembled the damage we had seen in the motor areas of mice used to model ALS–PDC of Guam and, in addition, resembled the pathological outcomes in human ALS [23].

The available data on GWS thus seem to point at aluminum in vaccines as one of the strongest links to ALS in GWS. The neurological signs and symptoms, especially

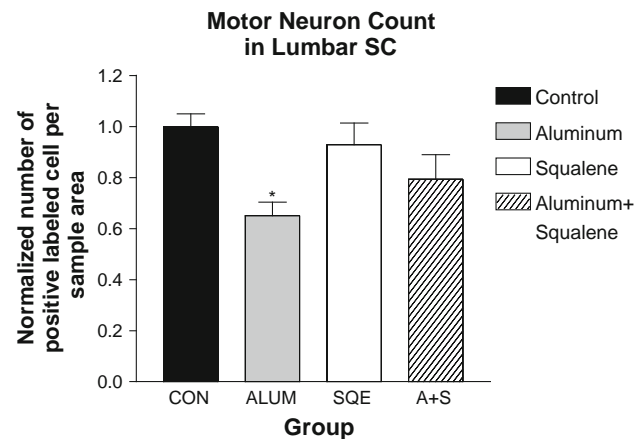


**Fig. 2** Behavioral outcomes in outbred male colony mice injected with two vaccine adjuvants. The studies used aluminum hydroxide, the most common vaccine adjuvant, or squalene a precursor to cholesterol. A third treatment group combined aluminum and squalene. Control mice were injected with saline. All injections were subcutaneous. The data show the outcomes of the wire-mesh hang test for motor strength. Mice injected with aluminum hydroxide showed a significant and sustained decrease in muscular strength and endurance (~50 %) compared with the controls mice. Mice injected with squalene or both adjuvants did not show a significant decrease in muscular strength. A = first injection, B = second injection. (\* $p < 0.05$ , \*\* $p < 0.01$ , \*\*\* $p < 0.001$ ; one-way ANOVA). (Adapted from [43])



**Fig. 3** Water maze test as an evaluation of learning and memory. Mice injected with aluminum hydroxide (6 injections) on average took significantly longer to complete the maze compared to saline-injected mice (two-way ANOVA. \* $p = 0.0389$ ). (From [44])

those for the ALS subgroup, are also a good match to other signs and symptoms of aluminum neurotoxicity. For example, dialysis solutions containing aluminum have been linked to an Alzheimer's-like disorder termed "dialysis-associated encephalopathy/dementia" (DAE) (see below). In animals, aluminum neurotoxicity appears to be particularly harmful to neurons that make the neurotransmitter



**Fig. 4** Motor neuron death following aluminum hydroxide injections in outbred male colony mice. Mice injected with aluminum hydroxide showed a statistically significant decrease in motor neuron number (35 %) compared with the controls. There was no significant difference in motor neuron counts between all other groups compared with the controls. Data are mean  $\pm$  S.E.M \*\*\* $p < 0.05$  versus control mice using one-way ANOVA. (From [43])

acetylcholine, for example, motor neurons in the brain and spinal cord.

Recently, two other groups have reported similar findings using aluminum hydroxide injections in mice (R. Gherardi; N. Agmon-Levin pers. comm.). Also, recent veterinary studies of apparent neurological disorders in Spanish sheep have linked the various behavioral deficits and CNS pathologies observed to aluminum-adjuvanted vaccines [45].

Macrophagic myofasciitis and the fate of aluminum adjuvants in the body

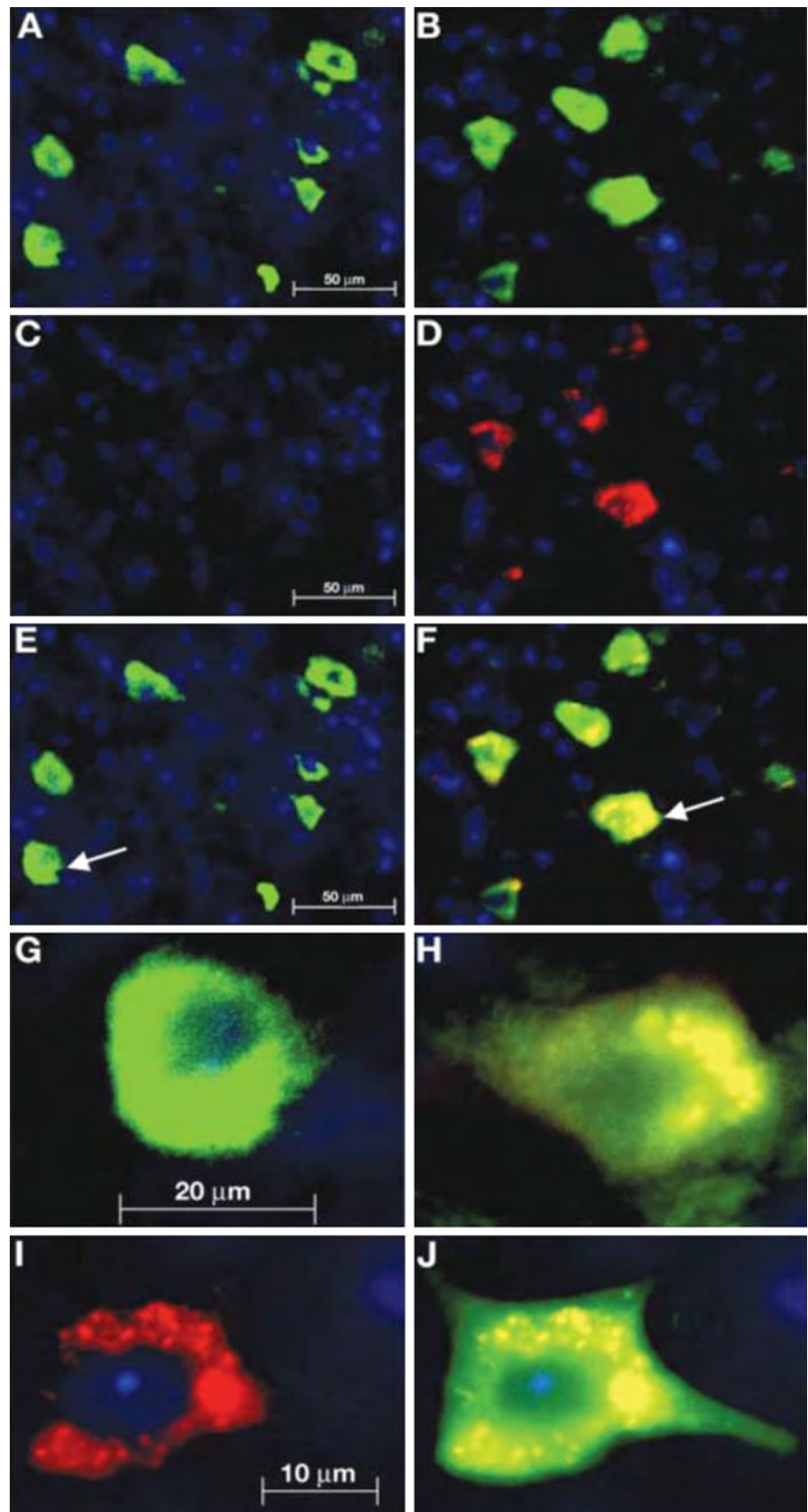
Additional evidence exists for aluminum's role in various central nervous system disorders, including multiple sclerosis associated with aluminum hydroxide injections that produce a persistent muscle inflammatory response termed macrophagic myofasciitis [22, 46, 47]. Other studies using even smaller amounts of aluminum hydroxide describe the pathway of aluminum from the muscle into the brain. In brief, these studies show that aluminum nanoparticles are carried from the site of injection in the muscle to the draining lymphatic system. Once there, the aluminum is carried into the central nervous system by circulating macrophages [46].

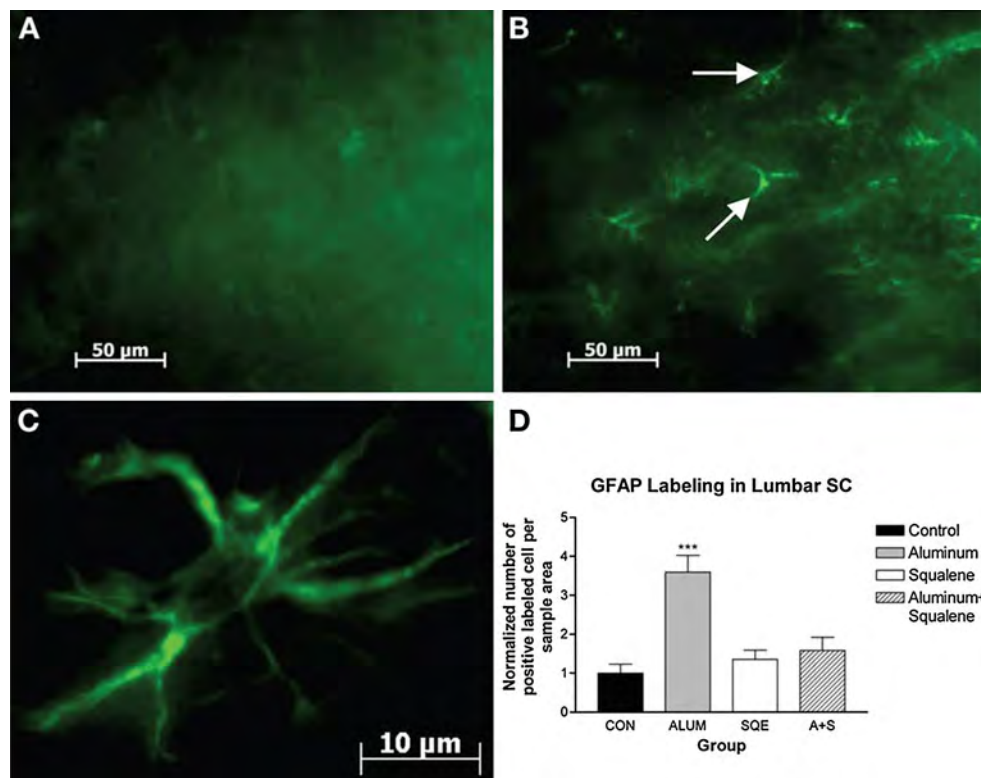
Alzheimer's disease

The potential link between aluminum, in various forms, and Alzheimer's disease has been the subject of speculation for decades. The first case of Alzheimer's disease was



**Fig. 5** Histological evaluation of aluminum hydroxide injection in mouse spinal cord. Control (a) and aluminum-injected (b) mouse motor neurons are fluorescently labeled with NeuN (green) and activated caspase-3 (red) (c, d, respectively) in the ventral horn of lumbar spinal cord. Yellow labeling is a merged image showing colocalization (e, f). The blue fluorescence is the nuclear marker DAPI. The data show that aluminum-injected motor neurons are undergoing programmed cell death (apoptosis). Magnification  $\times 40$  A–F. White arrows indicate neuron enlarged in (g, h). Enlargement of neurons e, f at  $\times 100$  magnification. i, j, Enlargement of another activated caspase-3-positive motor neuron at  $\times 100$  magnification. j Scale bar = 50  $\mu\text{m}$ . g, h, Scale bar = 20  $\mu\text{m}$ . i, j, Scale bar = 10  $\mu\text{m}$ . (From [43]) (Color figure online)





**Fig. 6** Activated astrocytes labeled with glial fibrillary acidic protein (GFAP) in ventral horn of lumbar spinal cord of control (**a**) and aluminum-injected mice (**b**). Sections from mice injected with aluminum hydroxide show increased GFAP labeling and a greater number of astrocytes (*white arrows*) compared with controls (**a**, **b**  $\times 40$  magnification). *Scale bar* = 50  $\mu$ m. **c** Astrocyte from

aluminum-injected mouse observed under  $\times 100$  magnification. *Scale bar* = 10  $\mu$ m. **d** Normalized cell counts for GFAP labeling of astrocytes in ventral horn of lumbar spinal cord ( $n = 32$ , eight per group). The largest increase in GFAP-positive cells occurred in the aluminum treatment group. Data are mean  $\pm$  S.E.M. \*\*\* $p < 0.001$  versus control mice using one-way ANOVA. (From [43])

reported in Frankfurt, Germany, about 20 years following the initial widespread use of aluminum products [9].

A rare disease as late as the 1920s, Alzheimer is now one of the most prominent neurodegenerative disorders and a leading cause of dementia, impacting some 24.3 million people worldwide (see [9] for references), with the increase is not solely attributable to a burgeoning aging population. Alzheimer's disease is characterized by a general loss of cognitive function, including memory. The brains of Alzheimer's victims contain amyloid "plaques" and neurofibrillary tau protein "tangles," and in various parts of the brain, there is significant neuronal loss. Various studies have shown the presence of aluminum associated with neurofibrillary tangles of neurotoxic tau protein [7, 48]. Although such association could be coincidental, the link certainly suggests a role somewhere in the disease process. Although discounted in recent years, the notion that aluminum could be a contributing factor in Alzheimer's disease has begun to regain momentum. An extensive review published in 2011 [9] documents the extent to which aluminum is toxic to plants, animals and humans.

An example of the potential role of aluminum in Alzheimer's disease arose with descriptions of "dialysis-

associated encephalopathy" (DAE) where patients with insufficient kidney function received dialysis fluids inadvertently contaminated with high levels of aluminum [49]. The overall list of DAE features included, in sequence, speech abnormalities, tremors, impaired psychomotor control, memory loss, impaired concentration, behavioral changes, epileptic seizures and coma [49–52]. The condition generally progressed to coma and death within 3–7 years following the sudden overt manifestation of clinical symptoms in patients who had been on long-term dialysis treatment [9, 49]. High levels of aluminum in the brain were demonstrated in DAE patients as well as amyloid  $\beta$  accumulation [53, 54].

Patients showed rapid improvement when aluminum was removed from the dialysis fluid. It is significant that DAE as a clinical syndrome vanished once aluminum was removed from the dialysis solutions [49, 51]. It is of interest that later epidemiological studies examining ground water and Alzheimer's incidence levels found a link between dietary consumption of aluminum and the disease [55–57].

A number of studies have linked elevated aluminum levels to an increased risk of cognitive impairment and Alzheimer-type dementia [55, 57–59] especially in



conditions where silica content is low [59, 60]. Campbell et al. [61] showed that exposure to even low levels of aluminum (0.01, 0.1 and 1 mM) in drinking water for 10 weeks increased inflammatory processes selectively in mouse central nervous system. Other animal studies by Walton and others in aged rats showed significant cognitive impacts and pathological features following prolonged exposure to aluminum chloride. Other behavioral changes in rats exposed to aluminum at human dietary levels included confusion and repetitive behaviors [12, 62, 63].

### Aluminum and Autism Spectrum Disorders (ASD)

The term “Autism spectrum disorders” describes a range of brain disorders that arise in infants or young children. Autism is typically characterized by delays in speech development and social functioning [64] that may never reach “normal” levels of function. By some estimates, in North America, there has been a sharp increase in the prevalence of autism by as much as 2000 % since the early 1990s [18]. A countervailing viewpoint is that autism has not changed in its yearly incidence over the last 20 years and that any apparent increases are due to (a) new and broader diagnostic criteria, (b) physicians more adept at diagnosing the condition [65] and/or (c) enhanced awareness by parents and pediatricians leading to a tendency to characterize unrelated conditions as ASD, (d) an increase in the general population, and (e) a changing gene pool. Of these, we note that (a) diagnostic criteria have not changed yearly although ASD has increased yearly; (b, c) the evidence to support these assertions appears to rests on assumptions rather than solid data; (d) the increase in the population of the United States since 1992 is closer to 35 %, not 2000 %; and (e) the occurrence of a massive shift in the genetics of the general population in a time span of only a few decades is highly unlikely.

The most conclusive data clearly show that autism prevalence has been increasing with time as shown by higher prevalence among younger groups [64, 66]. If autism rates have indeed increased since 1992, it seems reasonable to believe that some environmental factor, in combination with various genetic factors, may be responsible. What that environmental factor(s) remains largely unknown, but the increase in various toxins in the human environment seems a likely starting point.

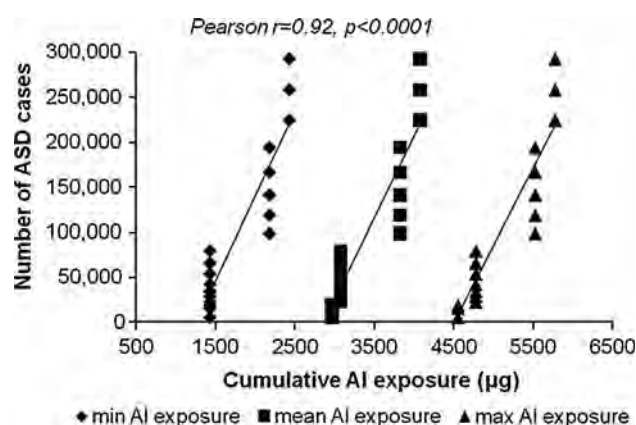
Clearly, as with GWS, there will be many such toxins to consider with a focus on those to which children might reasonably be exposed. Given the almost universal increase in the number of vaccines children routinely receive during their formative years [9, 18], and given the demonstrated neurotoxicity of at least some vaccine ingredients, much speculation has focused on two key vaccine components. These include mercury in the form of the preservative ethyl

mercury (trademarked as thimerosal) and aluminum, the most common vaccine adjuvant as documented above [18, 67–69]. As mercury’s potential role in ASD has been widely discussed in the literature [70–74], it will not be further discussed in the present review.

According to the Food and Drug Administration (FDA), vaccines represent a special category of drugs since they are generally given to healthy individuals, thus placing special emphasis on vaccine safety. The FDA sets an upper limit for aluminum in vaccines at no more than 850 µg (microgram)/dose; however, this amount was selected from data showing that aluminum in such amounts only enhanced the immunizing power of the vaccine (as cited in [18]). The FDA does not appear to have done any testing on the toxicological and safety issues of aluminum in vaccines [75].

Recently, Tomljenovic and Shaw [18] conducted a study to compare the Centers for Disease Control and Prevention (CDC) recommended vaccine schedules for children’s vaccines in the United States (1991–2008) to changes in autism rates during this same period (US Dept. of Education) (original references in [18]).

The data sets, graphed against each other, show a pronounced and statistically highly significant correlation between the number vaccines with aluminum and the changes in autism rates (Fig. 7). Further data showed that a significant correlation exists between the amounts of aluminum given to preschool children and the current rates of autism in seven Western countries. Those countries with the highest level of aluminum-adjuvanted vaccines had the highest autism rates. This correlation was the strongest at 3–4 months of age, a period of rapid growth of the child’s central nervous system, including synaptogenesis, maximal



**Fig. 7** Correlation between the number of children with ASD (6–21 years of age) and the estimated cumulative aluminum exposure (µg) from pediatric vaccines in the period from 1991 to 2008 (US data, see citations 18; adapted from [18]). The data satisfied eight of nine of the so-called Hill criteria for causality [81]

growth velocity of the regions of the brain responsible for short-term memory and the onset of growth of the amygdala, the latter involved in social interactions [76]. In addition, the period between 2 and 4 months in human infants also sees the development of neural systems regulating sleep, temperature, respiration and brain wave patterns [77]. Many of these brain functions are impaired in autism [78–80].

The observed correlation between the number of aluminum-adjuvanted vaccines and ASD was further tested using Hill's criteria and met eight of nine of these indicating that vaccines containing aluminum are highly likely to be at least partially causal for autism [81].

There are other links between aluminum exposure/toxicity and ASD. These include the following: A pilot study showed higher than normal aluminum levels in the hair, blood and/or urine of autistic children [6]; children are regularly exposed to higher levels of aluminum in vaccines per body weight than adults [18]; practically, nothing is known about the pharmacokinetics and toxicodynamics of aluminum in vaccines in children [82]; and aluminum in vaccines has been linked to serious neurological impairments, chronic fatigue and autoimmunity [26, 27, 83–85].

Animal studies support the human results. For example, as also cited above, injection of aluminum adjuvants at levels comparable to those that are administered to humans in vaccines has been shown to cause motor neuron death impairments in motor function and losses in spatial memory capacity in young mice (as cited above in [43, 44]). As well, injections of aluminum vaccines in 4-week-old mice were followed by a transient peak in brain aluminum levels on the second and third days after injection [86].

A common assertion made about aluminum in children's vaccines is that children obtain much more of this element from their diets, and hence, the small amount in most vaccines does not represent a significant risk factor for ASD [87]. However, this assertion contradicts basic toxicological principles because injected aluminum bypasses the protective barriers of the gastrointestinal tract and thus will likely require a lower dose to produce a toxic outcome [18]. In the case of aluminum, only ~0.25 % of dietary aluminum is absorbed [88], while aluminum hydroxide (the most common form of aluminum used in vaccines) when injected may be absorbed by the body at nearly 100 % efficiency over time [89]. In addition, although the half-life of aluminum consumed through the diet is short (approximately 24 h), the same cannot be assumed for aluminum in vaccines because the molecular size of most aluminum in vaccines (24–83 kDa (137)) is higher than what the human kidney or other bodily filtering systems can process (~18 kDa [44] and indeed is contradicted by the results of Gherardi et al. [47].

Autoimmunity: do aluminum adjuvants play a role?

It is of interest to note that a typical vaccine formulation contains all the necessary components for the induction of an autoimmune disease. For example, vaccines contain antigens that may share mimetic epitopes with self-antigens ("molecular mimicry") and immune adjuvants, the most common of which is aluminum. Injection of an antigen itself in the absence of an adjuvant is typically insufficient to trigger an autoimmune reaction as noted by Glenney et al. years ago. In fact, in the absence of aluminum, most vaccine antigens (with the exception of live-attenuated viruses) fail to elicit an adequate immune response [20, 90, 91], suggesting that a significant part of vaccine-induced immune stimulation is driven by the aluminum adjuvant itself.

While the potency and toxicity of aluminum adjuvants should be adequately balanced so that the necessary immune stimulation is achieved with minimal side effects, such a balance can be difficult to accomplish in practice. This is because the same mechanisms that drive the immune stimulatory effect of adjuvants have the capacity to provoke a variety of autoimmune and/or inflammatory adverse reactions including those associated with the ASIA syndrome [26, 27, 67]. Indeed, the immunotoxic effects of vaccine adjuvants are generally recognized to be a consequence of hyperstimulation of immunological responses [92, 93].

It is perhaps not surprising then to find that a potent "adjuvant effect" can overcome genetic resistance to autoimmunity. For example, while coadministration of coxsackievirus B3 (CB3) and *E. Coli* lipopolysaccharide (LPS) induces severe autoimmune myocarditis in C57BL/10 mice which are genetically resistant to autoimmunity, LPS alone has no such effect [94]. Similarly, while injection of C57BL/10 mice with myosin in combination with complete Freund's adjuvant fails to induce heart disease, coadministration of myosin, complete Freund's adjuvant and LPS has the opposite effect [94]. The fact that coadministration of as little as 2–3 immune adjuvants can overcome the genetic resistance to autoimmune diseases is often overlooked in the current design of vaccination schedules. For example, 2-month-old infants receive a total of 22 viral bacterial antigens (most of which are adsorbed onto aluminum) and 4 attenuated viruses following the current US vaccination recommendations for preschool children [67].

As noted above, autism incidence appears to have increased dramatically in the last few decades, and this increase is strongly correlated with an increase in the number of required pediatric vaccinations, most of which contain some form of aluminum. Autoimmune manifestations, particularly those affecting the CNS, are prevalent in autistic

individuals and are not restricted to only few CNS antigens. For example, Vojdani et al. [95] demonstrated elevated levels of autoantibodies against nine different neuron-specific antigens in autistic children. Such widespread manifestation of autoimmunity is indicative blood–brain barrier (BBB) disruption, as this would enable unrestrained access of immunocompetent cells to many different CNS antigens. There is substantial evidence that the BBB is indeed disrupted in autism and that this disruption, thought to be caused by environmental inflammatory stress triggers, leads to neuroinflammation and autoimmunity. Aluminum is known to damage the BBB and can increase its permeability by increasing the rate of transmembrane diffusion and by selectively altering saturable transport systems [96–98]. The breakdown of the BBB by aluminum may also result from excessive release of pro-inflammatory cytokines from aluminum-stimulated microglia [99, 100]. The ability of aluminum adjuvants to cross the BBB [47, 86] and up-regulate chemoattractants such as MCP-1 [91] could promote active recruitment of immunocompetent cells to the brain, leading to both widespread autoimmunity and deleterious inflammatory processes.

Compelling evidence for a causal role of aluminum adjuvants in triggering serious autoimmune disorders has been presented by Quiroz-Rothe et al. [92] who described a case of postvaccination polyneuropathy resembling Guillain-Barré syndrome in a dog. In this case, there was an apparent cause–effect relationship between vaccination and onset of clinical signs associated with the presence of antibodies against myelin. The authors noted that the vaccines used were obtained by cultures in renal cells and did not contain nervous tissue antigens. Thus, either viral or other vaccine antigens, or the adjuvants included in the vaccines, might have triggered the formation of anti-myelin antibodies by over stimulation of the dog's immune system. However, the fact that two different vaccines from two different manufacturers were involved strongly suggests a polyclonal activation induced by the vaccine adjuvants without the participation of myelin as the more probable pathogenesis.

Other controlled studies in dogs vaccinated with commercially available rabies and canine distemper vaccines showed a significant increase in the titers of IgG antibodies reactive with 10 autoantigens, an effect not observed in unvaccinated dogs [101]. Although molecular mimicry or a “bystander activation” of self-reactive lymphocytes could be the cause for these autoimmune manifestations, the relatively large number and variety of autoantigens observed (as in the cases of autistic children) point to a polyclonal activation or adjuvant reaction. Moreover, this adjuvant effect, associated with the development of a wide range of autoantibodies, has been typically associated with vaccines containing higher levels of adjuvants [102].

Altogether, these observations are consistent with both the neurotoxic and immunotoxic properties of aluminum. First, aluminum can compromise the integrity of the BBB, thus exposing the CNS to circulatory immunocompetent cells and pro-inflammatory mediators. In turn, aluminum stimulates the recruitment of these same immune mediators to the brain. As shown by the recent studies of the Gherardi group, aluminum adjuvant nanoparticles, taken up by monocytes after injection, first translocate to draining lymph nodes, then travel across the BBB and eventually accumulate in the brain where they can cause significant immune-inflammatory adverse reactions [47].

In summary, the above research clearly shows that hyperstimulation of the immune system by various adjuvants, including aluminum, carries an inherent risk for serious autoimmune disorders affecting the CNS. In this regard, the fact that the levels of adjuvants typically administered to vulnerable populations (i.e., infants and preschool children) have never undergone appropriate toxicity evaluations in animal models may be a cause for concern as highlighted by the various reevaluations of the clinical literature [67].

#### Emerging issues

The current review has demonstrated a range of neurological disorders that might arise due to exposure to aluminum. Two broad categories have emerged from this analysis: neurodevelopmental and age-related neurodegenerative. While these outcomes appear to be temporally distinct, there are clear caveats to both category and time of occurrence. For example, although ASD is clearly a neurodevelopmental disorder, neuronal damage may also occur. In regard to this aspect, we do not yet know whether such neuronal damage will serve as a precursor to the neurodegenerative diseases associated with aging.

One aspect that separates the two ends of the aluminum-induced neurological disorder spectrum is the route of administration, for example, injection versus oral. The first can be expected to have relatively rapid effects that, depending on age, can range from days to years. The latter may take years to reach a critical body burden or to trigger the end-state outcomes that are likely the result of a cascade of various pathological events. But, as above, these may not be stringent distinctions. For example, injected aluminum adjuvants in adults can trigger forms of cognitive impairment [103].

It is not really a matter of much debate that aluminum in various forms can be neurotoxic. Rather, the questions that remain are these: How crucial to the various age-related neurological deficits are routes of administration and genetic susceptibility? What role does gender play in sensitivity to aluminum toxicity and why? And, finally, can the

forms of aluminum-induced neurological deficits discussed be subsumed under the broad rubric of autoimmune disorders?

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# Aluminum's Role in CNS-immune System Interactions leading to Neurological Disorders

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## Abstract

Multisystem interactions are well established in neurological disorders, in spite of conventional views that only the central nervous system (CNS) is impacted. We review evidence for mutual interactions between the immune and nervous systems and show how these seem to be implicated in the origin and progression of nervous system disorders. Well-established immune system triggers leading to autoimmune reactions are considered. Of these, aluminum, a known neurotoxicant, may be of particular importance. We have demonstrated elsewhere that aluminum has the potential to induce damage at a range of levels in the CNS leading to neuronal death, circuit malfunction and ultimately, system failure. Aluminum is widely used as an adjuvant in various vaccine formulations and has been implicated in a multisystem disorder termed "autoimmune/inflammatory syndrome induced by adjuvants" (ASIA). The implications of aluminum-induced ASIA in some disorders of the CNS are considered. We propose a unified theory capturing a progression from a local response to a systemic response initiated by disruption of water-based interfaces of exposed cells.

**Keywords:** Aluminium; CNS; Neurological disorders; Autoimmunity

## Introduction

One characteristic of conventional reductionist approaches in the biological sciences is that various systems tend to be viewed in isolation from each other. That this occurs generally is not really in dispute, but the impact of such an approach often obscures relationships that almost certainly would prove seminal to a clearer understanding of various disease states. Examples from the neurological disease literature abound. For example, Lou Gehrig's disease (ALS), Parkinson's disease and Alzheimer's disease are frequently viewed as totally unrelated and completely distinct from each other, even though there are extremely clear cases that prove the opposite: ALS-parkinsonism dementia complex (ALS-PDC) of Guam and the Western Pacific often combines the features of all, albeit with the ALS phenotype usually preceding the loss of neurons in other CNS fields [1]. Parkinson's disease and ALS frequently feature aspects of Alzheimer's like dementia [2-5]; fronto-temporal dementia can have motor neuron loss, etc., as part of the long-term spectrum of disease expression [6]. The risk of ALS is significantly increased in people who suffer from asthma, celiac disease, early diabetes, multiple sclerosis, myasthenia gravis, hypothyroidism, Sjögren's syndrome, systemic lupus erythematosus (SLE) and ulcerative colitis [7].

Even within a particular disorder, entirely different organ systems may be involved. In ALS, patients often exhibit changes in skin characteristics, in addition to motor neuron losses, features that have been known since Charcot's seminal work in 1880 [8]. A series of studies by Japanese investigators have examined in detail the possible links between changes in dermis and epidermis and motor neuron loss in ALS [9,10]. Western scientists have generally ignored these data, in spite of the obvious linkage provided by embryology that both systems are ectodermal in origin [11-14]. These skin changes can even be demonstrated in animal models of ALS [15].

Other organ disorders can also be features of Parkinson's, Alzheimer's and ALS-PDC. The extent of central nervous system

(CNS) involvement of clearly multisystem disorders, chronic fatigue syndrome, Gulf War Illness, etc. shows just how widespread such multisystem effects may be [16-18].

Nowhere is the possible link more obvious than in disorders that involve both the immune system and the nervous system, and we will argue in this paper that more than their just being juxtaposed in the same disorder, there are powerful interactions between natural immune and abnormal autoimmune functions and normal development and pathologies, respectively, of the CNS. Moreover, we will argue that some common triggers of autoimmunity may be key contributors to neurological disease by direct toxic actions, as well as indirectly *via* autoimmune responses. In particular, we will focus on the clear multi-level multisystem toxicant role of aluminum (Al) [19].

## Evidence for CNS-immune interactions

A recent review by Besedovsky and A del Rey [20] describes in detail how immune/CNS interactions may occur through the release of various cytokines. Cytokine release in the periphery can directly impact neurons in the CNS by binding to a range of cytokine receptors on neural cells, resulting in changes in neuronal activity. The relationship is reciprocal, such that cytokine release from neural cells of all types can serve signaling functions to immune cells outside the CNS [21].

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During CNS development, cytokines released in the periphery are thought to shape neuronal circuitry and function [22]. Such impacts of the immune system have been linked to both normal and abnormal CNS development [19], in the latter case to autism spectrum disorder (ASD). Such stimulation could arise naturally by way of immune system activation by various pathogens, or by iatrogenic immune activation through vaccination.

### Autoimmunity in neurological disease

Although space precludes a full description of the literature, there is now abundant evidence for an autoimmune component to the classical age-related neurological diseases, including ALS [23-27], Alzheimer's [25,28-32] and Parkinson's diseases [28]. It remains, however, uncertain whether the immune markers found in affected regions of the CNS are causal or secondary to the resulting loss of neurons [23-32]. This same consideration applies to the typical presence of activated microglia at the site of most CNS lesions and whether the neuroinflammatory response is primary or secondary to neuronal degeneration. In the case of microglia, the situation is doubtlessly complicated by microglia's dual roles as neuroprotective cells or scavengers, a role that depends on a range of other factors [21].

### Multiple sclerosis and gut bacteria

There has been a recent surge in interest in the concept of gut bacterial dysbiosis as a mediator of autoimmune disease [33]. Multiple sclerosis (MS), an inflammatory disease that leads to demyelination in the CNS, is mediated by autoreactive T cells that become antigenic towards myelin [34]. The immune cell attack on myelin leads to altered axonal conductance and the slowing or failure of neuronal signaling [35]. It has been proposed that the autoimmunity in MS might arise out of molecular mimicry from a pathogenic protein with sequence homology to peptide sequences in myelin [36]. However, extensive search has not yet produced a candidate pathogen for MS. A recent study searched a database of reported sequences from all known human bacterial and viral agents for possible matches to three established encephalitogenic peptides. Intriguingly, mimics were detected for several bacteria that are ordinarily benign residents in the gut [37]. These data may suggest that a leaky gut syndrome, in conjunction with distressed microbiota, may lead to MS via antigenic exposure to DNA debris from common gut bacteria.

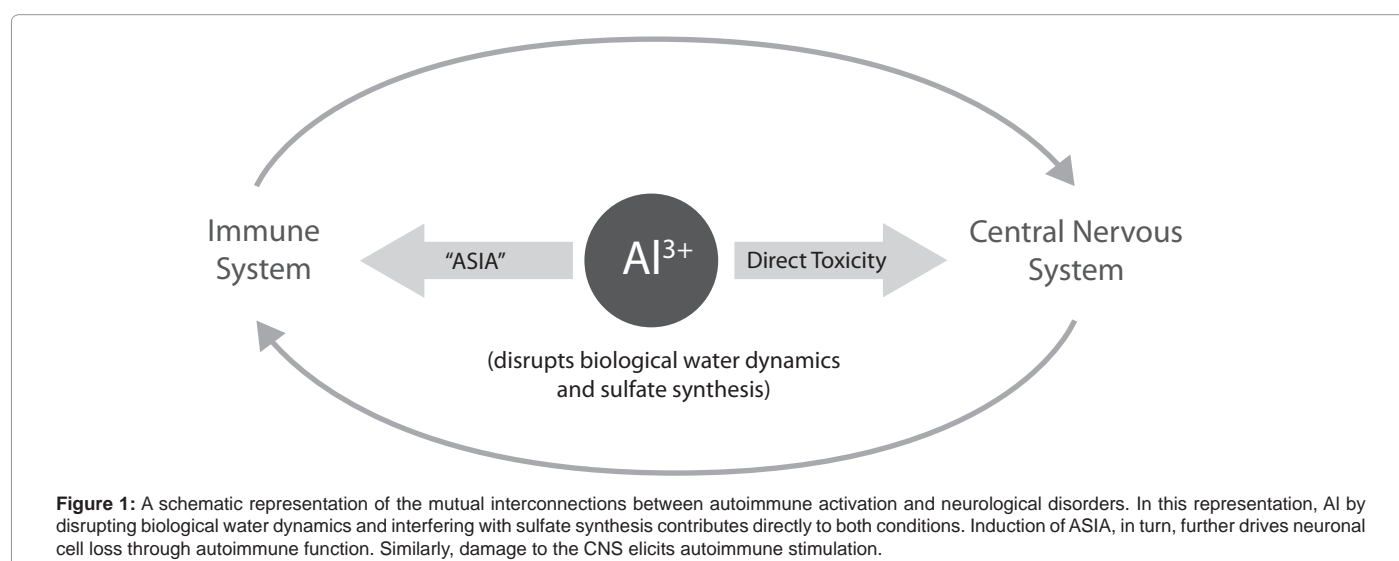
Guillain-Barre disease is a disorder involving the Schwann cell myelin sheath of peripheral nerves. As the nerves become progressively demyelinated, neural conductance may slow and then cease. A now large literature suggests that the overall mechanisms of action are autoimmune in nature. In the case of Guillain-Barre, a well-known trigger appears to be vaccination [38-40]. If correct, Guillain-Barre would be part of the spectrum of disorders, now termed "autoimmune/ autoinflammatory syndrome induced by adjuvants" or "ASIA".

### Autoimmune/autoinflammatory syndrome induced by adjuvants (ASIA)

Shoenfeld et al. [41,42] reviewed the large body of evidence that clearly demonstrates adjuvant administration preceding the onset of immune-mediated diseases, including siliconosis, Gulf war syndrome and a rapidly emerging entity termed macrophagic myofasciitis (MMF) (Figure 1) [43]. Collectively, these illnesses present similar clinical features, which are now designated being part of the ASIA syndrome. Many of these appear to arise due to the use of Al adjuvants, e.g. MMF in humans [43]. Similar outcomes have been reported in sheep also following Al adjuvant exposure from vaccines. Concerning the latter, Lujan et al. reproduced an autoinflammatory illness experimentally among sheep immunized against blue tongue and showed that Al was present in the CNS of affected animals. Notably, the impact of the adjuvant Al was more severe in winter months, suggesting an interaction with other stress factors.

Other vaccine adjuvants appear capable of inducing autoimmune reactions in humans, as well. Nohynek et al. provided evidence of a significant increase in adolescent narcolepsy in Finland, following vaccination with a lipid-based adjuvant in the H1N1 influenza vaccine [44,45], data that have now apparently been reproduced in several other northern European countries. Whether these outcomes truly reflect negative impacts of the particular adjuvant on the CNS or whether other components of the vaccine alone or in combination with the adjuvant were responsible, remains uncertain. As above with the Lujan et al. results, it may be notable that such impacts occurred during winter months.

Shoenfeld et al. [41,42] have also demonstrated that a variety of other compounds apart from vaccine adjuvants are also capable of inducing ASIA syndrome.



## Aluminum adjuvants: History of use and impact on CNS structure and function

Aluminum has been used in vaccine formulations since 1926 after the discovery that it potentiates the immune response to the target pathogen [46,47]. Perceptions of Al safety that abound in the medical literature are largely based upon a lack of recognized adverse events over the past 70 years [48], rather than randomized, true-placebo-controlled clinical trials, or the now abundant experimental animal literature [49]. A meaningful conclusion that unlimited use of Al is safe in vaccines cannot be made. Adverse events are significantly under-reported, and physician bias often influences the reporting process. Quite often, the requisite inquiry as to whether a vaccination preceded an acute illness is not asked. Autoimmune reactions to aluminum in vaccines are not of sufficient frequency to facilitate prospective randomized control trials. Causation is difficult to establish in general, when so many factors could be in play, although the use of the Hill criteria certainly helps the process of sifting causality from coincidence [49,50]. Some researchers have opined that the latency period of autoimmune disease makes it difficult to infer causation retrospectively, but this may not be a valid critique, since there is still a clear sequence of events from presumed causal factor to disease outcome.

Al adjuvants are used in childhood vaccines against diphtheria, tetanus, pertussis, hepatitis B, anthrax, *Haemophilus influenza* and human papilloma virus, amongst others [48,51]. A child may be injected with as much as 4.225 mg of elemental Al by the age of 12 months [52]. Our review of currently licensed vaccine package inserts in the United States is consistent with this figure. Mitkus et al. [52] reported that this dosage is within the U.S. Agency for Toxic Substances and Disease Registry's minimum risk levels for infants, extrapolating data from a volunteer study of adults using radioactive aluminum tracer [53], and a toxic autokinetic study performed on rabbits [54]. Mitkus et al. [52] used the creatinine clearance differential between children and adults to estimate total Al body burden of infants following vaccination. The estimation is based upon an assumption that Al excretion parallels creatinine clearance, an assumption that is unlikely to be correct either on theoretical or experimental grounds. In the first instance, rapid excretion of Al would nullify the very basis of having it as an adjuvant in the first place. Experimentally, the notion that Al adjuvants are rapidly excreted is challenged by the recent work of Khan et al. [55].

There is a growing body of data to suggest that Al is *biosequestered* by albumin, transferrin and macrophages of the reticuloendothelial system after intramuscular injection. According to Ganrot [56], insoluble metal hydroxides are thought to mainly be taken up by the reticuloendothelial cells, while soluble salts of the trivalent ions are mainly bound to the skeleton or excreted in the urine. Ubiquitous heparan sulfate proteoglycans (HSPGs), which decorate the glycocalyxes of our cells membranes, are likely to act as multi-dentate chelators--biosequestrants--of Al [57-59]. Moreover, "cationized" bovine serum albumin (cBSA) and "cationized" human serum albumin (cHSA) have long been known to have enhanced endocytic uptake via adsorptive transcytosis by the blood brain barrier [60-62]. cBSA has been found to be present in subepithelial immune deposits in children with idiopathic membranous glomerulonephropathy [63]. The Flarend rabbit study [54] showed that absorption following intramuscular Al particulate injections into the blood is not instantaneous, and only some of the Al was absorbed from the injection depot over the first 28 days. These data are supported by the recent study by Khan et al. [55] suggesting that the initial trajectory for Al hydroxide is into the lymphatic system. There has been a concerted effort to reduce the Al burden in parenteral

feedings to premature infants due to the observation that 4-5 µg/kg per day of Al can induce neurodevelopmental delays [64]. In spite of this, there seems not to be an equal or adequate concern about the potential risks of injected Al whose clearance from the CNS may be extremely slow [55]. The overall impact of Al used as an adjuvant in vaccines has been addressed in detail elsewhere [51]. In addition, these same authors have provided some evidence for a causal role in ASD based on anecological study of US government databases [65].

## Outline of the article

In the remainder of this paper, we will develop what we believe to be a novel proposal for an inflammation cascade subsequent to exposure of tissues to Al and other neurotoxicants. Briefly, the cascade can be outlined as follows:

- (a) Aluminum disrupts water-based cellular homeostasis and causes a crisis for the exposed cell.
- (b) The cell sends out "death alarm" messages, which draw in macrophages and other immune cells, initiating an inflammatory cascade.
- (c) The highly stressed cell dies *via* necrosis rather than a "programmed cell death," and releases its DNA into the interstitial tissues.
- (d) This extracellular DNA is picked up as an antigenic signal by immune cells and leads directly to autoimmune disease.
- (e) In parallel, sulfate synthesis and sulfate transport are disrupted due in part to Al contamination of the pineal gland and other sensitive nuclei in the midbrain.
- (f) The entire biological system switches from a sulfate-based to a phosphate-based management strategy for maintaining water interfaces, leading to hyperparathyroidism.

In the following three sections, we will introduce the three principle components of this cascade, the local disruption of cellular homeostasis, the systemic cascade response leading to widespread sulfate deficiency, and the calcium-signaling-based switch from sulfate to phosphate as an anionic buffering solution. We first briefly review in Section 4 the literature on the biophysical role of water in biological systems, emphasizing how this role gets disrupted by Al. Section 5 will address the systems level cascade response to such disruption, leading to impairments in the supply of biosulfates to the tissues, systemically. We will discuss various disease manifestations of this impairment and propose an essential role for the pineal gland. Section 6 describes the final stage of the cascade when calcium phosphate based signaling cascades launched by a hyperactive parathyroid gland replace magnesium sulfate for the role of buffering water and maintaining its homeostasis in the cells, in the vasculature and in the tissues.

Section 7 will provide some specific examples from the literature of various diseases and conditions that we think also fit the model, we are proposing here. The Discussion will review the sequence of events and summarize our main findings and conclusions.

## A Biophysically Based Pathway to Immune Dysfunction and Autoimmune Disease (Section 4)

There is a vast and growing literature on the special physical properties of water, and we have selected for the brief review here only some of the most compelling papers on this subject.

## Biological water is an active participant

It is well established that water is essential to life. However, it is the unique biophysical properties of water that make it essential. It is becoming increasingly clear that water is an active participant in most biochemical reactions, rather than simply the medium in which the reaction takes place. Sulfates are members of a distinguished class of molecules-kosmotropes-which have the property that they order neighboring water molecules into a dynamically-structured arrangement that is far more viscous than the bulk water (variously referred to as the “exclusion zone” or the “coherence domain”), and that also exhibits other unusual properties with respect to responses to electromagnetic fields, exclusion of solutes and the mobility of protons and electrons [66]. The interface between this dynamically structured water and the bulk water has interesting physical properties, as do the dynamically-structured water itself, and biological systems almost certainly exploit these properties to energize their reactions. There is not space here to provide anything other than a brief overview of this vast topic.

In 1987, Bak et al. [67] showed that dynamical systems with spatial degrees of freedom naturally evolve into a self-organized/ordered critical structure—a metastable state—a state which is barely stable. Such systems often, but perhaps not always, demonstrate power-law behavior over vastly different time scales [67]. Biological water dynamics fits the criteria for such self-ordered/self-assembling systems in that it demonstrates the combination of dynamical minimal stability and spatial scaling predicted to lead to a power law for temporal fluctuations [68-70].

## A novel hypothesis for dynamically-structured water at the interphase

In the remainder of Section 4, we will incorporate by reference and expand upon the data reviewed in two recent review articles [71,72]. We will briefly present a novel hypothesis in which the cumulative disruption of dynamically-structured biological water at the interphase [73] of neurolemmal membranes, induced by certain *polycationic* inorganic surfactants, e.g. various  $Al^{3+}$  species eventually exceeds a critical threshold, resulting in loss of macromolecular recognition, immune dysfunction and autoimmune disease.

Nanoclusters of biological water at the interphase are thought to represent clusters of minimally-stable states, which are defined dynamically as the spatial regions over which small local perturbations, e.g. induced by exogenous interfacial water stressors, such as  $Al^{3+}$ , will propagate. The neurotoxicity of  $Al^{3+}$  begins with the disruption of hydrogen-bond cooperativity and quantum coherence of water at the interphase of neurolemmal membranes, which consequently exceeds the threshold of self-ordered criticality necessary to maintain membrane potentials and action potentials [71,72]. The minimally stable states of interphase water at neurolemmal membranes are upset by the “noise” or “turbulence” propagated through the scaling clusters by means of a “domino” effect.  $Al^{3+}$  is thought to induce long-wavelength perturbations, which cause a cascade of energy dissipation on all length scales. Nanoclusters of water and ensembles of coherence domains comprise the “clusters” of minimally-stable states, which can be defined dynamically as spatial regions over which a small local, long-wavelength perturbation, e.g. induced by an exogenous interfacial water stressor, such as  $Al^{3+}$ , will propagate.

Many researchers have long sought data to show that the brain operates at a critical state to benefit from the maximal dynamic range

of processing, fidelity of information transmission, coherence between multiple “nested” biosemiotic levels [19] and information capacity [74-80]. A very appropriate marker of criticality may prove to be the percolation transition of interphase water at neurolemmal membranes, e.g. at the interphase of neuronal myelin [81,82]. Experimental studies of the conductivity of hydrated biosystems provide direct evidence for the formation of a spanning network of hydration water *via* the percolation transition [83]. The percolation transition and charge transfer of water may play crucial roles in biological function [71]. Several instances have been reported where the percolation transition of water occurs at the hydration level where various forms of biological activity develop. Based on percolation theory, the percolation transition of water at the interphase of myelin is likely to be the point at which neurological conductivity of charge occurs [82,83], with similar albeit shorter range conductivity occurring with unmyelinated axons.

## Water dynamically couples the neuronal network to the environment

A widely-held orthodox view of the etiology of immune dysfunction and autoimmune disease is that a combination of environmental, genetic and immunological factors may play roles in their pathogenesis. Today, environmental exposures, molecular mimicry and genetic predisposition [84,85] are frequently invoked etiologies. By itself, however, genetic reductionism utterly fails to explain most of the autoimmune diseases of today, including those for neurodevelopment disorders, such as ASD. Similarly, most age-dependent neurological disorders as cited in the Introduction cannot be reduced to gene mutations, in spite of prolonged efforts to do so.

In regard to ASD, Vargas et al. [86] published evidence of innate immune cell activation in brain tissue of autism patients; in particular, activated glial cells were identified microscopically, indicating that innate neuroimmune reactions play a pathogenic role in an undefined proportion of autistic patients. Of note, the Vargas microscopic data appears to provide support for a much earlier study by Gallez and Coakley [87], who demonstrated that interfacial instability at cell membranes accompanied cell “activation.” Specifically, the average number of waves per wavy cell rim “...decreased when cell surface charge was depleted, when polyvalent cations were in the suspending phase, and when cationic drugs were present and increased in the presence of anionic drugs”.

According to our novel alternative biophysical view of the etiology of autoimmune disease, various  $Al^{3+}$  species cumulatively induce exogenous interfacial water stress (EIWS) [71], which causes:

- (a) Immune cell activation, phagocytic activity, inflammatory cytokine release;
- (b) Decrease in neurolemmal membrane potentials and failure of action potentials [88,89];
- (c) Loss of macromolecular recognition [68];
- (d) Loss of proton and charge conduction of neurolemmal membranes [90];
- (e) Unfolded DNA Response (UDR) [72];
- (f) Unfolded Protein Response (UPR) [72];
- (g) Thrombohemorrhagic phenomena [91];
- (h) Loss of self-ordered criticality [74,76,77].



## The self-ordered criticality of biological water

Branching cascades of neuronal network activity have been likened to chain reactions and avalanches, such as those seen in events like earthquakes, forest fires, landslides, power grid collapses and nuclear chain reactions. In 2003, Beggs and Plenz [75] showed that *in vitro* propagation of spontaneous activity in cortical networks obeys a power law, and is described by equations that govern avalanches [62]. They proposed that these so-called “neuronal avalanches” may represent new modes of neuronal network activity [74-76], which differ profoundly from oscillatory, synchronized or wave-like network states. They further proposed that *in the critical state*, the branching network may satisfy competing demands of information transmission and network stability [75]. Previously, Paczuski et al. [78] showed that the spatial and temporal distribution of similar cascades or avalanches were well-described by power laws. The power law dependency indicates that the systems are in a critical state [67], and that the dynamics can be seen at many different scales [78]. Today, it seems clear that actual neuronal networks display critical behavior, and that criticality is a robust feature of neuronal organization. The percolation transition of biological water at the interphase of neurolemmal membranes is, in our opinion, very likely to be the minimum requirement for a neuronal system to show criticality.

Neuronal networks are thought today to be dynamically-coupled to their environment [77]. Biological water at the interphase of neurolemmal membranes is the likely mediator of the dynamical coupling between neuronal networks and their environment. Dissipative structures are not true organizational systems [77].  $\text{Al}^{3+}$  directly impairs self-ordered criticality of biological water dynamics and increases entropy [71]. According to Taylor et al. [74], a brain at or near criticality would have maximum dynamic range, enabling it to react and adapt to the dynamics of the surrounding environment and maintain balanced neuronal activity [74]. Quantum coherent, cooperatively hydrogen-bonded, nanoclusters of water at the interphase of biological membranes necessary for the percolation transition of neurolemmal membranes. Dynamically-structured water at the interphase is essential in (a) capturing and transducing extremely low frequency EM energy from the environment, (b) dynamically-coupling the neuronal network to the environment, and (c) maintaining the network in a metastable critical native state. Myelin is endowed with sulfoglycolipids such as sulfatide and HSPGs, which are essential in generating current and separating charge. Myelin lipids and proteins demonstrate surface fractality over many scales [69,81].

The point of criticality occurs at the percolation transition of interphase water at neurolemmal membranes. The detailed spatial and temporal embedding may be found in the ultrafast electron crystallography of interfacial water by Pal and Zewail [68], where it was found that macromolecular recognition is dependent on biological water dynamics in the 20-40 picosecond range [68,82]. Loss of macromolecular recognition would logically be expected to precede molecular mimicry, immune dysfunction and the onset of autoimmune disease. Neuropathological states can thus be conceptualized as the breakdown of, or deviation from, the metastable critical state of biological water dynamics at the interphase of neuronal membranes.

The notion has been proposed that the brain may self-organize to a critical state [63]. A new marker of criticality, which may have considerable utility in the neurosciences, is the self-ordered criticality [77] of biological water dynamics at the interphase of neuronal membranes. Support for this proposal is found in the work

of Johansson and Sukhotskaya [69], who showed that self-organized water demonstrates an allometric power law scaling.

## Pioneering studies with implications for neuroimmune disease

Inoue et al. [88] and Ueda et al. [89] conducted a series of experiments, which suggested remarkably that water, properly maintained, can contain solutes and hold cellular resting potentials even in the absence of a plasma membrane. A complex coacervate of protoplasmic droplets obtained from *Nitella* cells were shown to have an interfacial tension of 0.04 dyne/cm. These protoplasmic droplets not only exhibited an inside-negative resting potential of from -70 to -90 mV, equal to those seen in many normal excitable living cells, but they were also electrically excitable, generating an action potential in response to a short pulse of electric current. According to Ling [92], in his polarized multilayer (PML) theory of cell water (including his subsidiary hypothesis of coacervation), coacervates have exceedingly low interfacial tension because the coacervate surface contains a great deal of water, albeit polarized and oriented in parallel arrays.

Ling [92] cites the low interfacial tension of living sea-urchin eggs (0.08 dyne/cm or even lower), of *Nitella* endoplasm droplets (0.04 dyne/cm), and of gelatin-gum Arabic coacervate (0.0023 dyne/cm), which when viewed in toto, strongly suggest that the living cell membrane is “just like the bulk-phase protoplasm comprising in the main fully-extended proteins and multilayers of polarized-oriented water”. With increasing temperature, Ueda et al. [89] showed that the interfacial tension of the protoplasmic droplets isolated from *Nitella* cells decreased discontinuously from 10 dynes/cm to the order of  $10^{-4}$  dynes/cm at about 34°C. These changes were reversible. Ueda et al. [89] also observed that the addition of multiply-charged inorganic cations in the test solution led to an abrupt depolarization of the membrane potential at a definite concentration for each ion species, wherein the critical salt concentration was inversely-related and strongly dependent on the valence of the cations ( $\text{Th}^{4+}$ ,  $\text{La}^{3+}$ ,  $\text{Ba}^{2+}$ ,  $\text{Sr}^{2+}$ ,  $\text{Na}^{+}$ ) added. When the drop was allowed to stand for 10 minutes in the depolarized state, for example, in a 10 mM solution of various polycation inorganic salts ( $\text{UO}_2^{2+}$ ,  $\text{Fe}^{3+}$ ,  $\text{Hg}^{2+}$ ,  $\text{Cu}^{2+}$ ,  $\text{Cd}^{2+}$ ,  $\text{Ag}^{+}$ , etc.), the protoplasmic “streaming” movement in the drop was suppressed and led to an irreversible change of the drop, an unambiguous sign of toxicity. In 1991, Tsuchiya et al. [93] noted that interactions of actin and myosin molecules participate in generation of the motive force for protoplasmic streaming. If these experiments were repeated with  $\text{Al}^{3+}$ , it seems likely that similar depolarization of the membrane potential and loss of motive force for streaming would be observed. Supportive evidence from plants comes from the observation that Al depolymerizes microtubules and depolarizes the membrane in root cells of intact Arabidopsis seedlings [94].

The aforementioned studies have clinical ramifications for toxicant exposures of mammalian species, whose neuronal motility is intrinsic to the formation of the central and peripheral nervous systems during development [95]. Herein, lies in substantial measure, the clinical relevance of the preclinical electrophysiology research by Ling and others. Similar inorganic polycationic surfactants to  $\text{Al}^{3+}$  with high charge-densities are also toxicants under the definition of the National Cancer Institute. The novel hypothesis presented in this section gives a short overview of how such metal ions synergistically and cumulatively induce inflammation, immune dysfunction, autoimmunity and cancer.

Taken together, the aforementioned research of Ling, Inoue, and Ueda suggests that coacervation and phase transitions in aqueous heterogeneous media may provide much of the physical basis for

water-driven, coherent, dynamical, multiscale, cellular self-assembly, self-ordering and biosequestration, which enables the generation and maintenance of the membrane potential and action potential in the neurological tissue of mammals, including humans.

Evidence presented here and elsewhere [71], suggests that a metastable self-ordered critical state of neural tissue ensues once a certain threshold of hydration occurs in a relatively “dry” environment, such as that found sheltered within the blood-brain and blood-cord barrier [83]. As will become apparent later in this paper, we believe that biosulfates play a critical role in maintaining this healthy state. The 3D percolation transition of interfacial water within the interphase of aqueous myelin is predicted to be the threshold of criticality within neural tissue, throughout the entire human nervous system, both central and peripheral.

In summary, the hypothesis outlined herein, is not *per se* incompatible with the widely held conventional view of the etiology of immune dysfunction, and autoimmune disease being a result of molecular mimicry and genetic predisposition. However, it differs substantively in the view towards the role of biological water in the disease process. This distinction is not insignificant, and it should be said that the distinction has a large and *rapidly* growing published physical basis.

## A Systems Biology Pathway to Immune Dysfunction and Autoimmune Disease (Section 5)

We believe, based on the observed effects of Al-containing vaccines and in consideration of the known biophysical and biochemical properties of Al, that one of the most devastating consequences of exposure to the ions or complexes of this element in certain cell types is a near-permanent switch from sulfate synthesis to nitrate synthesis. This switch will have systemic consequences, as discussed below. There is not sufficient space here to cover all the details.

The affected cell types are those that contain nitric oxide synthase (NOS), which include epithelial cells [96], endothelial cells in the vasculature [97], red blood cells (RBCs) [98], skeletal muscle cells [99,100] and neurons [101]. NOS play an important role in the pathophysiology of many diseases and conditions, such as the metabolic syndrome and cardiovascular disease [102-104]. Endothelial NOS (eNOS) is found in epithelial and endothelial cells, RBCs and muscle cells, whereas neuronal NOS (nNOS) is present in muscle cells and neurons.

There is a large literature on eNOS [105-109], with respect to complex regulation of its synthesis of nitric oxide (NO), a signaling gas that regulates vascular tone. It has recently been proposed that eNOS is a “moonlighting” [110] enzyme, which synthesizes sulfate when it is attached to the membrane at caveolae upon sunlight exposure and synthesizes nitrate when it is free in the cytoplasm, serine-phosphorylated and activated by calmodulin following calcium binding [105,111]. Seneff et al. [111] argued that, with a single enzyme synthesizing both sulfate and nitrate, the cell can exercise tight control over titration between excess presence of kosmotropes (structure making molecules) or chaotropes (structure breaking molecules) [112] in the blood, as it is essential to keep these two influences in perfect balance.

Since sulfate is a kosmotrope and nitrate is a chaotrope, tight regulatory control over the synthesis of these two molecules can restore balance when other circulating molecules such as Al, disrupt it, which is a strong cationic kosmotrope. Al induces iNOS synthesis in the

cerebellum in rats [113], an effect that is not potentiated by iron. We hypothesize that it is due to Al's kosmotropic properties, which require balancing through immediate and intense production of the chaotrope, nitrate.

In the remainder of this section, we will first briefly review the argument that NOS produces sulfate when it is attached at the plasma membrane. We will discuss the important need for sulfate in maintaining levels of cholesterol sulfate and heparan sulfate in many tissues in the body. We will follow this with a discussion of how Al, both through its direct ability to mimic calcium and the ability of aluminum-fluoride complexes ( $AlF_x$ ) to mimic phosphate, induces NOS to switch from sulfate to nitrate synthesis, while simultaneously inducing many other metabolic adjustments in the cell.

### Sulfate synthesis by nitric oxide synthases

It has been very well established that the NOS isoforms synthesize NO, an important signaling gas, which is oxidized within a few seconds to nitrite and nitrate [114]. In the case of RBCs, this presents a puzzle [98], because hemoglobin is a potent NO scavenger and nitrosylation of hemoglobin, similar to the effect of carbon monoxide, would impair its ability to transport oxygen. RBCs, in fact, do not use their eNOS to synthesize NO, except perhaps under extreme pathological conditions. This is clear because (1) eNOS remains bound to the membrane rather than in the cytoplasm in RBCs, and (2) RBCs exclude the substrate for NO, L-arginine and have an enzyme that actively breaks down any minute amounts that gain entry.

The proposal in Seneff et al. [111] that eNOS is a dual-purpose enzyme solves two problems for RBC's: it explains (1) why they contain abundant Enos, and (2) how they can obtain sulfate to be combined with cholesterol, yielding cholesterol sulfate. Cholesterol sulfate is produced by RBCs, and it plays a vital role in their membrane by protecting them from hemolysis [115,116], and helping to maintain the blood's highly negative zeta potential [71,72,111,117].

Epithelial cells also produce abundant cholesterol sulfate, which becomes a major component of the corpus striatum--the outermost layer of skin composed of enucleated cells that maintains a tight barrier to protect from water loss and microbial invasion. Cholesterol sulfate stimulates the synthesis of filaggrin, an essential protein in the highly cross-linked mesh in the corpus striatum, essential to its proper functioning [118]. Deficiencies in filaggrin are associated with conditions like atopic dermatitis that are observed in adverse reactions to mercury-and Al-containing vaccines. Deficient filaggrin can explain skin-related pathologies associated with CNS disorders. Atopic dermatitis also has immune components in that IgE levels are elevated [119].

Endothelial cells need sulfate to produce sulfated proteoglycans that make up the glycocalyx, which is essential for protection from vascular leaks. The glycosaminoglycan (GAG) sulfate anions present in heparan sulfate, chondroitin sulfate and keratin sulfate are essential in maintaining the structured (gelled) form of water [120] in the region surrounding not only the cells lining the vascular walls, but also most cells in the body. Loss of sulfates in these GAGs results in extensive impairment in cell function. Under sulfated GAGs in the intestinal wall and the intestinal vasculature are implicated in intestinal disorders, such as colitis and Crohn's disease [121].

Both neurons and muscle cells require large amounts of energy and Seneff et al. [111] argued that these cell types take advantage of heparan sulfate in membrane-bound syndecans, as a way to temporarily store

excess glucose. The sulfation step is necessary to prevent glycation damage to vulnerable proteins in the vicinity. Heparan sulfate is constantly synthesized and stored outside the cell as GAGs, and then later endocytosed into lysosomes over an elapsed interval of 4 to 6 hours [122]. The subsequent breakdown of the glucose in the lysosome provides a buffered source of energy to the cell. The amount of eNOS found in muscle cells is inversely related to obesity [99], and to nutritive flow into skeletal muscles [99]. With insufficient sulfate, these cells become insulin resistant, because they can no longer store part of the glucose they take in these GAGs. A similar strategy probably exists in neurons and its impairment may be responsible for the "Type III" insulin resistance that has been proposed as an early indicator of dementia [123], and which has been linked to Alzheimer's disease [124].

Heparan sulfate in neurons also plays an important role in neurite outgrowth [125], which would, therefore, be impaired if sulfate supplies were insufficient, potentially contributing to the pathology in autism and in various dementias. It also participates in long-term potentiation in the hippocampus [126], a process thought by some to be part of memory formation. Mice engineered to be impaired in the ability to sulfate heparan-sulfate chains in the brain suffered from all of the pathologies associated with "mouse-autism" [127]. Structural pathologies in the hippocampi were associated with depletion of heparan sulfate in the lateral ventricles in the brains at autopsy of mice exhibiting a mouse-model of autism [128]. Similar heparan sulfate deficiencies were also observed in postmortem analyses of human brains of individuals with autism [129]. A study of Alzheimer's brains post-mortem assessing the distribution of various lipids found that sulfatide, the only sulfated lipid, was uniquely under-represented in the Alzheimer's brains compared to normal controls [130]. Sulfatide was depleted up to 93% in the gray matter. These studies point to a deficiency in sulfate in the brain as a contributing factor in both autism and Alzheimer's disease.

### Aluminum disrupts sulfate synthesis

As discussed above, the synthesis of sulfate by NOS when it is attached to the plasma membrane is highly plausible as a means for cells to supply themselves with adequate sulfate. Cells often need to supply their own sulfate due to sulfate's anionic kosmotropic property [111]. Because free sulfate transport is highly precarious, the body maintains an upper limit of less than 0.5 mM concentrations of free sulfate in the blood [131]. Any amounts above this level are exported through the kidneys. Cholesterol sulfate delivery by RBCs to the tissues during their passage through capillaries is likely an important means to supply the tissues with both cholesterol and sulfate. Unlike cholesterol, cholesterol sulfate freely migrates from one plasma membrane to another through water-based media because it is amphiphilic, i.e. both hydrophilic and lipophilic. In addition, the cholesterol in cholesterol sulfate supports a firm anchor within the membrane of an RBC during transit, ameliorating the kosmotropic effects of sulfate.

We can anticipate two ways in which Al would disrupt sulfate synthesis by eNOS, and in fact, cause eNOS to be locked into a nitrate-synthesis mode, with potentially devastating consequences. Most simply,  $\text{Al}^{3+}$  is a strong kosmotrope, which will influence the endothelial cells to switch to nitrate synthesis as a counterbalancing electrolyte. However, further considerations lead us to consider a more significant possibility.  $\text{Al}^{3+}$  is highly attracted, electrostatically to the negative charge of the sulfates in the GAGs of the glycocalyx.  $\text{Al}^{3+}$  would be expected to subsequently gain entry *via* calcium transporter channels, as a  $\text{Ca}^{2+}$  analogue. Once inside a cell,  $\text{Al}^{3+}$  binds to calmodulin with

a 10-fold higher affinity than  $\text{Ca}^{2+}$  [132]. Through a well-established signaling cascade, this would cause eNOS to detach from the membrane and stop producing sulfate [105].

Furthermore,  $\text{Al}^{3+}$  readily binds to fluoride to form  $\text{AlF}_x$  complexes (mostly  $\text{AlF}_3$  and  $\text{AlF}_4^-$ ). Fluoride is likely to be present in the blood due to nearly universal water fluoridation programs and fluoridated toothpaste.  $\text{AlF}_x$  is an excellent mimetic of phosphate, so much so that it has been effectively utilized to elucidate phosphorylation signaling cascades [133]. Like phosphate,  $\text{AlF}_x$  induces a GTP-mediated signaling cascade, through the mimetic  $\text{GDP-AlF}_x$ . Unfortunately, the  $\text{G}\alpha\text{GDP}^*\text{AlF}_4^-$  complex is a very stable molecule that resists deactivation by hydrolysis and remains in the active state indefinitely [134]. This initiates a pronounced inflammatory response that may partially explain Al's adjuvant activity to promote an antigenic response. What this means for eNOS is that it becomes and remains phosphorylated, and therefore, produces sustained excessive amounts of NO, at the expense of sulfate.

### Depression, Alzheimer's and the pineal gland

Seasonal affective disorder (SAD) may affect more than 10 million Americans [135]. In addition to depression, patients often experience fatigue, hypersomnia, carbohydrate craving and weight gain. Exposure to bright light, especially in the morning, is an established therapy [136].

SAD is likely tied to impaired melatonin synthesis in the pineal gland, a small gland located directly behind the eyes in the center of the brain. It produces the neurotransmitter melatonin, which plays an important role in the sleep-wake cycle. Melatonin is sulfated during transport, and we hypothesize that sulfate transport is a critical role of melatonin, such that it can supply sulfate to neurons distributed throughout the brain.

In a study of the amount of Al present in various brain tissues postmortem, more than twice as high a concentration of Al was found in the pineal gland, as in any of the other tissues examined, which included pituitary, cerebellum and cortex [137]. Mercury also accumulates in the pineal gland in occupationally exposed miners [138]. The amount of melatonin sulfate excreted in the urine is markedly reduced in association with occupational mercury exposure in miners, despite the fact that the amount of melatonin in the blood is sharply elevated [138]. Melatonin suppresses the synthesis of NO by NOS isoforms in the presence of calcium [106,108], which suggests that it enhances the synthesis of sulfate, which is needed for its transport.

An experiment on mice that involved exposing dams to Al orally during gestation and lactation at a level that did not noticeably impair their health was very informative in terms of the consequences to the offspring of the pregnancy [139]. The pups suffered from deficits in sensory motor reflexes, delays in the *opening of the eyes* and dose-dependent disturbances in serotonin and dopamine synthesis. Since serotonin is the precursor to melatonin, this translates into deficiencies in melatonin, which might be caused by impaired sulfate supply, as serotonin is also sulfated in transport. An experiment on rats exposed to Al with or without melatonin supplements demonstrated that melatonin protects from the oxidative damage in the cerebellum and cerebral cortex associated with Al exposure [140].

NOS activity exists in the pineal gland in both presynaptic nerve fibers and in pinealocytes, as well as in the endothelial cells of the blood vessels supplying the gland [106]. Norepinephrine is released at night from the nerve endings in the pineal gland, and such release is blocked by light exposure, which also markedly suppresses pineal NOS activity



[141]. Thus, NOS in the pineal gland produces NO mainly at night. We propose that during daylight and upon sunlight exposure, it produces sulfate instead. Strong support for this hypothesis comes from the fact that sunlight induces 3-O sulfation of heparan sulfate proteoglycans in the pineal gland, catalyzed by a heparan sulfate sulfotransferase [142]. The sulfate produced by day can be used to sulfate the melatonin produced by night.

The pineal gland may also supply sulfate to the *Substantia nigra*, a proximal midbrain nucleus that produces dopamine. Dopamine 3-O-sulfate is present in considerable amounts in mammalian plasma, and it is converted to norepinephrine through enzymatic action of dopamine- $\beta$ -hydroxylase, thus making the sulfate anion bioavailable [143]. Thus, the pineal gland may play a significant role in supplying sulfate to neurons in the brain, mediated by sunlight exposure and transport *via* melatonin and dopamine, a role that would be disrupted by Al accumulation. The pineal gland becomes calcified during aging, and it has been shown that such calcification is especially severe in association with Alzheimer's disease [144].

An accumulation of fluoride in the pineal gland has been identified in association with aging [145]. Excitotoxicity has been proposed as a central mechanism in fluoride toxicity, in part due to its ability to readily complex with Al to form  $AlF_x$  complexes [146]. Increased Al content was found in melanin-containing neurons of the *Substantia nigra* in two out of three Parkinson's disease patients compared to none in controls [147]. This midbrain nucleus lies in close proximity to the pineal gland in the mesencephalon. Al and fluoride, especially in combination, would be expected to disrupt sulfate synthesis in the pineal gland.

## A Neuroendocrine Pathway to Immune Dysfunction and Autoimmune Disease (Section 6)

Burnatowska-Hledin et al. [148] provide an excellent summary of the implications of hyperparathyroidism in the toxicity of Al, whether ingested in foods or in antacids, or present in dialysis fluid of patients with end-stage kidney disease. Hyperparathyroidism--excess production of parathyroid hormone (PTH)--leads to deposition of Al in brain and bone, as well as in the parathyroid gland itself. Al inhibits parathyroid hormone release, resulting in a euparathyroid state in dialysis patients with Al-related vitamin D-resistant osteomalacia. These authors argued that Al organ toxicity would be likely to occur not only in patients with impaired renal function, but also, more generally, in anyone expressing hyperparathyroidism. We develop this idea in this section, relating it, in particular, to vitamin D deficiency and insufficient sun exposure.

### Aluminum in vaccines and environment as an autoimmune stimulant

Modern vaccines, such as acellular pertussis, are highly processed antigens and hepatitis B contains a viral surface antigen mimic produced from recombinant DNA in yeast cells. However, these production methods render the processed antigens unrecognizable to the immune system as pathogenic. Thus, processed antigen does not reliably stimulate satisfactory acquired immunity. Therefore, adjuvants have become increasingly essential in vaccine formulations to maintain efficacy. For example, the processing of whole organism, *Bordetella pertussis* to an acellular antigen requires that the antigen be adsorbed on the surface of Al hydroxide or Al phosphate particles for the vaccine to be considered effective. Likewise, recombinant hepatitis B vaccine

antigen must be adsorbed on Al hydroxide or amorphous Al hydroxy phosphate sulfate (AAHS) particles, which are then injected.

### Mechanism of impaired excretion of aluminum

There is a strong link between Al toxicity and renal failure [149,150]. HogenEsch reviewed Al adjuvant safety and noted that Al toxicity is common in chronic kidney disease [48]. Aluminum causes renal dialysis dementia in part due to elevated parathyroid hormone activity in association with kidney disease [151]. Excess parathyroid hormone results in hypercalcemia and Al can be retained as well, as a consequence of its ability to mimic calcium. Parathyroid hormone inhibits normal urinary excretion and enhances gastrointestinal absorption of Al [152,153]. Therefore, it is not kidney disease per se that causes plasma Al to accumulate.

There are numerous reasons why parathyroid hormone activity or iPTH levels can be elevated besides chronic kidney disease. Thus, susceptibility to Al toxicity extends far beyond a select group of patients with chronic kidney disease. These include primary hyperparathyroidism, as well as physiologic hyperparathyroid state or secondary hyperparathyroidism. Causes of secondary hyperparathyroidism may include vitamin D insufficiency or deficiency, vitamin D resistant rickets, genetic variation of vitamin D receptor and others.

Parathyroid hormone levels can fluctuate physiologically relative to the availability of vitamin D in maintaining plasma calcium concentration within narrow bounds, and potentially impart a variable susceptibility to Al toxicity. Sun deprived populations, such as those who reside in Northern locations or those with darker skin [154,155] have incrementally higher prevalence of 25(OH)D3 insufficiency and secondary hyperparathyroid (2hPTH) state. Sufficient exposure to increasing Al dosage can ultimately intoxicate individuals with otherwise normal calcium homeostasis when parathyroid hormone becomes more predominant in maintaining plasma calcium.

Cannell [156] presented a compelling association of vitamin D deficiency and autism. Al is the only component listed in vaccine package inserts known to have a toxicokinetic profile modulated by parathyroid hormone activity. The causal cascade of aluminum toxicity in chronic kidney disease [157,158] would differ from sun deprivation only in that diseased proximal renal tubule cells are not able to convert 25(OH)D3 to 1,25(OH)2D3 [159,160].

Thus, the impaired ability to excrete aluminum may be more a function of parathyroid hormone activity than creatinine clearance. Movsas et al. [64] performed an experiment on 15 preterm infants at the age of 2 months by injecting 1200  $\mu$ g of aluminum in a single day and measuring serum and urine aluminum 24 hours before and after the injections. The investigators observed that the urine Al concentration remained unchanged. On that basis, they concluded Al in the vaccines is safe. Hillman et al. [161] found that parathyroid hormone is elevated in pre-term and full-term infants at 48 hours and up to 7 days. The pre-term infants had higher PTH than full term infants. Furthermore, Bishop et al. [162] found that feeding pre-term infants with solutions containing Al compared to Al-depleted solutions is associated with neurological impairment, using the Bayley Scales of Infant Development at 18 months of age. Therefore, Movsas et al. [64] view that urinary Al concentration remained unchanged after injecting 1200  $\mu$ g of Al into the infants is not reassuring when reviewing these papers.

PTH is the major systemic calcium regulating hormone, but it also induces both eNOS expression and eNOS activity, increasing the



production of NO from L-arginine, and therefore, of nitrate [163]. This is likely mediated by the protein kinase A and C pathways. Thus, this is entirely consistent with our prior discussion of a switch on the part of eNOS from sulfate synthesis to nitrate synthesis in association with calcium uptake in a cell. Any disease process that results in elevated PTH potentially renders individuals susceptible to Al toxicity, in part, perhaps even in large part, due to Al's ability to mimic calcium.

### Aluminum adjuvant specificity

Al hydroxide and Al phosphate are the most common adjuvants licensed for use in vaccines in most countries, including the United States. The selection of Al phosphate or hydroxide is based upon the electrostatic properties of the antigen [164]. Al phosphate particulates are known to have a neutral surface charge in a medium with pH of about 5.0, whereas Al hydroxide has a neutral surface charge in a medium with pH of about 11.4. The higher isoelectric point of Al hydroxide can be reduced using phosphate substitution by ligand exchange on the surface of the aluminum particles [165]. The degree of phosphate substitution creates an optimal isoelectric point for the given isoelectric point of a manufactured antigen to maximize electrostatic adsorption [166]. Negatively charged antigen has a higher electrostatic affinity for Alhydroxide particles having a more positive surface charge. Conversely, a positively charged antigen will have a higher electrostatic affinity for Al phosphate particles having a more negative surface charge. Aside from pH specificity, adjuvants are used with a variety of antigens to potentiate immunostimulation. We have not found a basis to assume the adjuvant effect of Al is specific only to manufactured antigens, or an explanation why self-antigens at the injection depot or distant sites of Al biosequestration would somehow be excluded from the effect [19].

Various versions of the DTaP vaccine allow us to examine any differences in the adverse reactions between Al hydroxide and Al phosphate. From comparing reactions to these two variants in the Vaccine Adverse Event Reporting System (VAERS) database, it can readily be seen that Al phosphate favors a systemic reaction (seizures, abdominal pain, diarrhea, nausea, throat irritation), whereas Al hydroxide favors a local reaction (edema and erythema at the injection site). We hypothesize that this difference reflects the fact that Al hydroxide tends to bind to negatively charged membrane-bound sulfates at the injection site, whereas Al phosphate, being negatively charged, is relatively more mobile and migrates through the lymph system to finally infiltrate midbrain centers that control homeostasis, such as the pineal gland.

### Aluminum clearance and kinetics

Crowther and Marriott [167] showed that, on oral ingestion, ions of higher valency, e.g.  $\text{Fe}^{3+}$  and  $\text{Al}^{3+}$  were bound with increasing avidity to a sulfate-bearing glycoprotein component of pig gastric mucosa. Jouhanneau et al. [168] studied the gastrointestinal absorption, tissue retention and urinary excretion of dietary aluminum in rats by using  $^{26}\text{Al}$  and found that (a) the median fraction of  $^{26}\text{Al}$  retained in the brain was  $3.8 \times 10^{-8}$  (range,  $0.8\text{--}6.5 \times 10^{-8}$ ; mean  $\pm$  SD,  $3.7 \pm 1.1 \times 10^{-8}$  ( $n=6$ ), (b) the amount of ingested Al retained by bones in young rats was as great as that excreted in urine, and (c) the accumulation in the skeleton appeared to be relatively permanent.

A very efficient phosphate binder, aluminum hydroxide, was introduced in the seventies as standard phosphate binder therapy in uremic patients receiving dialysis treatment, but was abandoned in favor of calcium-containing phosphate binders because of its *significant*

*negative effects on bone metabolism and cognitive function* [169]. In comparing the pharmacokinetics of aluminum and lanthanum, this group noted that absorption of orally administered aluminum from the gastrointestinal tract amounted to from 0.01% to 0.10%, and that aluminum was mainly eliminated *via* the kidney, with negligible biliary excretion. Also of note, this group found that when Al hydroxide (2.4 g/day) was co-administered with citrate, Al excretion increased from 70 to 120 mg/day up to 350 to 603 mg/day [170].

In a recent pilot study ( $N=15$ ), Movsas et al. [64] found significant declines post vaccination in serum iron (58.1%), manganese (25.9%), selenium (9.5%) and zinc (36.4%) levels, as well as a significant increase in serum copper level (8.0%). These authors noted that the trace elements play important roles in neurodevelopment and the immune system. Zinc and iron are both needed by iNOS, whose increased synthesis likely reflects an acute immune response to Al and would deplete serum stores of these minerals. The selenoprotein, iodothyronine deiodinase (DIO) catalyzes the conversion of thyroxine ( $\text{T}_4$ ) to triiodothyronine ( $\text{T}_3$ ), releasing iodide, which like nitric oxide, is a strong chaotrope [171]. An increased synthesis of DIO to further offset the kosmotropic influence of Al might explain selenium depletion, but these depletions may also represent competition by  $\text{Al}^{3+}$  for binding sites via molecular mimicry of  $\text{Fe}^{3+}$ ,  $\text{Mn}^{2+}$  and  $\text{Zn}^{2+}$ , thereby altering their pharmacokinetics post vaccination. Further research is warranted.

### *In vivo* phosphate substitution as a mechanism for autoimmune stimulation

Although elution of antigen from Al particles at the injection depot had been thought to diminish their efficacy [172], vaccines have been found to remain effective in inducing antibody titers despite desorption [173]. Furthermore, Al adjuvant more effectively potentiates immune stimulation after desorption of antigen in the interstitial fluid than if the antigen remains more strongly adherent to the adjuvant particles [174]. The strength of adsorption is greater by ligand exchange than by electrostatic attraction, and the force of attraction of phosphates to Al hydroxide adjuvant by ligand exchange greatly exceeds that of electrostatic repulsion forces [175]. Al hydroxide and to a lesser degree, Al phosphate, have free hydroxyls and are subject to ligand exchange with phosphorylated antigens [173]. It is conceivable that phosphorylated self-antigens can be substituted by ligand exchange *in vivo*, leading to autoimmune reactions following T-cell activation.

### Adaptations to environmental aluminum and toxic threshold

Although Al constitutes 8% of the earth's crust and is ubiquitous in the environment, living organisms are usually relatively well adapted to survive its toxic properties. Roots of plants have the ability to resist low concentrations of Al in more alkaline soil. This defensive mechanism can be overwhelmed when Al concentration in the soil exceeds a toxic threshold or the soil becomes acidic, following acid rain [176], leading to plant death and removal from the food chain.

Animals and humans, likewise have the ability to resist Al toxicity by ingestion. Ordinarily, gut absorption of Al is 0.1-0.4% [53]. However, this protective mechanism is limited and can be overcome by unnaturally high dietary aluminum, exposures such as Al-containing dialysis fluids, a hyperparathyroid state or concomitant ingestion of oral vitamin D and citrate. Kirschbaum and Schoolwerth [177] reported severe encephalopathy among women with renal failure who were given oral citrate and Al hydroxide as an antacid.

During July of 1988, the drinking water supply in Camelford,

England became contaminated with Al sulfate and many residents became ill [178]. They suffered from loss of concentration, memory loss and poor psychomotor performance [179]. The outbreak of illness that followed was initially dismissed as hysteria and heightened awareness by way of media publicity [180]. However, Altman et al. [179] performed a more rigorous evaluation of 55 affected residents three years after the incident. By comparing the results of psychological testing and visual evoked potentials with fifteen closely age-matched sibling controls living outside the area, they proved that affected individuals suffered cerebral dysfunction not related to anxiety [179]. Bondy [181] reviewed neurotoxicity of environmental Al and cited several epidemiologic reports, associating Al content of drinking water with increasing prevalence of neurological disease. Campbell et al. [157] proposed that long-term low dose oral Al exposure in drinking water that does not necessarily result in acute toxicity is associated with increased inflammatory response in the brain, and that minimal chronic exposure confers long-term risk of age-related neurodegeneration and neuro-inflammatory disease.

Aluminum has been found in pyramidal neurons in hippocampal tissue from confirmed Alzheimer's patients postmortem [182]. Impaired memory and attention disorder developed during old age in rats chronically exposed to aluminum in their drinking water, and the degree of impairment was highly correlated with the percentage of aluminum-loaded pyramidal cells in their entorhinal cortex ( $p < 0.05$ ) [183]. This was associated with an increased synthesis of amyloid precursor protein, a well-established marker of AD [184].

### Aluminum toxicity by inhalational and dermal exposure

Al can also be toxic by way of inhalational exposures. Inhalational exposures typically occur as a result of occupational activity. Inhalational exposures have been reported with Al smelting and among agricultural workers exposed to road dust. Al chlorohydrate is aerosolized in deodorants and can be inhaled. Dermal exposure to Al occurs with use of deodorants [158].

## ASIA: A Unifying Diagnosis (Section 8)

In this section, we will discuss two examples of autoimmune reactions that we believe can be explained by the reaction cascade we presented in the Introduction. We will first describe how three seemingly unrelated conditions, adverse reactions to vaccines, preeclampsia and autism, can be explained by nitrate overload and sulfate depletion subsequent to an acute reaction to Al exposure. Then, we show how physical somatic conditions that are often associated with neurological disease can be explained by a system-wide deficiency in cholesterol sulfate.

### Anaphylaxis, preeclampsia and autism

Anaphylaxis is an allergic reaction associated with severe hypotension as the initiating symptom. It is believed to affect from 1 to 15% of the US population, but the prevalence has increased significantly in recent times [185]. A study on a mouse model of anaphylactic shock used pertussis toxin plus Al hydroxide as the sensitizing agent [186]. Surprisingly, it was identified conclusively that eNOS rather than iNOS was the NOS isoform responsible for the excess synthesis of NO that induces hypotension and the subsequent acute cascade. The authors wrote: "In contrast to the unsubstantiated paradigm that only excessive iNOS-derived NO underlies cardiovascular collapse in shock; our data strongly support the unexpected concept that eNOS-derived NO is the principal vasodilator in anaphylactic shock".

In an example [115], an intricate relationship among preeclampsia, pernicious anemia, autism and acute adverse reactions to vaccines was demonstrated and supported by analyses of the Vaccine Adverse Event Reporting System (VAERS) database maintained by the US Centers for Disease Control. Preeclampsia is a condition characterized by hypertension, proteinuria and elevated serum homocysteine, which develops in the third trimester of pregnancy. Preeclampsia can be life threatening to the mother and the fetus, and is a strong predictor of autism in the fetus. It is commonly treated with magnesium sulfate, and/or heparan sulfate, both of which would help boost sulfate levels in the vasculature.

In the VAERS database, a comparison between reactions that contain mentions of anemia-related symptoms and those that do not reveal that the "anemic profile" in the reaction is predictive of autoimmune symptoms associated with autism, such as eczema ( $P = 0.01$ ) and asthma ( $P = 0.0005$ ), as well as being highly predictive of autism itself ( $P = 0.0007$ ). Seneff et al. [115] argued that excess nitric oxide released into the serum in response to the Al and the antigen leads to a dramatic drop in blood pressure and anaphylactic shock. Hemolysis is a natural *sequitur* and this releases hemoglobin, which can rapidly neutralize the excess NO. The bioavailability of sulfate is greatly reduced due directly to the loss in sulfate supply from both the switch in eNOS from synthesizing sulfate to synthesizing nitrate and the reduced population of sulfate-providing RBCs (via cholesterol sulfate)—the anemia arising from hemolysis. Those who are vulnerable are already deficient in sulfate, such that the added stress of the vaccine induces an acute reaction. The depleted sulfate supply may explain the eczema and asthma, as well as the increased risk of autism, as described above.

Since vitamin D3 synthesis and the metastable state of interphase water of neurolemmal membranes depend upon sunlight stimulus, as does eNOS' synthesis of sulfate [111], insufficient sunlight exposure would lead to impaired vitamin D3 synthesis and impaired sulfate supply. As shown in Table 1, there is a correlation between autism rates in the 50 states of the US and several different parameters related to climate, in such a way that exposure to UV light protects from autism. Autism rates were computed on the basis of data available on the Web at <http://nces.ed.gov/ccd/bat/> for individuals enrolled in the exceptional student education (ESE) autism category in grades 1-6 in 2007, with total student enrolment in grades 1-6 serving as the normalizing factor. These data were compiled according to the U.S. Department of Education (USDE), Individuals with Disabilities Education Act (IDEA). Weather information for the 50 states individually is readily available on the Web.

### Asthma, dermatitis and eosinophilic esophagitis

Aluminum hydroxide attracts eosinophils to the injection site, even in the absence of any antigenic stimulation, a response that is mediated

Parameter	Correlation
Latitude	0.22
Rainfall	0.16
RMS (Rainfall, Latitude)	0.34
Temperature	-0.16
Elevation	-0.28

**Table 1:** Pearson correlation coefficients (Correlation) for various measures of climate for the 50 states in the US compared with autism rates according to the US IDEA data. RMS() is the root mean square (geometric mean) of the two parameters. The larger correlation with autism shows that rainfall and latitude are largely independent (additive) factors. High elevation results in higher exposure to UV, which may be protective against autism.

by T cells [187]. It also elicits and activates IL-4 expressing eosinophils that prime B cell responses to generate antigen-specific IgM [188].

Dysphagia (difficulty swallowing) is a common problem affecting up to 22% of patients in primary care [189], and a characteristic feature of Alzheimer's, Parkinson's and ALS. Eosinophilic esophagitis (EE) is a newly recognized condition as of the mid 1990's [190]. It is characterized by eosinophil infiltration into the esophagus, which is manifested as dysphagia in adults and refractory reflux symptoms in children [191]. There has been an alarming recent increase in the incidence of EE in Western countries [192-195]. Yakoot [195] proposed that EE and allergic bronchial asthma may be two expressions of the same disease in two different organ systems.

EE is associated with a Th2 immune profile and synthesis of the cytokine IL-13, which has direct cytotoxic effects on epithelial cells. Eosinophils are characteristic of a Th2 response, and eosinophil recruitment is mediated by IL-13. Vaccination with formalin-inactivated respiratory syncytial virus (RSV) can lead to enhanced morbidity and mortality following a subsequent natural infection with the virus, due to enhanced eosinophil recruitment [196]. RSV is the leading cause of lower respiratory tract disease in children.

IL-13 down-regulates filaggrin expression in skin keratinocytes [197]. Perturbed barrier function, leading to increased skin permeability, microbial invasion and autoimmune diseases, can be explained by impaired filaggrin expression, and this can lead to increased susceptibility to atopic dermatitis (eczema) [198], EE [199], asthma and various food allergies [198].

Mutations in the gene encoding filaggrin play a significant role in ichthyosis vulgaris, eczema, and in other atopic diseases, such as asthma and allergic rhinitis [200]. Certain single nucleotide polymorphisms [SNPs] increased the risk for eczema by more than 3-fold, and of concurrent asthma. Filaggrin is strongly expressed in the cornified epithelium in the nasal vestibular lining.

Patients with atopic dermatitis have low levels of cholesterol sulfate in the skin, and this is associated with pathological desquamation (skin peeling), characteristic of dermatitis [201]. Mercury poisoning can also cause such desquamation, along with hypertension, failure to thrive and developmental regression [138]. This suggests that mercury may interfere with cholesterol sulfate synthesis in the skin. A case example of contact dermatitis from occupational exposure to Al supports our hypothesis that Al may induce atopic dermatitis via cholesterol sulfate inhibition [202]. Reduced filaggrin synthesis consequential to impaired cholesterol sulfate synthesis likely increases risk to these allergic conditions, especially in genetically susceptible individuals.

The lung epithelium possesses both constitutive and inducible NOS activity, and the synthesis of NOS isoforms is enhanced under inflammatory conditions [107]. Asthma is characterized by epithelial damage in the lung, along with increased cytokine production and increased synthesis of nitric oxide from iNOS, brought on by inflammatory agents [203]. Asthmatic patients produce significantly more nitric oxide in exhaled air compared to controls [204].

## Discussion

In this paper, we have developed a systems-level hypothesis to explain the commonly observed links between immune disorders and neurological disorders. Furthermore, we have implicated chronic and acute aluminum exposures as playing a critical role in the pathology of both of these systems level diseased states. We argue that the initial

exposure of cells localized to the site of an injection containing Al adjuvant can lead to a breakdown in their water-based membrane potential and electrical supply, as well as disrupting their ability to metabolize glucose. Distressed cells launch an immune response cascade, which causes the release of cytokines and inflammatory agents that can be destructive to neighboring cells. Membrane destruction of acutely stressed cells leads to the release of antigenic DNA debris into the tissues, which can eventually lead to autoimmune disease due to activation of T cells [205].

At the molecular biosemiotic level, in Section 4, we have presented a brief overview of a novel hypothesis wherein the onset of immune dysfunction and autoimmune disease is postulated to begin with exposure to EIWS, wherein local "unwetting", "stretching" and hydrophobic "collapse" of interfacial water occurs, and for which considerable support is currently provided by a large and rapidly-growing body of published scientific literature. Macromolecular recognition has been shown empirically to depend critically on biological water dynamics in the 20-40 picosecond timescale. We refer to long wavelength noise or turbulence by sub-nanometer scaled particles as manifestations of EIWS. EIWS is thought to impact multiple biosemiotic levels simultaneously. Biological water is proposed to quantum coherently and fractally mediate the dynamical-coupling between the neuronal networks and their environment on multiple scales of time and space.

While EIWS results on the macro-scale in immune dysfunction and autoimmunity, EIWS results on the micro-scale in disruption of the percolation transition of biological water at the interphase of neuronal membranes, thereby lowering membrane potentials, and in certain circumstances, completely eliminating action potentials. This model of immune dysfunction is based on biological water dynamics at the interphase of neuronal membranes, percolation theory and avalanche mathematics, which require for optimal function, the unique molecular level properties of both (a) sufficient hydration levels, and (b) the sulfoglycolipids and HSPGs at the neuronal membranes, to facilitate the storing of incident radiant energy from sunlight as entropy loss and charge separation [206]. We suggest that such a model will provide a potentially useful biophysical parameter for assessing the criticality of the native metastable critical state of neural function found in the CNS and peripheral nervous systems. We suggest further that there will be proven a strong correlation between loss of self-ordered criticality of biological water, with the polysystemic clinical manifestations described recently by Shoenfeld and others, as ASIA.

At the systems biology level in Section 5, we identified the molecule eNOS as coordinating an intricate balancing between sulfate and nitrate buffering in the blood in order to maintain a healthy ratio between chaotropic and kosmotropic influences. Al, as a strong cationic kosmotrope, is highly disruptive of blood homeostasis in this regard, as well as through its disruption of zeta potential. An important contributor to susceptibility is inadequate sun exposure to both the eyes and the skin, because, as argued [111], sunlight catalyzes the synthesis of sulfate by eNOS.

We have proposed here for the first time to our knowledge, a novel role for the pineal gland in synthesizing sulfate upon sunlight exposure and in transporting this sulfate to various parts of the nervous system via neurotransmitters such as melatonin and dopamine. Sleep disorders are associated with many neurological diseases, such as Alzheimer's disease, Parkinson's disease, autism and depression, and Alzheimer's is associated with both low bioavailability of sulfatide, a sulfated lipid and calcification of the pineal gland, which would impair its ability to



synthesize sulfate. Al accumulates in high concentration in the pineal gland, and this likely relates to calcification. Al gains entry by acting as calcium mimetic, as evidenced by the fact that the depolarization and disruption of microtubules observed in plant roots exposed to Al is prevented by calcium channel blockade [94].

The capacity to produce vitamin D3 in the skin decreases with aging [207], and we believe this can be attributed in part to the impaired ability to produce sulfate because of an increasing Al burden. Sulfate is needed for efficient transport of vitamin D3 and of cholesterol, which is also produced in the skin. We have argued that Al disrupts this function by its biophysical effects on water. The overuse of Al-containing high-sun protection factor (SPF) sunscreens contributes to the problem both by blocking the UV light and by Al's role in disrupting eNOS' sulfate synthesis. Correlations between reduced sun availability and autism rates in the 50 states of the US are consistent with this hypothesis. Impaired sulfate synthesis leads to systemic dysfunction manifested not only as neurological impairment, but also as diverse somatic conditions such as eczema, asthma, impaired gut function, diabetes, kidney disease and heart disease, due to deficiencies in cholesterol sulfate and other sulfated biomolecules. This provides a direct link between somatic and neurological aspects of autoimmune diseases.

Depending on a combination of genetic predisposition and the cumulative burden of environmental toxic exposures, the brain may or may not be spared when sulfate supplies become deficient. Even within the brain, it depends on which parts of the brain are most affected as to which neurological disease will emerge. Parkinson's disease defects are mostly concentrated in the *Substantia nigra* (the source of dopamine) [208], whereas Alzheimer's affects mainly the cortex, at least initially [209], and ALS may focus on the motor neurons in the spinal cord, brain stem and motor cortex [210]. However, all of these conditions have somatic complications that are explained by deficiencies in sulfate and by excessive activation of calcium phosphate pathways through an overactive parathyroid gland.

As discussed in Section 5, an increase in bone fragility and parathyroid function follows directly from vitamin D3 insufficiency [207]. We propose that this is due directly to the need to replace sulfate with phosphate as an ionic kosmotrope for maintaining water homeostasis in the cells. Patients suffering from hyperparathyroidism have a higher incidence of impaired glucose tolerance, along with elevated serum levels of calcium [211]. These are connected by the fact that excessive PTH leads to a leaching of calcium phosphate from the bones in order to supply it to the tissues as a substitute for magnesium sulfate [212]. Sulfate supply is depleted due to the interference of toxic chemicals like Al on sulfate synthesis and sulfate transport [213]. In addition, sulfate depletion then leads to glucose intolerance due to the important role sulfate plays in the storage of glucose in the extracellular matrix [111].

In this article, we have demonstrated the multiple deleterious roles that Al plays across all levels of organization, beginning at a molecular level and culminating in systems-wide dysfunctions. Of particular relevance for the etiology of CNS disorders, Al acts directly to alter neural cell function. As well, Al disturbs immune function, and thus indirectly attacks the nervous system through autoimmune actions. The combined weight of these two actions may explain the diverse forms of many developmental and age-related neurological diseases. These observations may provide more than sufficient reasons to consider how we can limit human exposure to this element from whatever source. Of particular concern in this regard is to limit the exposure to the most vulnerable populations: the very young and the very old.

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## Review Article

# Aluminum-Induced Entropy in Biological Systems: Implications for Neurological Disease

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Over the last 200 years, mining, smelting, and refining of aluminum (Al) in various forms have increasingly exposed living species to this naturally abundant metal. Because of its prevalence in the earth's crust, prior to its recent uses it was regarded as inert and therefore harmless. However, Al is invariably toxic to living systems and has no known beneficial role in any biological systems. Humans are increasingly exposed to Al from food, water, medicinals, vaccines, and cosmetics, as well as from industrial occupational exposure. Al disrupts biological self-ordering, energy transduction, and signaling systems, thus increasing biosemiotic entropy. Beginning with the biophysics of water, disruption progresses through the macromolecules that are crucial to living processes (DNAs, RNAs, proteoglycans, and proteins). It injures cells, circuits, and subsystems and can cause catastrophic failures ending in death. Al forms toxic complexes with other elements, such as fluorine, and interacts negatively with mercury, lead, and glyphosate. Al negatively impacts the central nervous system in all species that have been studied, including humans. Because of the global impacts of Al on water dynamics and biosemiotic systems, CNS disorders in humans are sensitive indicators of the Al toxicants to which we are being exposed.

## 1. Introduction

Aluminum (Al) is the most common metal and the third most abundant element in the earth's crust [1–3]. However, it seems to have no beneficial role in the biochemistry of any biota [1]. Until the 1820s when the industrial extraction of Al, primarily from bauxite ore [4], made it possible to bring Al into food processing, manufacturing, medicines, cosmetics, vaccines, and other applications, Al was almost completely absent from the biosphere [5]. Concerns about the

toxicity of ingesting Al were expressed over 100 years ago [6]. Today, biologically ingested or injected forms include salts of Al in processed foods [7] and medicinal products [8] such as antacids, glossy coatings for pills, and vaccine *adjuvants*. The last use, which portrays Al compounds as “helpers”—the English translation of the Latin root of *adjuvants*—is supposed to shock the recipient's immune defenses into action, ostensibly to enhance the immunogenicity of the pathogen(s) in the vaccine(s) [9]. Al salts are also found in dyes [10], cosmetics [5], antiperspirants [11–14], sunscreens

[15, 16], and thousands of material products including foils, food containers, and utensils.

In this paper, we will show that Al is harmful to the CNS, acting in a number of deleterious ways and across multiple levels, to induce biosemiotic entropy [17]. A countervailing view exists [18–20], but the assertions of safety are invariably based on weak epidemiological designs, ones that overwhelm significant negative signals with irrelevant noise factors. Such studies that fail to detect significant negative outcomes neither stand up to rigorous scrutiny nor outweigh better designed research, in a vast and growing literature, showing significant negative impacts sustaining the central hypothesis of this paper. Irrefutable research evidence shows that Al exposure is harmful. Further, results discussed in this paper show that it is counterfactual for researchers to argue that Al is universally safe or beneficial even in trace amounts.

Al is used extensively in food processing, for example, in Al-mordanted dye lakes for food coloring, in coatings for pharmaceutical tablets and vitamin capsules, for emulsifying, as a rising agent, to thicken gravies, and in meat-binders, stabilizing agents and texturizers [18]. Even drinking water is a source of Al exposure, although the amount contained in drinking water is typically far below concentrations in common antacids [21]. However, there is concern that the Al in drinking water may be more easily absorbed than at mealtime, due to the fact that an empty stomach promotes absorption [21]. Alum (Al sulfate or Al potassium sulfate) is commonly used in water treatment plants as a coagulant to allow negatively charged colloidal particles to clump together for easy removal. Epidemiological studies have shown that people living in districts with higher Al burden in drinking water are more likely to be diagnosed with Alzheimer's disease [22].

Because tea plants contain a higher concentration of Al than many other plants, and, because tea beverages are consumed in large quantities worldwide, a high incidence of Al exposure comes through drinking tea [23]. Al content in tea ranges from 2 to 6 mg/L [24]. Tea infusions have been analyzed for the speciation of Al content, and it has been determined that it is typically bound to large organic molecules such as polyphenols or to citrate [24, 25]. Tea typically contains much more Al than water, and so tea becomes a significant source of Al for heavy tea drinkers. An experiment to estimate oral Al bioavailability from tea involving 8 rats was conducted by injecting Al citrate into tea leaves, delivering approximately the same amount of Al as is inherently found in tea leaves (0.5 to 1 mg/gm) [26]. The brewed tea was administered through intragastric infusion. Following infusion, peak serum levels of Al were up to 1500-fold above mean pretreatment values.

In a substantial and recent review of research, Walton [27] concludes that Alzheimer's disease is a manifestation of chronic Al neurotoxicity in humans. Because Al is similar to iron, it gains access to iron-dependent cells involved in memory. As it accumulates over time in such cells, it causes microtubule depletion and disables neuronal afferents and efferents resulting in the multiregion atrophy characteristic of Alzheimer's pathology [27]. Table 1 highlights some of the Al compounds to which humans are commonly exposed which

are known to have deleterious effects on the central nervous systems (CNS) of both animals and humans [28], whereas Tables 2 and 3, respectively, present Al intake data, and its physical properties compared to other metals. Table 1 also shows dosage and known effects of each source on animals and/or humans.

Al in all of the forms studied, as Table 1 shows, produces harmful effects in living organisms: it especially harms the CNS. In studies involving *in vitro* cultures of neuronal-glial cells, the ROS-generating capabilities of several physiologically relevant neurotoxic factors were compared [29, 30]. It was found that Al-sulfate was the most potent single metal sulfate inducer of ROS, as well as the most potent combinatorial inducer in conjunction with Fe. Nanomolar concentrations of Al were sufficient to induce ROS and proinflammatory gene expression. Nanomolar concentrations of Al-sulfate upregulated the expression of several genes implicated in Alzheimer's disease, including proinflammatory and proapoptotic gene expression [30].

Given the fact that there are no known biochemical reactions that require Al, should it be surprising that introducing it into living organisms commonly leads to pathological outcomes [31–46]? Because of its +3 charge, Al attracts negatively charged ions and electrons, but because it cannot transition to other oxidation states besides +3, it is not a component in any redox reactions. Oxygen, carbon, hydrogen, nitrogen, calcium, and phosphorous constitute 99% of human body mass, with the remaining 1% consisting of potassium, sulfur, sodium, chlorine, and magnesium, as well as trace elements such as fluorine, selenium, and zinc, and xenobiotic (biologically foreign and usually toxic) elements such as titanium, mercury, and lead [47]. Thus, Al can end up in many biochemical contexts in theory, but in fact some atoms and molecules are far more likely to react with Al compounds [48]. Among the most vulnerable molecules are those most directly involved in self-ordering, self-assembling systems of biosemiotics that work like multilayered, interrelated languages. The best known macromolecules that are susceptible to minute but often disabling injuries by Al compounds are DNA molecules that must be translated via the assistance of a growing multitude of RNA molecules into proteins. The latter in turn are essential to the structure and functions of the whole society of cells [49], tissues, and organ systems. Formerly, it was thought, following the Crick dogma [50], that communications were essentially a one-way street from DNA to RNA to protein, but it has more recently been argued [17, 51, 52] that communications involve more complex bidirectional interactions among those macromolecules, such that the genome is informed concerning what is going on in the environment. The dynamical matrix of negative charge densities in heparan sulfate proteoglycans (HSPGs), as modulated in time and space by interfacial water, exchanging between the first few solvation layers and bulk, might prove to be the supramolecular physical basis for *informing* the genome over distance [53].

There are estimated to be 20,000–25,000 protein coding genes in the human genome [54] and even more variant proteins possible through posttranslational modifications estimated to be upwards of 100,000. Thus there are many



Table 1: Common sources of Al compounds and their immunoneurotoxicological effects in humans and animals.

Aluminum source/compound	Dose & duration	Route	Species	Adverse effects
Standard infant feeding solution	~20 µg/kg/day; >10 days	Intravenous (parenteral)	Human, premature infants	Reduced developmental attainment at the corrected post-term age of 18 months, as evidenced by significantly lower Bayley Mental Development Index (BMDI) scores (mean loss of one point on the BMDI/day of full intravenous feeding, after adjustment for potentially confounding factors) compared to infants fed with Al-depleted solutions [31].
Al-containing dialysis fluid (derived from Al-sulfate treated tap water)	1ppm, chronic (2–5 years)	Intravenous	Human, dialysis patients (15–61 years old at the start of the dialysis treatment)	Speech impairments (stuttering, dysarthria, dyspraxia, and motor aphasia), movement disorders (twitches, tremors, myoclonic jerks, seizures, and motor apraxia), cognitive impairments and behavioural changes (progressive dementia, paranoia, confusion, and psychosis), and death [32].
Al-containing antacids	Chronic	Oral	Human infants	Craniosynostosis (premature ossification of the skull and obliteration of the sutures) [33].
Various dietary	Chronic	Oral	Elderly human subjects	Impaired visuomotor coordination, poor long-term memory, and increased sensitivity to flicker (correlated with high Al-serum levels) [34].
Al sulfate (present as flocculant in potable water supplies, accidentally released in high amounts)	500–3000 x the acceptable limit under European Union Legislation (0.200 mg/L), chronic (15 years)	Oral	Human adult (female, 44 years old)	Sporadic early-onset $\beta$ amyloid angiopathy (Alzheimer's-related disease), difficulty in finding words, progressive dementia, visual hallucinations, headache, anxiety, cerebral ischemia, and death [35].
Al-containing food pellets	0.5–1.7mg/kg/day (typical human), chronic (22–32 months)	Oral	Rats, 6 months old at the start of treatment	Cognitive deterioration and impaired performance in learning tasks, impaired concentration, and behavioral changes including confusion and repetitive behaviour [36].
Al lactate	500–1000 ppm, chronic (during gestation and lactation)	Oral	Mice dams	Hind limb paralysis, seizures, and death (dams), lower neurobehavioral development and altered performance on a neurobehavioral test battery in pups (foot splay, forelimb, and hind limb grip strengths reduced) [37].

Table 1: Continued.

Aluminum source/compound	Dose & duration	Route	Species	Adverse effects
Al hydroxide as a vaccine adjuvant	1–17 doses of Al-containing vaccines (hepatitis B, hepatitis A, and tetanus toxoid) in the period of 10 years prior to disease diagnosis	Intramuscular injection	Human adult macrophagic myofasciitis (MMF) syndrome patients (mean age 45 years)	MMF typical clinical manifestations: myalgia, arthralgia, chronic fatigue (disabling fatigue >6 months), muscle weakness and cognitive dysfunction (overt cognitive alterations affecting memory, and attention manifested in 51% of cases) [38–41]. Typical histopathology: presence of granulomatous myopathological lesion comprised of Al-hydroxide-loaded macrophages at the site of vaccine injection (usually deltoid muscle); persistence of Al long-term, up to 8–10 years in postinjection mice [38, 39, 42]. 15–20% MMF patients concurrently develop an autoimmune disease, most frequently being multiple sclerosis-like demyelinating disorders, Hashimoto's thyroiditis, and diffuse dysimmune neuromuscular diseases (i.e., dermatomyositis, necrotizing autoimmune myopathy, myasthenia gravis, and inclusion body myositis); even in the absence of overt autoimmune disease, low titres of autoantibodies, increased inflammatory biomarkers, and abnormal iron status commonly detected in exposed mice [38, 39].
Al hydroxide as a vaccine adjuvant	14 injections over a 6-month period	Subcutaneous	Sheep, male 3 month old lambs	“Sheep adjuvant syndrome” first identified following mass-vaccination for bluetongue; experimentally reproduced by repetitive injection with Al-containing vaccines [14]; observed in acute form (affecting 25% of exposed flocks, 0.5% animals within a flock) and chronic phase form (affecting 50–70% of all exposed flocks and up to 100% of animals within a given flock). Acute phase symptoms: lethargy, reluctance to move, bruxism (teeth grinding), transient blindness, nystagmus (rapid abnormal eye movements), stupor, abnormal behavior, disorientation, and a low response to external stimuli, seizures, and occasionally death; histopathological lesions mainly consisting of acute meningoencephalitis (similar to those observed in humans postvaccination) and demyelinating foci Chronic phase symptoms: severe neurobehavioral outcomes including restlessness, compulsive wool biting, generalized weakness, muscle tremors, loss of response to stimuli, ataxia, tetraplegia (paralysis of all four limbs), stupor, coma, and death. Inflammatory lesions (multifocal neuronal necrosis and neuron loss in both dorsal and ventral column of the gray matter) and presence of Al in CNS tissues [43].



Table 1: Continued.

Aluminum source/compound	Dose & duration	Route	Species	Adverse effects
Al hydroxide as a vaccine adjuvant	2 injections, 2 weeks apart	Subcutaneous injection (behind the neck)	Mice, 3 months old CD-1 male	Motor neuron degeneration and apoptosis, motor function deficits, decrease in strength, cognitive deficits, and decreased performance in learning tasks, decrements in spatial memory, activation of microglia [44, 45].
Al oxide fumes, occupational exposure	0.13–1.95mg/m <sup>3</sup> , chronic	Inhalation	Human, adults (mean age 39 years)	Headache, emotional irritability, concentration difficulty, insomnia, mood lability [46].

macromolecules with which  $\text{Al}^{3+}$  species can interact, either directly or indirectly. Eukaryotic proteins are polymers of various combinations and lengths consisting of an array of 23 amino acids joined by peptide bonds. Each of the 23 amino acids has a unique side chain consisting of various organic substituents. Al can interact with the side chains [55], some of which—serine, threonine, and tyrosine—are phosphorylated, enabling phosphoregulation of enzyme activity and binding with other proteins. Al can disrupt all of these side chains and the processes dependent on them [56]. Cysteine, methionine, homocysteine, and glutathione contain sulfur, and they are intermediaries instrumental in methylation and transsulfuration pathways, as well as in heavy metal detoxification. These processes can be disrupted by Al [57] because of the strong binding affinity of Al with sulfur oxyanions. Glutamic and aspartic acids have negatively charged carboxylate side chains. Al has a much stronger binding affinity to these side chains, for instance, than the nontoxic cation, magnesium [58].

Therefore, Al is ineffective in redox reactions, though its +3 charge makes it likely to adsorb to suspended colloids (e.g., complex proteinaceous polymeric molecular structures or clusters suspended in fluid) in nonliving systems, resulting in its kosmotropic character (see Table 4), which enables the salting-out known as “floculation.” This useful tendency, for example in public water systems, can, however, be catastrophic in the blood and fluids of living organisms, where building blocks of necessary proteins are apt to be turned into useless debris linked to Al salts [59, p. 1410] and [60]. According to its Lewis acidity classification [61],  $\text{Al}^{3+}$  belongs in Class A, a small (hard) metal ion with low polarizability (deformability), preferentially forming ionic complexes with similar nonpolarizable ligands, particularly oxygen donors such as oxyanions of carbon, phosphorus, and sulfur—all of which are plentiful in living organisms—giving Al the potential to wreak havoc in living systems. For these reasons, Al is certainly not “inert,” nor is it biologically harmless [29–48]. As Table 1 shows, Al is causally linked to disorders in plants, animals, and humans [9, 28, 57], especially in the CNS of animals and humans.

Among the CNS problems in humans attributed to Al are dialysis associated encephalopathy (DAE) [32, 62], autism spectrum disorders [9, 63, 64], Alzheimer’s disease, Parkinson’s disease, and related dementias [28, 36] including those typical in Down syndrome [18]. Experimental and clinical data show the CNS as the most sensitive organ system negatively impacted by Al. Toxic effects manifest in impaired psychomotor control, altered behavior (i.e., confusion, anxiety, repetitive behaviors, sleep disturbances, deficits of speech, concentration, learning, and memory), and in potentially fatal seizures [18, 28, 38]. Al has been identified as the efficient cause of a whole class of immune dysfunctions directly involving the CNS and known as “autoimmune-inflammatory syndrome induced by adjuvants” (ASIA) [65–68]. As will be seen in this paper, the disorders with which Al has been associated as a causal factor are pervasive because they begin with the disruption of fluid structures involving water. Also, although Al negatively affects every layer of the body’s biosemiotic systems, on which health depends,

the symptoms of Al poisoning are often noticed when they inevitably reach and impact the CNS.

*1.1. Aluminum in the Nervous System.* As Table 2 shows, humans get about 95% of their Al burden from food [69] though estimates vary between 2 and 25 mg per day amounting to 14–175 mg per week [70–73]. In urban societies, the intake can exceed 100 mg per day, between 4 and 50 times the averages shown in Table 2. Because of increasing consumption of Al-containing convenience foods [74], in 2006, the Food and Agriculture World Health Organization Joint Expert Committee on Food Additives (FAO/WHO-JECFA) amended their provisional tolerable weekly intake (PTWI) for Al from 7 mg per kilogram of body weight (amounting to 490 mg per week for an average 70 kg human) to 1/7 of that amount. The Committee concluded that “aluminum compounds have the potential to affect the reproductive system and developing nervous system at doses lower than those” previously supposed [74]. Interpreting the averages in Table 2, using the estimated intake in urban settings as the higher end of the actual range, referring to the supposedly tolerable weekly intake based on the post-2006 numbers, average consumers weighing 70 kilograms are consuming between 2 to 100 times the provisionally estimated safe amounts of Al.

Given that severe toxic effects of Al occur in animal models at a concentration of 1.5 to 5 mg/kg of wet weight, independent of the mode of administration [75], it may be inferred that lethal poisoning of humans can occur at about 3–10 times the average amounts estimated to be absorbed by adult consumers studied. This leaves a narrow margin between the estimated average uptake and the lethal threshold of Al in the human CNS. Experiments on cats involved injecting Al into the brain and monitoring the response both behaviorally and physiologically [76]. Measured tissue levels of Al averaging 14 micrograms/gram were associated with extensive neurofibrillary tangles, which are a common feature of AD. This level is only marginally higher than the 9–11 micrograms/gram that have been detected in some regions of AD brains postmortem. This physiological effect was associated with observed impairment in short-term memory and acquisition of a conditioned avoidance response [77]. Al also causes a condensation of brain chromatin disrupting DNA transcription [78]. Animal models of neurological disease plainly suggest that the ubiquitous presence of Al in human beings implicates Al toxicants as causally involved in Lou Gehrig’s disease (ALS) [44, 45], Alzheimer’s disease [20, 21, 28] and autism spectrum disorders [9, 63].

*1.2. The Toxic Effects of Aluminum as a Vaccine Adjuvant.* Al salts (hydroxide and phosphate) are the most commonly used vaccine adjuvants and, until recently, the only adjuvants licensed for use in the USA [79–89]. In the absence of Al, according to their manufacturers, antigenic components of most vaccines (with the exception of live attenuated vaccines) fail to elicit the desired level of immune response [66, 80]. Although Al is neurotoxic, it is claimed by proponents that the concentrations at which Al is used in the vaccines do not

Table 2: Estimates of daily and weekly intakes of Al in humans [28, 74].

Major sources of Al exposure in humans	Daily Al intake (mg/day)	Weekly Al intake (mg/day)	÷PTWI <sup>†</sup> (1mg/kg/bw; for an average 70 kg human PTWI = 70 mg)	Amount delivered daily into systemic circulation (at 0.25% absorption rate)
Natural food	1–10 [2, 8, 23–26]	7–70	0.1–1	2.5–25 $\mu\text{g}$
Food with Al additives	1–20 (individual intake can exceed 100) [3, 5, 18]	7–140 (700)	0.1–2 (10)	2.5–50 $\mu\text{g}$ (250 $\mu\text{g}$ )
Water	0.08–0.224 [2, 8, 21]	0.56–1.56	0.008–0.02	0.2–0.56 $\mu\text{g}$
Pharmaceuticals (antacids, buffered analgesics, antiulceratives, and antidiarrheal drugs)	126–5000 [1, 2, 8]	882–35,000	12.6–500	315–12,500 $\mu\text{g}$
Vaccines (HepB, Hib, Td, DTP)	0.51–4.56 [9]	NA	NA	510–4560 $\mu\text{g}$ <sup>‡</sup>
Cosmetics, skin-care products, and antiperspirants <sup>§</sup>	70 [1, 9]	490	NA	8.4 $\mu\text{g}$ (at 0.012% absorption rate) [10, 11]
Cooking utensils and food packaging	0–2 [2]	0–14	0–0.2	0–5 $\mu\text{g}$

<sup>†</sup> PTWI (provisional tolerable weekly intake) is based on orally ingested Al, generally only 0.1–0.4% of Al is absorbed from the GI tract, however, Al may form complexes with citrate, fluoride, carbohydrates, phosphates, and dietary acids (malic, oxalic, tartaric, succinic, aspartic, and glutamic), which may increase its GI absorption (0.5–5% [70, 82]). Coexposure to acidic beverages (lemon juice, tomato juice, and coffee) also increases Al absorption as well as conditions of  $\text{Ca}^{2+}$ ,  $\text{Mg}^{2+}$ ,  $\text{Cu}^{2+}$ , and  $\text{Zn}^{2+}$  deficiency [70, 83–85].

<sup>‡</sup> A single dose of vaccine delivers the equivalent of 204–1284 mg orally ingested Al (0.51–5.56 mg), all of which is absorbed into systemic circulation [86, 91]. Al hydroxide, a common vaccine adjuvant has been linked to a host of neurodegenerative diseases; it also induces hyperphosphorylation of MAP tau *in vivo* [44, 45, 87].

<sup>§</sup> The risk of antiperspirants is both from dermal exposure and inhalation of aerosols. Al is absorbed from the nasal epithelia into olfactory nerves and distributed directly into the brain [88, 91].

Table 3: A comparison of the physical properties of metallic Al with those of its common competitors in biological systems [89]. Crystal ionic radius source: [92]. Magnetic susceptibilities source: [47, pp. 4-131 to 4-136]. Viscosity  $B$  coefficient source: [93]. Standard molar electrostriction volume source [94].

	Mg	Al	Ca	Mn	Fe	Co	Zn
Atomic number	12	13	20	24	25	27	30
Electron configuration	$[\text{Ne}]3s^2$	$[\text{Ne}]3s^23p^1$	$[\text{Ar}]4s^2$	$[\text{Ar}]4s^23d^5$	$[\text{Ar}]4s^23d^6$	$[\text{Ar}]4s^23d^7$	$[\text{Ar}]4s^23d^{10}$
Ionization energies (kJ/mol)	737.7 1450.7 [7732]	577.5 1816.7 2744.8 [11577]	589.8 1145.4 [4912.4]	717.3 1509 [3248]	762.6 1561.9 [2957]	760.4 1648 [3232]	906.4 1733.3 [3833]
Crystal ionic radius (pm)	86	67.5	114	97	92	135	88
Electron affinity (kJ/mol)	0	42.5	2.37	0	15.7	63.7	0
Electronegativity (eV)	1.31	1.61	1.0	1.55	1.83	1.88	1.65
Magnetic susceptibility ( $X_m/10^{-6} \text{ cm}^3 \text{ mol}^{-1}$ )	+13.1	+16.5	+40	+511	<i>Ferro-magnetic</i>	<i>Ferro-magnetic</i>	−9.15
Charge density (coulombs·mm <sup>−1</sup> )	120.1	372.6	51.6	143.7	98.1	154.9	112.1
Viscosity $B$ Coefficient (dm <sup>3</sup> mol <sup>−1</sup> , 298.15K)	0.385	0.75	0.289	0.390	0.42	0.376	0.361
Standard molar electrostriction volume ( $-\Delta_{\text{elstr}} V_i / (\text{cm}^3 \text{ mol}^{-1})$ )	52.5	59.3	38.5	30.7	—	38.5	—

Table 4: Summary comparisons of chaotropic versus kosmotropic ions.

Chaotropes (water-structure breakers)	Kosmotropes (water-structure makers)
Typically larger radius, singly charged ions with low charge density	Typically small radius, often multiply charged ions with high charge density
Interact more weakly with waters than water molecules interact with each other	Interact more strongly with waters than water molecules interact with each other
Interfere little with hydrogen bonds of the surrounding waters	Capable of weakening and breaking hydrogen bonds of the surrounding waters
Decrease surface tension	Increase surface tension
Reduce viscosity	Increase viscosity
Increase nonpolar solubility	Decrease nonpolar solubility
Unfold proteins	Stabilize proteins
Destabilize hydrophobic aggregates	Stabilize hydrophobic aggregates and bonding
Increase solubility of hydrophobic solutes	Reduce solubility of hydrophobic solutes
Salt in proteins	Salt out proteins
Net positive entropy of ion solvation	Net negative entropy of ion solvation

represent a health hazard [19]. For that reason, vaccine trials often treat an Al adjuvant-containing injection as a harmless “placebo” (a comparison benchmark or control treatment) or they use another Al-containing vaccine to treat a “control group,” despite evidence that Al in vaccine-relevant exposures is universally toxic to humans and animals [9, 90, 91]. Its use in a supposed “placebo” or in any “control” treatment in vaccine trials is indefensible [95]. It is precisely analogous to comparing fire A against fire B, to make the argument that since A is no hotter than B, A is therefore not a fire.

During the last decade, studies on animal models and humans have shown that Al adjuvants by themselves cause autoimmune and inflammatory conditions [19, 79–81, 90, 95–103]. The animal models show that subcutaneous injections of Al hydroxide induced apoptotic neuronal death and decreased motor function in mice [2, 37–39] and sheep [43]. In newborn mice they were associated with weight increases, behavioral changes, and increased anxiety [2]. All these findings plausibly implicate Al adjuvants in pediatric vaccines as causal factors contributing to increased rates of autism spectrum disorders in countries where multiple doses are almost universally administered [9]. Also, as shown by Goldman and Miller in studies published in 2011 and 2012, strong correlations between infant mortality rates and the number of doses of vaccines administered also suggest deleterious impact of multiple exposures to their components [104, 105].

Follow-up experiments focusing on Al adjuvants in mice by Khan et al. [106] have shown that the adjuvants do not stay localized in the muscle tissue upon intramuscular injection. The particles can travel to the spleen and brain where they can be detected up to a year after the injection. Such findings refute the notion that adjuvant nanoparticles remain localized and act through a “depot effect.” On the contrary, the Al from vaccine adjuvants does cross the blood-brain and blood-cerebrospinal fluid barriers and incites deleterious immunoinflammatory responses in neural tissues [1–3, 9]. Tracking experiments in mice reveal that some Al

hydroxide nanoparticles escape the injected muscle inside immune system cells such as macrophages, which travel to regional draining lymph nodes, where it can exit to the bloodstream gaining access to all organ systems, including the brain. As Khan et al. [106] have warned, repeated doses of Al hydroxide are “insidiously unsafe,” especially in closely spaced challenges presented to an infant or a person with damaged or immature blood brain or cerebrospinal fluid barriers [2]. Given macrophages acting as highly mobile “Trojan horses” [107], the Khan et al. warning suggests that cumulative Al from repeated doses in vaccines can produce the cognitive deficits associated with long-term encephalopathies and degenerative dementias in humans [40, 99].

The latest research by Luján et al. [43] described a severe neurodegenerative syndrome in commercial sheep linked to the repetitive inoculation of Al-containing vaccines. In particular, the “sheep adjuvant syndrome” mimics in many aspects human neurological diseases linked to Al adjuvants. Moreover, the outcomes in sheep were first identified following a mass-vaccination campaign against blue tongue and have now been successfully reproduced under experimental conditions following administration of Al-containing vaccines. Notably, the adverse chronic phase of this syndrome affects 50–70% of the treated flocks and up to 100% of the animals within a given flock. The disorder is made worse by cold weather conditions, suggesting synergy with other stress producing factors. The disorder is characterized by severe neurobehavioral outcomes—restlessness, compulsive wool biting, generalized weakness, muscle tremors, loss of response to stimuli, ataxia, tetraplegia, stupor, inflammatory lesions in the brain and the presence of Al in the CNS tissues, coma, and death [43]. These findings confirm and extend those of Khan et al. [106] who demonstrated the ability of Al adjuvants to cross the BBB, and they show that Al in the brain can trigger severe long-term neurological damage. The findings by Luján et al. [43] and Khan et al. [106] also show how and why reported adverse reactions

following vaccinations are most commonly neurological and neuropsychiatric [6, 7].

**1.3. Aluminum Disrupts Biosemiosis.** The nervous system utterly depends on coherent signaling from the genome upward to psychological and social behaviors and is suited to induce entropy at these and the levels in between them. The long-term consequences involve many minute injuries, leading to inflammation, disorders, diseases, and the ultimate death of certain neuronal elements and possibly of the whole organism. As documented by Gryder et al. [108] in reference to cancer, disruptions in gene signaling and/or RNA transcription mechanisms induce a range of deleterious outcomes on protein formation. In turn, altered proteins impact cellular function. As Al moves in the body and CNA, it can create dysfunctional cells that foul signaling systems and neural circuits leading to additional dysfunctions and even behavioral aberrations. Immediately and cumulatively, Al-induced injuries tend to be expressed as abnormalities in the CNS trending toward ultimate fatality [109].

## 2. Biophysics of Aluminum Toxicity and Impact on Cellular Processes

The concepts of kosmotropic and chaotropic solutes (water structure makers and breakers), introduced by Collins and Washabaugh in 1985, have been used extensively by the biochemical and biophysical communities [110]. These concepts are highly relevant to this section. The reader is referred to Table 4 (above) for a summary of the concepts. According to Marcus (2012), when “the structural entropy according to [Barthel and] Krestov (1991) was compared by Collins (1997) to the entropy of pure water...for the alkali metal and halide ions, and  $\Delta S = \Delta_{\text{struc}}S - S^*(\text{H}_2\text{O})$ . Those with  $\Delta S < 0$  have large surface charge densities and are called kosmotropes (water structure making) whereas those with  $\Delta S > 0$  have small surface charge densities and are chaotropes (water structure breaking)” [111–113].

**2.1.  $\text{Al}^{3+}$  Disrupts Water Dynamics of Biological Exclusion Zones.** Al is a reactive element existing abundantly in nature but almost exclusively bound as mineral salts. Al salts are relatively insoluble except under acidic conditions, which are created by organic acids *in vivo* and adjacent to the exclusion zones (EZs) of biomembranes [114]. Concerning EZs, as argued by Ling [115] (also see his references), “under an ideal condition, an idealized checkerboard of alternatingly positively, and negatively charged sites of the correct size and distribution could polarize and orient deep layers of water molecules *ad infinitum*. Based on the quantitative data thus obtained and a relevant simple statistical mechanical law, the new theory predicts that a thin layer of water held between two juxtaposed ideals or near-ideal nanoprotoplasm (NP) surfaces will not freeze at any (attainable) temperature. On the other hand, water polarized and oriented by an ideal or near-ideal NP-NP system may also not evaporate at temperature hundreds of degrees higher than the normal boiling temperature of water” (p. 91). However, as Ling

has also shown, Al has the power to alter these crucial EZs, disrupting their unique biophysical properties [116]. Or, as argued more recently by Davidson and colleagues, toxicants such as Al are invariably disposed to contribute to exogenous interfacial water stress (EIWS) in the critical EZs precipitating in vast numbers of minute toxic injuries, and leading to disorders, diseases, and sometimes catastrophic changes ending in fatalities [57, 59, 68, 117–119]. Concerning the many ways that toxicants in both their near and distant effects can increase biosemiotic entropy also (see arguments developed by Oller [17, 51], Gryder et al. [108], and Ho [52]). Shaw et al. (2013) have also presented data showing that biological water dynamics crucially enable quantum coherence across all biosemiotic systems [68].

**2.2.  $\text{Al}^{3+}$  Speciation, Solubility, and Adsorption Are pH-Dependent.** Conventional beliefs about Al safety [19] are rooted in the knowledge that, in the absence of citrate, insoluble Al compounds are poorly absorbed even if ingested [91]. However, the fact that Al hydroxide and phosphate solutions remain nearly saturated at neutral pH and standard temperature in pure water suggests that their poor solubility does not make them benign in living systems. Many other ligands besides water molecules can interact with Al when it is inhaled, ingested, topically absorbed, or parenterally injected. Acidic beverages such as soft drinks have a pH < 3; most fruit drinks have a pH < 4. Al in drinking water in concert with chemical agents that literally pull it out like claws—as suggested by the term *chelation*—can increase gastrointestinal absorption [107] and thus the biosemiotic entropy-inducing tendency of Al. Moreover, precipitates of Al need not be soluble to be toxic, especially in low pH compartments, *in vivo*, which favor more mobile hydrated  $\text{Al}^{3+}$  aqua ion,  $[\text{Al}(\text{H}_2\text{O})_6]^{3+}$ , as opposed to inner sphere contact ion pairs. According to Martin, the octahedral hexahydrate  $[\text{Al}(\text{H}_2\text{O})_6]^{3+}$  dominates at pH < 5, and the tetrahedral  $[\text{Al}(\text{OH})_4]^-$  at pH > 6.2, while there is a mixture of species from 5 < pH < 6.2 [120, p. 12]. Adsorption and desorption of  $\text{Al}^{3+}$  species have long been known to demonstrate pH dependence [121–122]. The aluminum aqua ion,  $[\text{Al}(\text{H}_2\text{O})_6]^{3+}$ , is well characterized in solution and the solid state [123]. In 1994, Marcus provided data indicating that, while  $[\text{Al}(\text{H}_2\text{O})_6]^{3+}$  behaved like a typical strong kosmotrope, with a negative structural entropy value and enhancement of the H-bond structure of water,  $[\text{Al}(\text{OH})_4]^-$  demonstrated the properties of a chaotrope, with a positive structural entropy value and lessening of the H-bond structure of water [93]. Thus, it is clear from these data that pH has a major influence in determining the speciation, solubility, adsorption, and Hofmeister behavior [58, 59] of Al *in vivo*.

**2.3. Glyphosate—A Ubiquitous  $\text{Al}^{3+}$  Chelating Agent.** Being a modified form of glycine with both phosphonyl and carbonyl groups, glyphosate is already known to chelate metal cations [124]. Moreover, Al caged by glyphosate dimers and trimers [125] bears a certain resemblance to chelation complexes of Al citrate. Given its biocidal effects on gut biota [126, 127], leading to inflammatory intestinal disorders commonly



treated by Al-containing antacids [128], Al interacting with glyphosate is likely to increase its crossing of the endogenous intestinal biofilm barrier into the blood stream [129, 130]. Such Al-induced *leaking* of the endogenous biofilms of the gut and blood brain barrier could increase Al accumulation in the CNS. Glyphosate impairs the bioavailability of both tryptophan and methionine [126], and significantly reduced plasma concentrations of these amino acids have been found in Alzheimer's disease patients [131, 132].

Given the escalating use of glyphosate worldwide and the increasing incidence of inflammatory bowel disease [133] and gastroesophageal reflux disease [134], studies with animal models [135] are needed to assess the potential of glyphosate to specifically chelate and distribute Al compounds *in vivo*. High precision adsorption calorimetry may prove to be useful means of studying the thermodynamics of Al biosequestration, generally, and glyphosate Al chelation complexation, *in vitro* [136–138], specifically as suggested in Figure 2 from Guo and Friedman [139] which shows how Gadolinium ( $Gd^{3+}$ ) serves in biological cation sequestration. CNS delivery is known to occur, at least in part, via adsorptive transcytosis of cationized proteins and peptides [140]. This empiric observation, therefore, begs the questions: does glyphosate promote adsorptive transcytosis of Al, and *vice versa*; does Al promote adsorptive transcytosis of glyphosate, across the protective biofilms of the gut and blood brain barrier?

**2.4.  $Al^{3+}$  Induces Oxidative, Genotoxic, and Interfacial Water Stress—A Triple Threat.** A well-recognized effect of  $Al^{3+}$  is the induction of oxidative stress [141] and though it has prooxidant [142] effects through its impact on water dynamics as Ling has shown [143–145], it disrupts enzymes involved in the methylation pathway, increasing EIWS [59]. As a consequence, Al impacts epigenetic interactions and everything dependent upon them. As early as 1968, Riddick showed that  $Al^{3+}$  generally promotes agglomeration and precipitation even of anionic colloidal finely ground silica (minasil) [146]. Evidently, it does so in the same way that, in living organisms,  $Al^{3+}$  disrupts interfacial hydrogen bond (H-bond) cooperativity and the quantum coherence of water essential for cellular homeostasis.

**2.5.  $Al^{3+}$  Disrupts H-Bond Cooperativity of Biological Water.** The disruption induced by  $Al^{3+}$  can be seen as a “red shift” of the stretching bands in the absorption spectra of water to longer wavelengths—thus a “bathochromic” shift—on both infrared and Raman spectroscopy. In 1985, Newton and Friedman employed a neutron diffraction method [147] to show that the dominant isotope effect of +3 ions is associated with the O–H stretch of the water. The shift to lower frequencies is proportional to the square of the ionic charge  $z$  in  $Na^+$ ,  $Mg^{2+}$ ,  $Al^{3+}$  (or, resp., 1, 4, and 9), while the oscillatory motion—the “libration” frequency—increases linearly with  $z$  in the same series (or, resp., 1, 2, and 3). More recent confirmation of this expectation has been produced in a series of papers by Probst and Hermansson (1992), Desiraju and Steiner (2001), Joseph and Jemmis (2007), and Jemmis and Parameswaran (2007) [148–151].

Light and electron microscopy also show that cell morphology is sensitive to EIWS [152]. Tielrooij et al. (2010) [153] employed both terahertz and femtosecond infrared spectroscopy showing that the effects of ions and counterions on water can be strongly interdependent and nonadditive, and, in certain cases, extend well beyond the first solvation shell of water molecules directly surrounding the ion [153]. They also found that, “if strongly hydrated cations and anions are combined, the dynamics of water molecules are affected, wherein the *hydrogen bond network is locked in multiple directions* (italics, ours)” as shown in Figure 1.

**2.6.  $Al^{3+}$  Disrupts the Critical Metastable State of Neurolemmal Membranes.**  $Al^{3+}$  dangerously shifts the intracellular balance that normally keeps macromolecules of DNA, RNA, and proteins from breaking up and disintegrating into an incoherent, disordered chaotropic mixture. This can lead to the disintegration of blood cells for example in hemolysis or, with equal harm, bioactive molecules combining in biologically useless ways into kosmotropic precipitates, forming dysfunctional molecular debris deposited on the walls of blood vessels (as in atherosclerosis, e.g.) or disabling neurons (as seen in the beta amyloid and/or hyperphosphorylated tau deposits characteristic of Alzheimer's plaques and tangles). To the extent that the membranous (plasmalemmal) material of all cells, along with the material linings of mitochondria, neurons, and neurofibrils, can be depolarized by  $Al^{3+}$ ; the loss of cytoskeletal conduction, much like an electrical circuit that “shorts-out” and burns, is certain to be injurious to macromolecules and to cells.

Some molecular damage can result in the orderly, and usually safe, disassembly of cells by apoptosis [154] or, with  $Al^{3+}$  toxicity, the disorderly disintegration which may release formerly contained pathogens and/or additional toxic debris, leading to necrosis and disease-enabling conditions. The noted effects of  $Al^{3+}$  can graduate from destroying macromolecules, plasmalemmal membranes, and whole cells to the destruction of tissues, organs, and even the death of the whole organism [155]. Studies on plant seedlings have shown an immediate effect on the cytoskeleton in which  $Al^{3+}$  causes a calcium channel blockade by its depolarization of membrane potential [156]. In both plants and animals,  $Al^{3+}$  blocks voltage-gated calcium channels and interferes with normal metabolism [157–162]. It also disrupts the stable water clusters found in highly structured multilayered EZs that serve as vehicles for storing incident radiant energy, as Chai et al. have shown [161].

Platt et al. (1993) demonstrated that extracellular pH modulates the Al blockade of mammalian voltage-activated calcium channel currents [163] at concentration range  $<200 \mu M$ . Platt and Büsselberg (1994) then investigated the extracellular and intracellular effects of Al on voltage-activated calcium channel currents (VACCCs) in rat dorsal root ganglion neurons [164] and found that (a) Al applied extracellularly reduces VACCCs in a concentration-dependent manner, (b) the effect of Al was highly pH dependent in the investigated range (pH 6.4 to 7.8), and (c) there was evidence of intracellular as well as extracellular



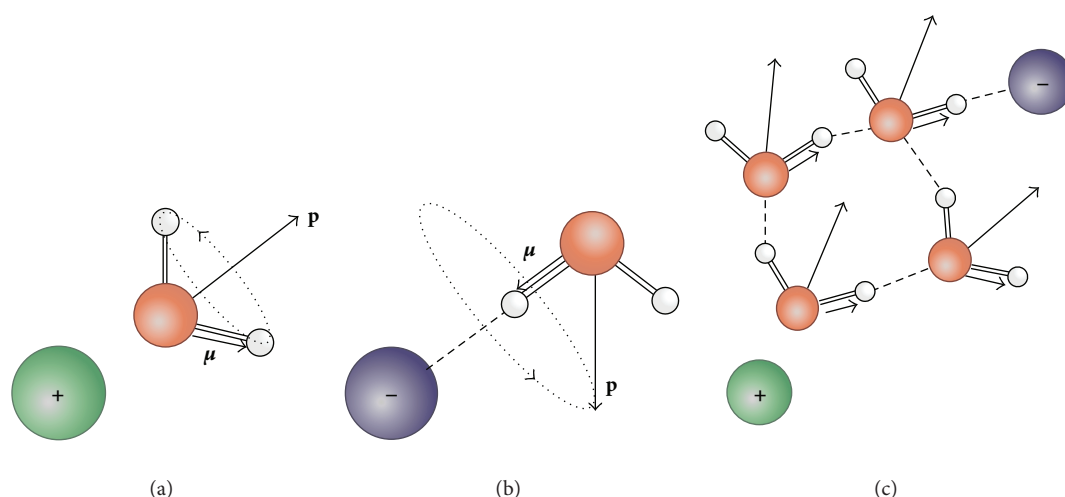


Figure 1: Semirigid hydration and cooperativity ((a) and (b)) a water molecule in the solvation shell of a cation (a) and an anion (b). Dielectric relaxation measurements probe the reorientation of the permanent dipole vector  $p$ . Femtosecond infrared spectroscopy is sensitive to the reorientation of the OD-stretch transition dipole moment  $\mu$ . The dotted arrows indicate reorientation in a cone, in the case of semirigid hydration. (c) Proposed geometry, in which the water dynamics are locked in two directions because of the cooperative interaction with the cation and the anion. Figure 1 is reproduced here from (Tielrooij et al. 2010) [153] with permission of the American Association for the Advancement of Science.

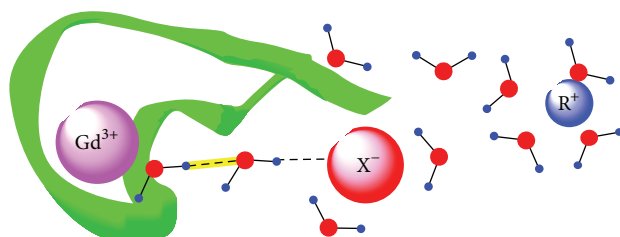


Figure 2: Depiction of how Gadolinium ( $Gd^{3+}$ ) vibronic side band luminescence spectroscopy (GVSBLS) acts as a probe of the coordination of biologically-relevant sites of cation sequestration. The figure is reproduced here from (Guo and Friedman 2009) [139] with permission of the American Chemical Society. Copyright 2009 American Chemical Society.

binding. They concluded that irreversibility, use dependence, and pH dependence, as well as binding sites for Al inside cells, contribute to its neurotoxicity. Platt and Busselberg also examined the combined actions of  $Pb^{2+}$ ,  $Zn^{2+}$ , and  $Al^{3+}$  on VACCCs [164] showing that each of these metals reduced VACCCs, for all possible combinations, independent of the order of application. The impacts were additive and consistent with two metals acting at the same site as well as independent actions at different locations of the ion channel. Trombley (1998) demonstrated selective disruption of class A gamma-aminobutyric acid, the ligand gated ion channels ( $GABA_A$ ) receptors, by Al occurred with a minuscule concentration of  $<100 \mu M$  in a culture of rat olfactory bulb neurons [165].

At the same time, and for some of the same reasons, ultrafast electron crystallography of interfacial water by Pal and Zewail (2004) as followed by Oliveira et al. (2010) showed that recognition at the macromolecular levels of DNA, RNA, and protein is dependent on biological water dynamics in the 20–40 picosecond range [159, 160]. Based on the biosemiotic functions of such macromolecules, loss of

such recognition would invariably lead to molecular mimicry, immune dysfunction, and the onset of autoimmune disease. Neuropathological states involving immune disorders can thus be conceptualized to arise from the breakdown of, or deviation from, the metastable critical state of biological water dynamics at the interphase of neuronal membranes. Similarly, with respect to neurological damage, Al has been shown to induce neuronal apoptosis *in vivo* as well as *in vitro* [166].

Sadiq et al. (2012) found that metal ions such as  $Al^{3+}$  tend invariably to target signaling pathways and may interact with various targets simultaneously. The long-range consequences show that ions interacting with any given molecular target can disrupt all of the processes dependent on it [162]. With respect to developmental neurological and other communication disorders, Oller and colleagues (2010a, 2014) have described this phenomenon as a domino or cascading effect [167–169] and Seneff et al. produced the same sort of argument for the biophysical level [57]. Likewise, Shaw et al. (2013) show how minimally stable states of interphase water at

neurolemmal membranes can be upset by “noise” from  $\text{Al}^{3+}$  producing a “domino” effect [68] inducing long-wavelength perturbations leading to a cascade of energy dissipation on all scales [170].

**2.7. Biological Water Modulates Biosemiotic Entropy at Multiple Levels Concurrently.** Underlying all of the foregoing evidence, there is sound theory and a growing body of research (partially summed up in Figure 1) showing that water, rather than being a passive medium in which biological reactions take place, is an active participant [59, 60, 171]. With that in mind, it is plain that  $\text{Al}^{3+}$  must disrupt long-range, dynamical, interfacial H-bond cooperativity and that it must interfere with the quantum coherence of water, both of which are essential for cellular homeostasis. The geometry proposed by Tielrooij et al. (Figure 1), in which the water dynamics are locked in two directions, shows how the cation and anion produce the polarized-oriented multilayer (PML), confirming the theory of Ling (2003) [115], the exclusion zones (EZs) of water reported by Zheng and Pollack (2003) [172, 173], and the H-bond cooperativity implicit in the EIWS theory [59]. Because of their chemical properties and affinities,  $\text{Al}^{3+}$  species tend to disrupt the hydrophobic surfaces of water based biofilms of all kinds.  $\text{Al}^{3+}$  disrupts such films by breaking down the complex hydrophobic forces binding the liquid. This kind of breakdown can be seen in its impact on the liquid films containing the peculiar colloids known as “coacervates” studied for the last 150 years by Lillie [174], Oparin and Synge [175], and numerous others, the recounting of which is found in Ling’s work as cited. It also has the same disintegrative effect on the neurolemmal membranes throughout the body, showing how protoplasmic poisoning is invariably induced at many levels by the  $\text{Al}^{3+}$  species. The barriers between the blood and the brain and blood and the spinal cord, as well as the barriers protecting the blood and the rest of the body’s tissues from the contents of the gut can be thought of as analogous to “exclusion zones” or differentiated “coherence domains” [172, 176], consisting in part or in whole of polarized-oriented multilayers of biological water as described by Ling [115] (and see his references).

Because of stretching and reorientation of H–O bonds, generalized from the dynamics illustrated in Figure 1, the local “unwetting,” “stretching,” and hydrophobic “collapse” of interfacial water can also disrupt signaling systems, leading to immune dysfunctions and autoimmune diseases, all beginning with EIWS [59, 68]. Also, for reasons already partially explained, the CNS is particularly susceptible to Al toxic damage, especially considering the critical role of biosulfates, both the HSPGs and, especially, the sulfoglycolipids such as sulfatide [57, 177, 178] in the CNS. The latter are crucially involved in the formation of myelin, which is essential for healthy neural tissue and functions of the CNS and peripheral systems. Myelin, in turn, depends on HSPGs, which are essential in generating current and separating charge. But because myelin lipids and proteins demonstrate surface fractality over many scales [170, 178], toxic impact from Al and its compounds can do far-reaching harm. Also, it is

known that  $\text{Al}^{3+}$ ,  $\text{F}^{1-}$ ,  $\text{Hg}^{2+}$ , and  $\text{Pb}^{2+}$  are synergistically toxic and particularly so because of their affinity for biosulfates, such as the HSPGs.

The anion in Figure 2 may be generalized conceptually to include the biosulfates,  $\text{ROSO}_3^{1-}$  or  $\text{SO}_4^{2-}$ , fluoride ( $\text{F}^{1-}$ ), carboxylates, oxyanions of nitrogen, and the biophosphates. The cation in this figure may also be generalized conceptually to include high charge density polycationic metals, such as  $\text{Al}^{3+}$ ,  $\text{Hg}^{2+}$ , and  $\text{Pb}^{2+}$ , as well as oxycations. If vectors (arrows with direction and magnitude) are employed, as in Figure 1 [153], the dynamical reorientation of the OD-stretch transition dipole moment vectors and permanent dipole vectors will result in polarization and orientation of multiple layers of water along the lines explained by Ling in 2003 [115].

**2.8. Protoplasmic Poisoning via Cooperative Adsorption of Polycationic Metal Toxicants.** In 2008, Harrison et al. found that certain heavy metal cations exert synergistic bactericidal and antibiofilm activity against *Pseudomonas aeruginosa* [179]. In May 28, 2008, Harrison et al. filed patent (U.S. 2008/0118573 A1) for use of heavy metals in the treatment of biofilms, including metal cations such as  $\text{Mn}^{2+}$ ,  $\text{Co}^{2+}$ ,  $\text{Ni}^{2+}$ ,  $\text{Cu}^{2+}$ ,  $\text{Zn}^{2+}$ ,  $\text{Al}^{3+}$ ,  $\text{Ag}^{+}$ ,  $\text{Hg}^{2+}$ ,  $\text{Pb}^{2+}$ ,  $\text{Cd}^{2+}$ ,  $\text{Sn}^{4+}$ , and metalloid oxyanions. In 2010, Renslow et al. employed pulsed-field gradient nuclear magnetic resonance to study *in situ* effective diffusion coefficient ( $D_{rs}$ ) profiles in live biofilms [180] and observed distinctive spatial and temporal variation in  $D_{rs}$  for various locations in the biofilm. In 2013, Davidson et al. reviewed literature showing that, in several neurodegenerative and neuroimmune diseases, loss of anisotropy, loss of curvature, increase in diffusion magnitude, and loss of stiffness (softening), may be directly attributed to destructuring of interfacial water, which precedes overt signs and symptoms of oncologic, neurologic, and infectious disease [181, pp. 3851–3852].

Ling (1991) has argued as follows.

In autocoperative adsorption, the adsorption of an *i*th solute favors the adsorption of more *i*th solute; in a heterocoperative adsorption, the adsorption of an *i*th solute favors the adsorption of the alternative *j*th solute. Autocoperative behaviors, like those of a school of swimming fish and the sentinels guarding the Great Wall of China, tend to be all-or-none. . . autocoperative adsorption is the backbone of coherent behavior in living cells including the maintenance of the living state [181 pp. 135–58].

Heterocoperative adsorption of  $\text{Hg}^{2+}$  solute would favor the adsorption of an alternative solute, such as  $\text{Al}^{3+}$  and vice versa, in a manner which tends to be all-or-none. Cumulative heterocoperative adsorption of cationic neurotoxicant metals, for example,  $\text{Hg}^{2+}$ ,  $\text{Al}^{3+}$ , and  $\text{Pb}^{2+}$  explains their neurotoxic synergy and biosequestration.

**2.9. EIWS Promotes Both Structural and Biosemiotic Entropy.** The fact that  $\text{Al}^{3+}$  species are potent exogenous interfacial

water stressors per the EIWS theory was elaborated by Davidson et al. [57, 59, 68, 117, 119, 177]; Marcus (2013) found, in his study of the incremental surface tensions of various elements, that  $\text{Al}^{3+}$  has one of the largest individual ionic surface tension increments (second only to  $\text{La}^{3+}$ ) [94]. This finding explains why  $\text{Al}^{3+}$  along with  $\text{Hg}^{2+}$  and  $\text{Pb}^{2+}$ , as well as various cationic and nonionic surfactants, are potent factors in producing EIWS. Such observed facts explain how aluminum/phosphate and aluminum/sulfate species, either as the  $\text{Al}^{3+}$  aqua ion form at low pH or the inner sphere contact ion pairs at higher pH, by exceeding the incremental surface tension threshold of exclusion zones, can disrupt H-bond cooperativity [123]. In doing so they must augment biosemiotic entropy *in vivo*, tending toward dehydration as described by Sharma and Debenedetti (2012) [182].

In 1966 and 1967, Selye had already provided a comprehensive exposition of the toxicity of polyvalent metal ion salts [183, 184], particularly those with high charge density, leading to serial sensitization, resulting in both local and systemic thrombohemorrhagic phenomena, with microvascular ischemic and immune sequelae, in a highly stereotyped, pluricausal manner. The earliest events in the toxicity of  $\text{Al}^{3+}$  are biophysical, mediated by water, through disrupted interfacial H-bond cooperativity and quantum coherence [185–190]. Consistent with the red shift in Raman vibrational absorption frequencies discussed earlier and demonstrating it, in part, Falk (1984) had already found that a lowering of the bending frequency of water is associated with increasing cation charge and decreasing cation size [19]. Much more recently, Imoto et al. (2013) studied the origin of the difference in the H–O–H bend of the infrared spectra between liquid water and ice [192]. Furthermore, as suggested by Exley (2004) [142] and Mujika et al. (2011) [193],  $\text{Al}^{3+}$  may be predisposed to react *in vivo* with toxic impact on endogenous reactive oxygen species, such as the superoxide radical anion to form an Al-superoxide semireduced radical cation complex  $[\text{AlO}_2^*]^{2+}$ .

**2.10 Distinctive Physical Properties of Al Species Determine Their Toxicity.** Another unique property of Al ions is their high charge density. Ionic charge densities are reported in Table 3 using the methodology described by Rayner-Canham and Overton (2010) [194]. Also reported in the table are the crystal atomic radii as published by Shannon (1976) for the various ions [92]. The charge density of  $\text{Al}^{3+}$  is  $372.6 \text{ C}\cdot\text{mm}^{-1}$  as compared to that of  $\text{Gd}^{3+}$  ( $91.5 \text{ C}\cdot\text{mm}^{-1}$ ),  $\text{F}^{1-}$  ( $16.2 \text{ C}\cdot\text{mm}^{-1}$ ),  $\text{Na}^+$  ( $24.5 \text{ C}\cdot\text{mm}^{-1}$ ), and  $\text{Ca}^{2+}$  ( $51.6 \text{ C}\cdot\text{mm}^{-1}$ ).

The high charge density of Al is a consequence of its relatively small radius and its fixed 3+ charge. These factors impact the solubility of the individual Al salts and their incremental impact on the surface tension of water [94, 195, 196]. With respect to biological impact, the vast array of enzymes and signaling proteins inhibited by Al species shows that Al toxicity is not limited merely to diffusion. The interaction of the various Al species with long-range, dynamical H-bond networks and the coherence domains of interfacial water suggests the involvement of nonthermal, magnetic [47], and quantum effects that are no doubt generalizable to many

Table 5: Selected hydration enthalpies of common biologically relevant ions [89].

Symbol	$\Delta H_{\text{hydr}}$ ( $\text{kJ mol}^{-1}$ )	Source
$\text{NO}_3^-$	−312	[198]
$\text{K}^+$	−321	[197]
$\text{NH}_4^+$	−329	[198]
$\text{HSO}_4^-$	−368	[198]
$\text{Cl}^-$	−371	[197]
$\text{HCO}_3^-$	−384	[198]
$\text{Na}^+$	−413	[197]
$\text{OH}^-$	−520	[198]
$\text{H}_2\text{PO}_4^-$	−522	[198]
$\text{SO}_4^{2-}$	−1035	[198]
$\text{H}^+$	−1100	[197]
$\text{Ca}^{2+}$	−1650	[197]
$\text{Mg}^{2+}$	−1920	[197]
$\text{Mg}^{2+}$	−1949	[198]
$\text{Al}^{3+}$	−4690	[197]

toxics, particularly those with polycationic surfactants of high charge density (see Table 3).

Inorganic ions can be ranked on a chaotropic (disintegrative) to kosmotropic (colloid forming) gradient according to their enthalpy of hydration [197, 198] presented in Table 5 (above). The more negative the enthalpy of hydration, the more kosmotropic the solute. The opposite would indicate a chaotropic tendency. A formula that aids in understanding the relationship between charge density, radius, and enthalpy of hydration is given as follows:

$$H = -\frac{Ze^2}{2r} \left(1 - \frac{1}{\epsilon}\right), \quad (1)$$

where  $H$  = Hydration enthalpy,  $Ze$  = Charge of the ion,  $r$  = Ionic radius, and  $\epsilon$  = Dielectric constant of the solvent.

A smaller atomic radius and higher charge correlate with a more negative hydration enthalpy and greater kosmotropism—defined biologically as the tendency to cause macromolecular complexes in bodily fluids to form useless colloidal precipitates that are effectively sequestered from the water in organelles, cells, blood, lymph, protoplasm, or any bodily fluid. In biological systems, protein folding and unfolding (DNA also) depend on a delicate balance of chaotropic and kosmotropic forces on water [199]. Solutes sorted according to a chaotropic to kosmotropic gradient define the Hofmeister series [59]. In agreement with hydration enthalpies found in Table 5,  $\text{Al}^{3+}$  normally acting as a powerful kosmotrope plays havoc with the biological balance. In particular, the more kosmotropic a substance is, the more capable it is of salting-out proteins from an aqueous medium. Table 4 presents a comparison of the properties of chaotropic and kosmotropic ions.

The oxyphilic behavior of Al acting as a kosmotrope is shown in its avid binding to oxyanions of carbon, sulfur, and phosphorus [120]. Its lipophilicity, dose-dependence, time-dependence, and glial versus neuronal specificity have

been studied by Campbell et al. (2001) [200] and as early as 1996, Bondy and Kirstein had already shown how Al species can promote iron-induced generation of harmful reactive oxygen species [201]. Cations such as Al can bind to  $\pi$  electrons within biomolecules [202] *in vivo*, inciting lipid peroxidation, DNA damage, and disruption of essentially all the biosemiotic systems deploying molecules containing calcium and sulfur [203]. A *prima facie* indicator of its toxicity is inflammation shown in cerebral markers elicited by chronic exposure to Al in drinking water [204]. Kiss (2013) has reviewed the coordination chemistry of  $\text{Al}^{3+}$  with small and large biomolecules, including serum components, and also the role of time in the distribution of this “sluggish” metal ion in a biological environment [205]. The results agreed with the computer model of Beardmore and Exley (2008), showing that Al has kosmotropic effects at a greater distance and more quickly than the “depot” theories could possibly explain [206].

The magnitude of the kosmotropic property of  $\text{Al}^{3+}$  can be seen in bold relief by comparing the degree of H-bond strengthening required to cause  $\text{Al}^{3+}$  to behave as a chaotrope [207]. If the H-bond energy of water increases, then various kosmotropic ions behave as chaotropes and vice versa. The required change in strength of H-bonds to cause  $\text{Na}^+$  to behave as a chaotrope is 11% strengthening and for  $\text{K}^+$  to behave as a kosmotrope is 11% weakening. The gradient between  $\text{Na}^+$  and  $\text{K}^+$  is almost two orders of magnitude smaller in comparison with the hydration enthalpy of  $\text{Al}^{3+}$  ( $-4690 \text{ kJ mol}^{-1}$ ), in theory, the amount of energy released (as heat) when a mole of  $\text{Al}^{3+}$  dissolves into an infinitely diluted solution. The change of H-bond strength required for  $\text{Al}^{3+}$ , a kosmotrope, to behave as a chaotrope is 1260.75% H-bond strengthening. The required H-bond strengthening is calculated by dividing the hydration enthalpy of the solute by the estimated isotropic point ( $-372 \text{ kJ mol}^{-1}$ ). Table 5 shows selected hydration enthalpies of several common biologically relevant ions.

**2.11. Molecular and Cellular Biosemiotic Disruption by  $\text{Al}^{3+}$  Is Concomitant.** The foregoing facts and findings in this section help to show why and how  $\text{Al}^{3+}$  interacts synergistically with certain other toxic molecules and how it acts in producing or augmenting auto- and neuroimmune diseases. Kamalov et al. (2011) demonstrated the cytotoxicity on immune cells of environmentally common concentrations of Al (10–40  $\mu\text{M}$ ) in murine thymocytes and lymphocytes [208]. Nearly all thymocytes showed evidence of damage at 30  $\mu\text{M}$   $\text{AlCl}_3$  after only 5 minutes of incubation. A 60-minute exposure to 10  $\mu\text{M}$   $\text{AlCl}_3$  caused damage of about 5% of thymocytes, while 50% were injured after 10 minutes at 20  $\mu\text{M}$ . In lymphocytes, injury was observed at 15  $\mu\text{M}$   $\text{AlCl}_3$ , and less than 50% of cells were injured after a 60-minute exposure to 20  $\mu\text{M}$ . Injury only rarely proceeded to rapid cell death and was associated with cell swelling. These results demonstrated a rapid dose-dependent injury in murine thymocytes and lymphocytes resulting from exposure to Al, as indicated by an increase in the entry into the cell of the DNA-binding dye, propidium iodide. The data suggest direct damage to the

plasma membrane, manifested as an increase in membrane permeability, consistent with the EIWS theory.

Likewise, with respect to the synergistic interaction of  $\text{Al}^{3+}$  with  $\text{Hg}^{2+}$  species, Kern et al. (2013) examined the action of low levels,  $\leq 1,000 \text{ nM}$ , of thimerosal (49.55%  $\text{Hg}^{2+}$  by weight) on immortalized B-cells taken, respectively, from autism spectrum disorder subjects, their fraternal twins, a sibling, and an age/sex matched control. Observed contrasts showed impaired sulfation chemistry owed to the thimerosal exposure [209, 210]. In 2009, Pogue et al. presented data which underscores the potential of nanomolar concentrations of Al to drive genotoxic mechanisms characteristic of neurodegenerative disease processes [211–212]. These data, combined with results reported earlier by Haley (2005), suggest toxic synergy between  $\mu\text{M}$   $\text{Al}^{3+}$  levels and nM thimerosal levels, *in vivo* [213].

While  $\text{Al}^{3+}$  can undoubtedly form complexes with proteins, nucleotides, nucleosides, RNAs, and DNAs, so too can stable nanoclusters of water, some of which are helical [214]. The presence of  $\text{Al}^{3+}$  could only create difficulties in such delicately balanced systems [215]. Also, given the growing body of empirical data suggesting that both gene structure and protein structure are dependent in part on interfacial water dynamics, it follows that the best known biological macromolecules depend in part on supramolecular systems [216, 217].

### 3. Corrupted Processes and Pathways Induced by Aluminum

**3.1. Effect of Al on Iron Toxicity and Interference with BH4 and Calmodulin Function.** Al is primarily transported in serum by transferrins [218]. Al may interact with transferrins at multiple candidate binding sites, including the transferrin receptors, thus influencing iron metabolism and transport. The fastest subunit of transferrins to react with iron is the tyrosinate complex [219]. Other amino acid residues with which Al may interact are aspartic acid, glutamic acid, and glutamine [220]. Al readily binds to apo-transferrin binding sites but does not compete with iron for binding with halo-transferrins. Al causes small conformational changes in transferrins without significant structural consequence [221], thus enabling transferrin receptors to actively transport Al across the blood brain barrier as if it were iron [222]. Once in the brain, displacement of iron from transferrins by Al results in iron toxicity and overproduction of reactive oxygen species by Fenton reactions [203, 223].

Six interactive cycles within the methylation pathway include (1) the urea cycle, (2) the tetrahydrobiopterin (BH4) cycle, (3) the folate cycle, (4) the methionine cycle (5) the S-Adenosyl methionine (SAM) cycle, and (6) the transsulfuration pathway. Dihydrobiopterin reductase (DHPR) is a critically important enzyme in the BH4 cycle that is inhibited by Al, and calmodulin (CaM) is critically inhibited in the urea cycle.

DHPR inhibition is implicated in Al induced encephalopathy [224]. Many accounts of Al toxicity are reported in the context of renal insufficiency. Al intoxication



associated with pediatric renal insufficiency causes progressive encephalopathy in children [225]. Furthermore, Al intoxication by any cause such as occupational exposures will have the same inhibitory effect on DHPR [226]. BH4/BH2 ratios are decreased as a result of DHPR inactivation. BH4/BH2 ratios are reported to be decreased in Alzheimer's disease [28] and in autism [227]. About 60% of children on the autism spectrum are reported to experience clinical improvement after BH4 replacement therapy [228].

The folate cycle [229] enables components of urea, BH4, and methionine cycles to adapt to varying oxidative conditions. The dihydrofolate reductase (DHFR) system is a means of BH4 supply in cases of dysfunctional or inactive DHPR [230]. In this process, DHPR becomes more active in recycling BH4 from BH2 instead of acting on dihydrofolate to synthesize tetrahydrofolate when DHPR is functional. Congenital DHPR deficiency, such as in phenylketonuria (PKU) is associated with folate depletion [231] and treatment for PKU includes dietary folate replacement [232].

In addition, BH4 is cofactor for production of dopamine from tyrosine. Dopamine, cyanocobalamin, and 5-methyl tetrahydrofolate are required for synthesis of methionine from homocysteine [233, 234]. In Al toxicity, as in autism [63], dopamine becomes depleted because BH4 is depleted, further limiting remethylation of DNAs, RNAs, lipids, and proteins [235]. Furthermore, methionine is required to methylate DNA. The brain malformations seen in autopsies of autistic subjects [236] suggest failure of DNA methylation during brain development and growth.

In the urea cycle, BH4 is a cofactor with arginine in the synthesis of nitric oxide (NO) under endothelial nitric oxide synthase (eNOS). Not only does Al inhibit DHPR and production of BH4, but it also out-competes calcium for binding sites on calmodulin (CaM) causing conformational changes [237]. Properly bound with calcium, CaM is an essential cofactor in coupled eNOS mediated production of citrulline and NO from arginine. If BH4 is depleted or Al binds to calmodulin, eNOS follows an uncoupled pathway that favors production of peroxynitrite and superoxide. NO levels are paradoxically high in BH4 depletion, because it continues to be produced by alternate pathways, and its release from endothelial cells is inhibited by the high level of accumulated homocysteine [238].

High NO levels are associated with increased vascular permeability. NO stimulates mast cells and macrophages to release proinflammatory cytokines including IL-1, IL-6 tumor necrosis factor (TNF), and vascular endothelial growth factor (VEGF) [239]. This is the inflammatory profile found in autistic encephalopathy [240]. Accumulation of both reactive oxygen and nitrogen species results in severe oxidative and nitrosative stress [241–243].

**3.2. Effects of Distinct Formulations of Aluminum Adjuvants: A Role for the Zeta Potential.** As already noted, Al adjuvants are predominant modulators used in vaccines, although relatively little is known about how they work [244]. It was formerly claimed that Al adjuvants directly stimulate antigen-presenting cells by forming an antigen depot at the

Table 6: Three different formulations of the DTaP vaccine and the number of reported adverse reactions available from VAERS for each one.

Formulation	Adjuvant	Adverse reactions
Tripedia	Aluminum potassium sulfate	11,78
Daptacel	Aluminum phosphate	8,786
Infanrix	Aluminum hydroxide	13,238

injection site [245]. Given the evidence that Al species used in adjuvants are readily transported throughout the body, the depot theory must be rejected. Others have proposed that Al stimulates dendritic cells, activates the immune complement system, and induces the release of chemokines [246]. It is generally agreed that Al hydroxide induces a Th2 type immune response [247, 248], whereas Al phosphate has been shown to induce a Th1 type response [249].

However, based on data from the CDC's Vaccine Adverse Event Reporting System (VAERS) database it is possible to compare the three distinct Al adjuvants used in the DTaP vaccine in particular (see Table 6): they consist of a hydroxide, a potassium sulfate, and a phosphate. The fact that all are used in the same multivalent vaccine minimizes the degree to which other factors, including the several antigens in the vaccine, might be influencing adverse reactions. Assuming only that all other factors excepting the Al adjuvants are held constant, an experimentally orthogonal comparison is possible among the three adjuvants. The method of comparison was a standard ratio of an expected value to the one obtained in each instance as susceptible to a standard chi-square distribution (the log-likelihood ratio) as described in [250].

The statistic in question expresses the likelihood that a given ratio of expected adverse reactions to actually observed adverse reactions could be attributed to chance. The critical probability for our tests was conservatively set at  $p < 0.05$ . The VAERS database for DTaP adverse reactions for the several formulations were compared with subsamples matched for age and number of cases. The comparison enabled the testing of experimental predictions concerning the relative mobility of charged particles in an electric field based on the Zeta potential (ZP) of the various Al adjuvants at issue. In blood—the most abundant fluid involved in transporting adjuvants from an injection site—the ZP reflects the negative charge of molecules attached to the membranes of suspended particles, such as red blood cells (RBCs) or lipid particles, which the  $Al^{3+}$  compound in any given case would be likely to link up with. A less negative ZP is associated with an increased tendency for RBCs to aggregate [251] that is, to form clots, whereas an even more negative ZP reduces that tendency.

The three DTaP formulations (Table 6) differ chemically only in their Al adjuvant component, as detailed by Caulfield et al. [244], and to that extent the vaccines differ in zeta potential (ZP). As those researchers found, ZP measured at pH 7.0 closely matching the value for blood, yielded a

Table 7: Adverse reactions reported in VAERS for sulfate versus hydroxide in age-matched samples, and the likelihood that the contrasts observed in these distributions could have occurred by chance ( $p < 0.05$ ).

Condition	Sulfate	Hydroxide	$p$ value
Swelling	2210	2665	0.0066
Cellulitis	445	617	0.020
Pain	622	815	0.020
Fever	2032	2296	0.034
Injection site reaction	393	520	0.038
Injection site swelling	7	33	0.045

Table 8: Counts of various adverse reactions reported in VAERS for sulfate versus phosphate in age-matched equal subsets of the sample space, and the likelihood that the contrasts observed in these distributions could have occurred by chance according to a log likelihood ratio test. Included are all the reactions for which phosphate was more common with a  $p$  value under 0.05.

Condition	Sulfate	Phosphate	$p$ value
Hospitalization	177	363	0.0044
Seizures	186	333	0.011
Rotavirus	3	47	0.013
Abdominal pain	6	53	0.014
Nausea	203	338	0.015
Diarrhea	95	174	0.028
Pneumonia	13	50	0.032
Dehydration	12	48	0.032
Throat irritation	81	147	0.036

ZP value for hydroxide at +30 mV, for sulfate at 0 mV, and for phosphate at -20 mV: the sulfate formulation, therefore, should have the least impact. Using its ZP value at 0 mV as the baseline, it provided a reasonable estimate of the “expected value” for the ratio comparisons with the other two adjuvants to assess the impact of ZP on the adverse reactions reported. Results shown in Tables 7 and 8 show the outcomes for phosphate and hydroxide adjuvants. Compared to phosphate, local adverse events are reported more often for hydroxide, which, as expected, should migrate less from the injection site owing to a higher positive ZP, while phosphate should show the opposite effect owing to its negatively displaced ZP value.

The negative charge induces mobility owing to the electrical field induced by the voltage difference between arteries and veins [99, 100] while the positive charge tends to prevent mobility through the blood. The voltage difference is partly because the veins have a lower pH because  $\text{CO}_2$  is more acidic than  $\text{O}_2$ . The lymphatic system, of course, as noted by Gherardi and colleagues [98–100], affords a bypass route that white blood cells (e.g., immune cells) can take (having penetrated the endothelial wall into the tissues) [252, 253]. However, this pathway also has the same voltage drop that would propel movement of negatively charged particles, as the lymph system returns to the venous system at the subclavian vein. On the other hand, positively charged

particles would be stalled in the tissues as shown by Davidson and Seneff [59].

Thus, with the Al hydroxide adjuvant, we expect and find relatively more edema (swelling) at the injection site accompanied by “injection site reaction” and cellulitis because both plasma and lymphatic transit are stalled. Al phosphate, in contrast, with higher mobility and easier migration through the lymphatic system into the venous system, is more likely to reach distant areas including the brain, resulting, as observed, in a greater likelihood of systemic responses such as throat irritation, nausea, diarrhea, abdominal pain, and seizures. As expected, Al potassium sulfate did not produce any reactions with a  $p$  value under 0.05, when compared against either of the other formulations.

Observed syndromes associated with Al hydroxide include “macrophagic myofasciitis” (MMF) characterized by diffuse myalgia, chronic fatigue, and cognitive dysfunction, termed “mild cognitive impairment” [38, 40]. In a relevant study of that disorder, it was determined that the Al hydroxide adjuvant led to an accumulation of Al-loaded macrophages at the site of a previous intramuscular immunization [39]. Given the results reported in Table 7, it must be inferred that macrophages lingering at the injection site on account of the elevated ZP associated with the hydroxide formulation are responsible for this observed syndrome. Likewise, the autoimmune syndrome recently identified by Shoenfeld and colleagues [65–67] is consistent with the generalized toxicity of the Al adjuvants.

**3.3. Aluminum Interactions with Fluorine.** Fluorine is the most chemically reactive nonmetal and the most electronegative element [254]. According to Martin (1996) [255],  $\text{Al}^{3+}$  binds  $\text{F}^-$  more strongly than 60 other metal ions tested. Even with micromolar concentrations of  $\text{Al}^{3+}$ , these two atoms react to form  $\text{AlF}_4^-$ , a molecule whose shape and physical properties closely resemble those of the phosphate anion,  $\text{PO}_4^{2-}$ . This feature has been exploited to help researchers understand phosphate-dependent reactions in signaling cascades [255–258]. For example, it has been shown, by exploiting  $\text{AlF}_4^-$ , that melatonin’s widespread signaling effects are mediated by G-proteins [259]. However, if  $\text{AlF}_4^-$  forms whenever these two elements are both present, it is known to interfere with regulatory GTP hydrolases which play an initiating role in phosphate-based signaling cascades [260, 261]. Should the  $\text{AlF}_4^-$  mimetic, which is not responsive to the GTPase, stick in the “on” position, an overresponsive cascade of transcription, motility, and contractility, as well as apoptosis would proliferate. If this were to happen, such interference, for which Al toxicity affords many alternative routes remaining to be explored, is certain in all cases to augment biosemiotic entropy.

Strunecká and Patocka proposed that the toxic role of Al in Alzheimer’s disease may be predominantly due to the formation of  $\text{AlF}_4^-$  [262]. The formation of that complex, according to experimental evidence, in quantities as little as 1 ppm of fluoride contamination of water supplied to rats led to greater uptake of Al into the kidney and brain along with the formation of amyloid deposits like those in Alzheimer’s



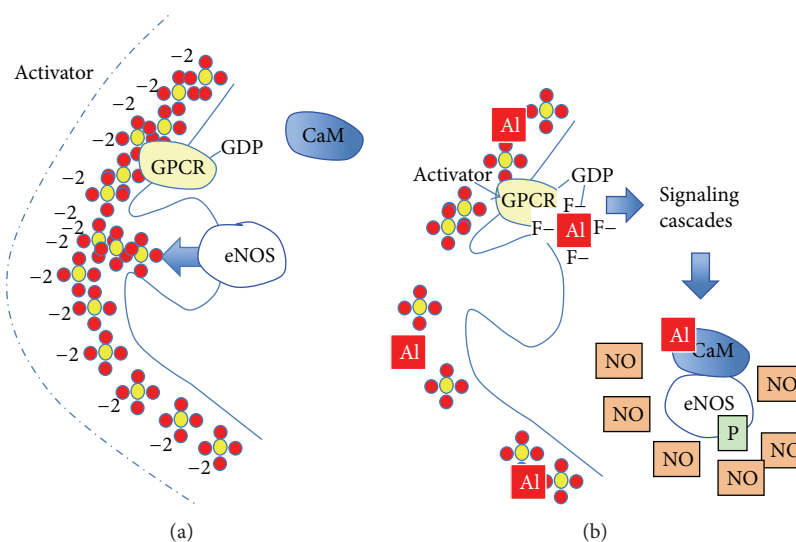


Figure 3: Illustration of the devastating effects of Aluminum on a typical cell related to sulfate inactivation, G-protein signaling, and calmodulin signaling. (a) A healthy cell without Al contamination. eNOS, attached to the membrane at a caveola, produces sulfate, which maintains a healthy glycocalyx with sufficient negative charge. (b) Al binds to the sulfates, eliminating the negative charge, which allows cytokines to penetrate through the glycocalyx, activating G-protein coupled receptor signaling cascades.  $\text{AlF}_4^-$  disrupts the signal, acting as a phosphate mimic, and Al binds to CaM, inducing eNOS detachment from the membrane. Phosphorylation cascades activate eNOS to produce abundant NO released into the cytoplasm, instead of producing sulfate to enrich the glycocalyx.

disease [263]. As proteins, RNAs, and DNAs become damaged through oxidation [264–267], if they cannot be repaired, failure of the lysosomal and mitochondrial organelles will lead to apoptosis [268–270] or, in worse cases, to necrosis. Al compounds can only contribute to such outcomes in a negative way.

Prior research has also shown that insufficient sulfate in the extracellular matrix of all the tissues, particularly the endothelial wall, plays a significant role in disorders and disease conditions [59, 117, 177, 199]. Heparan sulfate populates the glycocalyx in the capillaries [118, 271–273] and enables a low-resistance capillary wall permitting smooth blood flow [57, 59, 68, 117, 177, 199]. Sphingosine-1-phosphate-induced Rac activation, chemotaxis, and angiogenesis associated with endothelial cell migration are mediated by G-proteins [274].

With all of the above considered it may be notable that postmortem examination of Alzheimer's brains reveals severe deficiency in sulfatide, a myelin-specific sulfated sphingolipid, which normally makes up 6% of the lipid content and is especially concentrated in the myelin sheath [275]. Twenty-two subjects in the early stage of Alzheimer's disease showed a depletion of 93% in gray matter and up to 58% in white matter in all brain regions examined. Aside from an overabundance of ceramide, the precursor to sulfatide (ceramide was elevated threefold in white matter), all other lipid parameters appeared normal. This outcome was not associated with a defect in sulfatide synthesis, so the pathology appears to involve breakdown of sulfatide to provide sulfate to the vasculature, critical for maintenance of an adequate supply of oxygen and nutrients to the brain.

Seneff et al. previously suggested that endothelial nitric oxide synthase (eNOS), an enzyme present in endothelial

cells, RBCs, and platelets, among other cell types, is a “moonlighting” enzyme, which synthesizes sulfate when it is attached to caveolin in the plasma membrane and synthesizes NO (which is converted to nitrate within a few seconds) when it is phosphorylated and bound by a calcium-CaM complex in the cytoplasm [118]. These findings suggest that eNOS plays the dual-purpose of regulating the balance between kosmotropes and chaotropes in the cytoplasm of the cell and also enabling the proper folding and functions of cellular proteins [199], as detailed in Figure 3.

#### 4. Discussion

Considering all the ways  $\text{Al}^{3+}$  is known to impact biological systems negatively, as summed up in Table 1, exposure to that cation generally disrupts biosemiotic cascades. Its effects lead to minute cumulative injuries to DNAs, RNAs, cellular proteins, and lipids through glycation and oxidation damage, as well as impaired lysosomal recycling of debris, and, ultimately, in some cases, leads to cell death by necrosis. Death by apoptosis, the preferred alternative, may also follow Al-induced injuries and changes in DNAs, RNAs, proteins, and any downstream mediators. For example, MMF has been shown to manifest with Al retention at the injection site of vaccines containing Al hydroxide [38, 39] and far-reaching negative effects on the body's immune systems can be seen in ASIA owed to eventual migration of Al adjuvants away from the injection site [65–67]. Given its positive differential impact on ZP, Al hydroxide has been shown to linger at the injection site for many months, although it eventually is transported into brain by macrophages [77]. In that particular case, the normal apoptosis of injured cells is disrupted by

the high electrostatic attraction of the  $\text{Al}^{3+}$  ion towards the negatively charged sulfates in the glycocalyx actually forcing the  $\text{Al}^{3+}$  cation to penetrate and traverse the viscous water of the exclusion zone. The result is disruption of the structured water in the exclusion zone, compromising the glycocalyx barrier and allowing signaling molecules to gain access and launch a G-protein mediated cascade reaction.

This cascade is intensified by the effects of  $\text{AlF}_x$  on G-protein signaling, and the subsequent disruption of cellular metabolism follows. When the cell becomes necrotic, having skipped over any possibility of normally regulated and orderly apoptosis, it virtually disintegrates, releasing DNA and other cellular debris into the interstitial spaces to degenerate or to be carried away by the lymphatic system. In the case of the other less confined Al adjuvants that can more readily migrate away from the injection site, the confusion induced in biosemiotic systems is the predictable source of a confused and self-destructive autoimmune response as seen in ASIA. The downstream result is an immune attack on cells, tissues, and organs throughout the body but especially in the CNS, as seen in diseases such as multiple sclerosis and other demyelinating conditions.

It is clear that  $\text{Al}^{3+}$  toxicity, interacting synergistically with other toxicants such as solvated species of  $\text{Hg}^{2+}$ ,  $\text{Pb}^{2+}$ ,  $\text{F}^-$ ,  $\text{AlF}_x$  (aluminum fluoride),  $\text{SiF}_x$  (silicofluoride), glyphosate, and including chelation complexes, must directly increase biosemiotic entropy on multiple levels simultaneously by disrupting long-range, dynamical, interfacial hydrogen bond cooperativity and the quantum coherence of water. The outcome is widespread (systemic) and involves virtually simultaneous inhibition of many different enzyme systems. It is therefore unsurprising that  $\text{Al}^{3+}$  is associated with anaphylaxis and sudden death [59]. The data from the studies reviewed here show that the complex coacervate protoplasm, studied now for about 150 years [145, 174, 175], is susceptible to poisoning by high charge density polyvalent cations, for example,  $\text{Al}^{3+}$ ,  $\text{Hg}^{2+}$ , and  $\text{Pb}^{2+}$ . Empirical studies [93, 94] of ion solvation suggest that local order induction can result in loss of long-range, systemic coherence and cooperativity [185]. On a supramolecular biosemiotic level, EIWS induced by  $\text{Al}^{3+}$  disrupts interfacial hydrogen bond cooperativity and quantum coherence of biointerfacial water. At a critical threshold, the self-ordered criticality of biointerfacial water collapses. The most notable effects of this sort occur in the CNS [68, 276].

In the larger context, however, Al toxicants can themselves, or by synergistically interacting with other toxicants, destroy cells in any organ system, although none are more vulnerable than the CNS and the peripheral systems attached to it. While significant everywhere in the body, the impact of biosemiotic entropy in the CNS is critical because of the nested and highly interdependent systems connected to it. For example, the loss of neural cells (neurons or glia) in the CNS tends to disrupt circuits that depend on such cells. In turn, groups of neurons in functional nuclei can be rendered dysfunctional through the loss of individual neuronal elements. In the same way, the loss of functional nuclei can lead to catastrophic stress on the CNS itself and/or

on dependent organ systems. Fatality may be preceded by a cascading series of failures resembling the collapse of complex interdependent networks [277].

An additional factor that makes the nervous system uniquely vulnerable is the highly specified differentiation of neuronal activities due to sequenced developmental programs. These programs, acting in response to both genetic and environmental instructions, ensure that the loss of functional circuits cannot be easily replaced, since the very milieu into which they might be integrated (e.g., stem cells) differs from one stage of development to the next during which some window, or “critical period,” for neuroplasticity may have passed. While it is true that critical periods vary between neuronal regions (human association versus primary cortex, e.g.), younger nervous systems appear to have a greater capacity for recovery following injuries to organ systems provided stem cells remain intact. However, damage to the DNA of stem cells is apt to be irreparable even in early stages of development, and  $\text{Al}^{3+}$  can cause both injuries to organs and DNA damage directly impacting stem cells.

A third reason for the notable toxicity of  $\text{Al}^{3+}$  is that neurogenesis—that is, the birth of new neurons—is relatively rare in the adult CNS in most regions. Compared to the ability of other organs to regenerate, for example, the skin or liver, the CNS has limited capacity to do so, which renders it more vulnerable to irreversible damage at fairly early stages of development. Thus, Al and its compounds have remarkable power to harm neurons and to produce systemic damage. The observed impact may, in some instances, be sudden, as in anaphylaxis and sudden death syndrome, but in other instances, it may build slowly to a crisis level through chronic doses leading to systemic autoimmune responses as in the vaccine-induced disorders. The variable range of toxic effects in ASIA, for example, can best be explained in terms of the biodistribution and pharmacokinetics of the particular Al adjuvant used. Some of the observed differences depend predictably on ZP and its impact on interfacial water tension.

Figure 4 is a two-dimensional schematic showing some of the ways Al and its compounds can impact the biosemiotic systems encompassed by the CNS. The summary suggests a nested biosemiotic hierarchy of ranked systems communicating within and across levels. In ascending order, they range from molecules to genes, proteins, cells, circuits, CNS subsystems, and the CNS itself. Impacts at any level can induce changes in those above and below them. For example, Al actions at a cellular level will necessarily perturb protein structures and DNA (the levels below) and alter cell-to-cell communication at the circuit level (above). Although Figure 4 focuses on the deleterious effects of Al on the nervous system, it should be clear that its impacts are systemic.

## 5. Conclusions

Aluminum induces entropy in living organisms by disrupting all levels of structure from water molecules through all biosemiotic systems. Entropy-inducing cascades, feedback loops (positive and negative) within and across levels, can damage DNAs, RNAs, proteins, cells, tissues, and whole

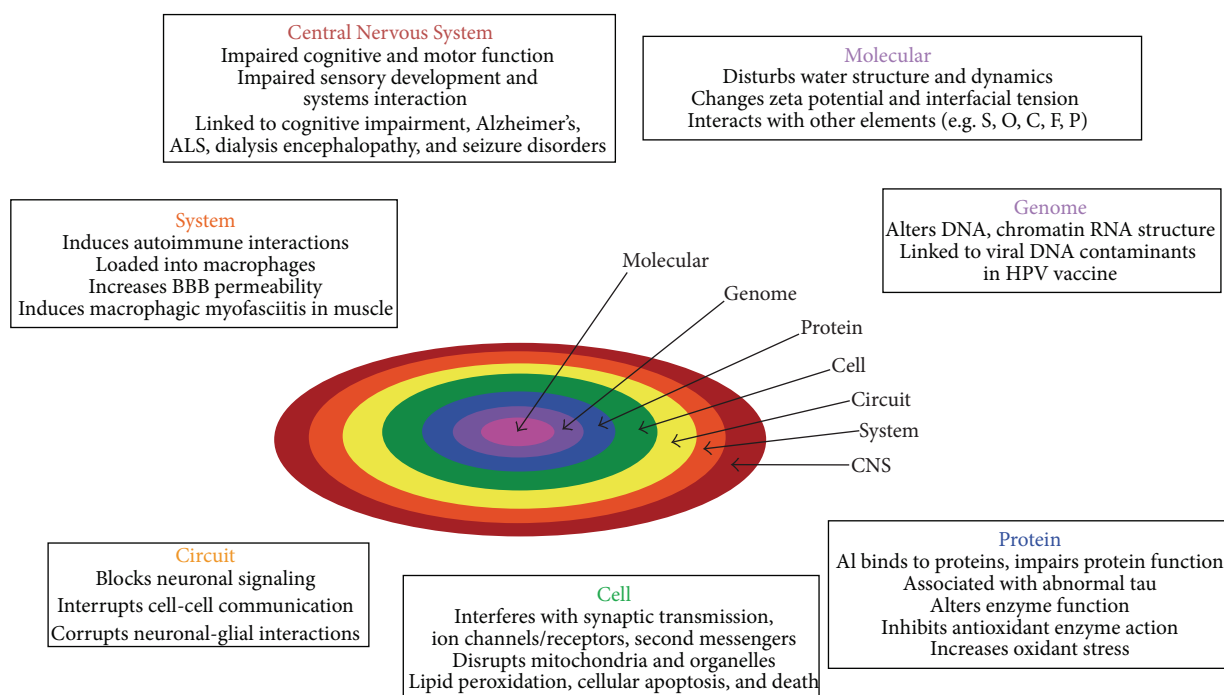


Figure 4: Schematic of the biosemiotic levels at which Al can impact the body and CNS.

organ systems. As a result of cellular damage caused by an Al compound, injured and dying cells will release proteases, excitatory amino acids, and ions (e.g., potassium, calcium), disrupting biosemiosis at many levels. Toxic effects of Al and its compounds thus tend to proliferate. Interactive results involving immune functions, for instance, make the impact worse than if only one system were involved. Of course, the dose-response of Al and its compounds must be considered, but even at low doses, especially with repeated exposures, Al can have cumulative deleterious effects that can be extreme and even fatal. For that reason, a repeated low dose exposure may prove more damaging than a single larger dose. Al and its compounds can cross biosemiotic levels, damaging genetic systems, proteins, cells, and all systems up through the CNS. While higher doses may rapidly affect multiple levels, as in dialysis-associated encephalopathy (DAE), low doses over time, for example, from vaccines, can degrade metabolism and disrupt repair and defense systems and can spiral out of control as in ASIA. Al adjuvants in vaccines may hyperdrive the immune functions of the body but they also directly disrupt biosemiotic systems. Sound theory, empirical research, and reasonable inferences from sources cited here show that Al and its compounds damage biological systems. Such conclusions warrant considerations at a policy level to limit human exposure to Al and its compounds.

## Highlights

- (i) Aluminum ( $\text{Al}^{3+}$ ), suspected as a toxicant for 100 years, injures the CNS and immune systems, individually and synergistically.

- (ii)  $\text{Al}^{3+}$  disrupts biological water dynamics and macromolecules: DNAs, RNAs, proteoglycans, and proteins.
- (iii)  $\text{Al}^{3+}$  disrupts H-bond cooperativity interfering with the quantum coherence of living systems.
- (iv)  $\text{Al}^{3+}$  interferes with biological signaling—biosemiosis—from the very lowest to the highest levels in the nervous system.
- (v) The effects are synergistic with other toxicants, including mercury, lead, fluoride, and glyphosate.

## Disclosure

Christopher A. Shaw, Stephanie Seneff, Stephen D. Kette, Lucija Tomljenovic, John W. Oller Jr., and Robert M. Davidson are equal first authors.

## Conflict of Interests

The authors declare that there is no conflict of interests.

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## Are there negative CNS impacts of aluminum adjuvants used in vaccines and immunotherapy?

In spite of a common view that aluminum (Al) salts are inert and therefore harmless as vaccine adjuvants or in immunotherapy, the reality is quite different. In the following article we briefly review the literature on Al neurotoxicity and the use of Al salts as vaccine adjuvants and consider not only direct toxic actions on the nervous system, but also the potential impact for triggering autoimmunity. Autoimmune and inflammatory responses affecting the CNS appear to underlie some forms of neurological disease, including developmental disorders. Al has been demonstrated to impact the CNS at every level, including by changing gene expression. These outcomes should raise concerns about the increasing use of Al salts as vaccine adjuvants and for the application as more general immune stimulants.

**Keywords:** adjuvant • aluminum • autoimmunity • CNS • neurodegeneration • toxicity

### Background

Immune stimulation can occur as the normal response to a foreign pathogen or as an artificial signal designed to stimulate the same immune response. In the latter case, some compounds used in vaccinology, termed ‘adjuvants’, have been widely used as immune stimulants and have conventionally been considered safe [1]. Of these, the most widely used have been the various salts of Al, used for almost 90 years (since 1926) in a great variety of vaccines [2]. Al salts have also been used in allergy therapy for many decades under the assumption of safety, although convincing data for the latter are still lacking [3]. Al adjuvants act as vehicles for the presentation of antigens not only in the benign sense since they are capable of stimulating pathological immune and inflammatory responses even in the absence of an antigen.

Al is both immuno- and neuro-toxic and in the last decade, studies on animal models and humans have indicated that Al adjuvants have an intrinsic ability to inflict immune and inflammatory responses [4–7]. Notably, the vast majority of adverse manifestations experimentally triggered by Al in animal models, and

those associated with administration of adjuvanted vaccines in humans, are neurological or neuropsychiatric [4,6–10]. In this context, recent experiments have revealed that Al adjuvant compounds have a unique capacity to cross the blood–brain and blood–cerebrospinal fluid barriers and incite deleterious immunoinflammatory responses in neural tissues [4,11]. In spite of these data, it is currently maintained by both the pharmaceutical industry and drug regulating agencies that the concentrations at which Al is used in vaccines does not represent a health hazard [12,13]. In the current review we have provided an overview of what is currently known about Al adjuvants, in particular, their modes of action and mechanisms of potential toxicity. We have further addressed the most common misconceptions regarding the safety of Al compounds as adjuvants and the implication of Al’s toxicity in the context of present vaccination and immunotherapy-based medicinal applications.

### Bioavailability of aluminum & its impact on the CNS

As widely cited, Al is the most common metal and the third most abundant element in the

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earth's crust [14,15]. In spite of this ubiquity, it has not been widely bioavailable until relatively recent historical periods [16–18] and, perhaps for this reason, seems to have no beneficial role in the biochemistry of any biota [19,20].

The industrial extraction of Al after the early 1800s, primarily from bauxite ore, made it possible to bring Al into a variety of applications from food processing, manufacturing, medicines, dyes, cosmetics, antiperspirants, sun screens and many others [21–23]. One notable addition in recent years has been the widespread use of Al cans and Al foil to store various beverages and food items. Some of these substances are quite acidic and in the absence of adequate or complete coating of the cans, can cause Al to leach into the liquid [24]. Similarly, parenteral nutrition solutions are liable to contamination with Al, particularly from acidic solutions in glass vials, such as calcium gluconate. Because of this, the UK Medicines and Healthcare regulatory Authority (MHRA) recently issued the advice that calcium gluconate in small volume glass containers should not be used for repeated treatment in children <18 years, including in the preparation of parenteral solutions [20]. The advice from the UK MHRA is particularly relevant in light of the findings by Fewtrell *et al.* who found that parenterally fed preterm infants retain >75% of the Al, with high serum, urine and tissue levels [20]. Notably, the same research group found that preterm infants exposed for >10 days to standard parenteral solutions had impaired neurologic development at 18 months [25]. At 13–15 years, subjects randomized to standard parenterals had lower lumbar spine bone mass; and, in nonrandomized analyses, those with neonatal Al intake above the median had lower hip bone mass [20,26]. Altogether, these studies demonstrated long-term adverse effects of Al on neural development and bone health.

Concerns about the toxicity of ingested Al were expressed over 100 years ago [27], long before it became as widely used as it is today. Nonetheless, it has long been assumed that dietary Al is the main risk source of exposure to biologically available Al. Such false assumptions naturally lead to under-estimation or, even worse, neglect of other sources and routes of Al exposure such as that through skin (i.e., via antiperspirants), nose (via aerosols), as well as medicinal applications (i.e., parenteral feeding solutions and vaccinations) [28,29]. There are thus clearly different routes of Al exposure and what must be emphasized is that these are not necessarily equivalent with regard to the amount of Al delivered per unit of time. For example, although it is commonly assumed that children obtain much more Al from diet than from vaccinations [12], this notion contradicts basic toxicological principles. For instance, the route of exposure that bypasses the

protective barriers of the gastrointestinal tract and/or the skin will likely require a lower dose to produce a toxic outcome [28]. In the case of Al, only approximately 0.25% of dietary Al is absorbed into systemic circulation [30]. In contrast, Al hydroxide (the most common adjuvant form) injected intramuscularly may be absorbed at nearly 100% efficiency over time [31].

Similarly to vaccine-derived Al compounds, Al absorbed across the lung or olfactory epithelia by default bypasses the liver and kidney clearance route before encountering the blood–brain barrier. In addition, Al that gains access into the human body through the olfactory system bypasses the defenses of the blood–brain barrier and has direct accesses to the entorhinal cortex and the hippocampal region of the brain, areas which are most vulnerable to neuronal degeneration associated with Alzheimer's disease [17,28]. Consistent with this observation, abnormally high levels of Al are routinely found in Alzheimer's brains, with up to fourfold the level of healthy controls. Indeed, sensitive quantifying techniques demonstrate that perikarya of pyramidal cells of the hippocampus and entorhinal cortex are foci where Al accumulation is most pronounced while interneurons are spared [32–34]. Furthermore, several studies have examined the effects of Al on the nervous system function in workers involved in Al production and thus chronically exposed to Al fumes. The findings of such studies suggested a likely role of the inhalation of Al dust in preclinical mild cognitive disorders, which might precede Alzheimer's disease or Alzheimer's-like neurological deterioration [35,36].

In regards to dietary Al intake, it is estimated that humans routinely ingest between 2–25 mg per day amounting to 14–175 mg per week [15,37–39]. In urban societies, the intake can exceed 95 mg per day [38], or, 665 mg per week. Because of an increasing consumption of Al-containing convenience foods, in 2006 the Food and Agriculture Organization/WHO Joint Expert Committee on Food Additives [40] amended their provisional tolerable weekly intake for Al from 7 mg per kilogram of bodyweight (amounting to 490 mg per week for an average 70 kg human) to one-seventh of that amount (70 mg per week for a 70 kg human). The Committee concluded that, “Al compounds have the potential to affect the reproductive system and developing nervous system at doses lower than those used in establishing the previous provisional tolerable weekly intake” [40]. Using the estimated intake in urban settings of the higher end of the spectrum of Al consumption referred to above (i.e., 175–665 mg Al per week), it would appear that the ‘average’ consumers weighing 70 kg consume between three- to ten-times the provisionally estimated safe weekly amount of Al according to the standard set by the WHO.

Among the adverse CNS issues in humans linked to Al exposure are: dialysis associated encephalopathy [41,42], autism spectrum disorders [10,43–46] and Alzheimer's, Parkinson's disease and related dementias [17,18,47,48], the latter usually seen in aged adults. Al's toxic effects can manifest as impaired psychomotor control, altered behavior (i.e., confusion, anxiety, repetitive behaviors, sleep disturbances, deficits of speech, concentration, learning and memory) and seizures [17]. Experiments on cats demonstrated that Al induces neurofibrillary degeneration when present at levels detected in Alzheimer's patients [49]. This physiological effect was associated with observed impairment in short-term memory and acquisition of a conditioned avoidance response [50].

At a genomic level, Al also causes alterations in DNA transcription. In particular, at nanomolar concentrations, Al inhibits brain-specific gene transcription from selected AT-rich promoters of human neocortical genes [51]. Al's repressive action on gene transcription is linked to its ability to decrease the access of transcriptional machinery to initiation sites on the DNA template by enhancing chromatin condensation [52,53]; or interfering with ATP-hydrolysis-powered separation of DNA strands either indirectly (by binding to phosphonucleotides and increasing the stability and melting temperature of DNA [51,54]) or directly (by inhibiting the ATPase-dependent action of RNA polymerase [51]). These effects were experimentally demonstrated at physiologically relevant Al concentrations (10–100 nm [51,55]) and at levels that have been reported in Alzheimer disease patients' chromatin fractions [56]. It is particularly interesting to note that in spite of its overall repressive action on some gene expression, Al can also promote transcription. Al promotes lipid peroxidation and oxidative stress and in this way activates the reactive oxygen species-sensitive transcription factors, HIF-1 and NF- $\kappa$ B, and augments specific neuroinflammatory and proapoptotic signaling cascades by driving the expression from a subset of HIF-1 and NF- $\kappa$ B - inducible promoters [57,58]. Out of eight induced genes upregulated in cultured human neurons by 100 nm Al sulfate (the same compound that is used as a flocculant in water), seven showed expression patterns similar to those observed in Alzheimer's, including HIF-1/NF- $\kappa$ B-responsive A $\beta$ PP, IL-1 $\beta$  precursor, NF- $\kappa$ B subunits, cPLA<sub>2</sub>, COX-2 and DAXX, the latter a regulatory protein known to induce apoptosis and repress transcription [58]. Both HIF-1 and NF- $\kappa$ B are upregulated in Alzheimer's disease where they fuel the proinflammatory cycle, which leads to further exacerbation of oxidative stress and inflammation, culminating in neuronal death [59].

In light of the above data, we selected 18 candidate genes that are involved in neural functions and

innate immune response [60]. In preliminary studies we measured the expression levels of these genes using semiquantitative RT-PCR in brain samples from three CD-1 male controls and three mice injected subcutaneously (s.c.) with Al. The CD-1 mouse model is a good model for toxicity studies as these are heterozygous outbred mice, which is thus representative of the heterozygous human population. In total, seven genes showed changes in expression. Some of the activators and effectors of immunoinflammatory response were significantly upregulated, including IFNG, TNF, chemokine CCL2 and LTB, while the inhibitors of immune reaction NF- $\kappa$ B (inhibitor of NF- $\kappa$ B), complement component C2 and a gene controlling the regulation of the degradative enzyme for the neurotransmitter acetylcholine (acetylcholinesterase), were significantly downregulated (**Figure 1A & B**). In five out of these seven genes, the analysis of the corresponding protein levels showed significant changes in expression: IFNG, TNF and CCL2 were upregulated while NF- $\kappa$ B and acetylcholinesterase were downregulated (**Figure 1C & D**).

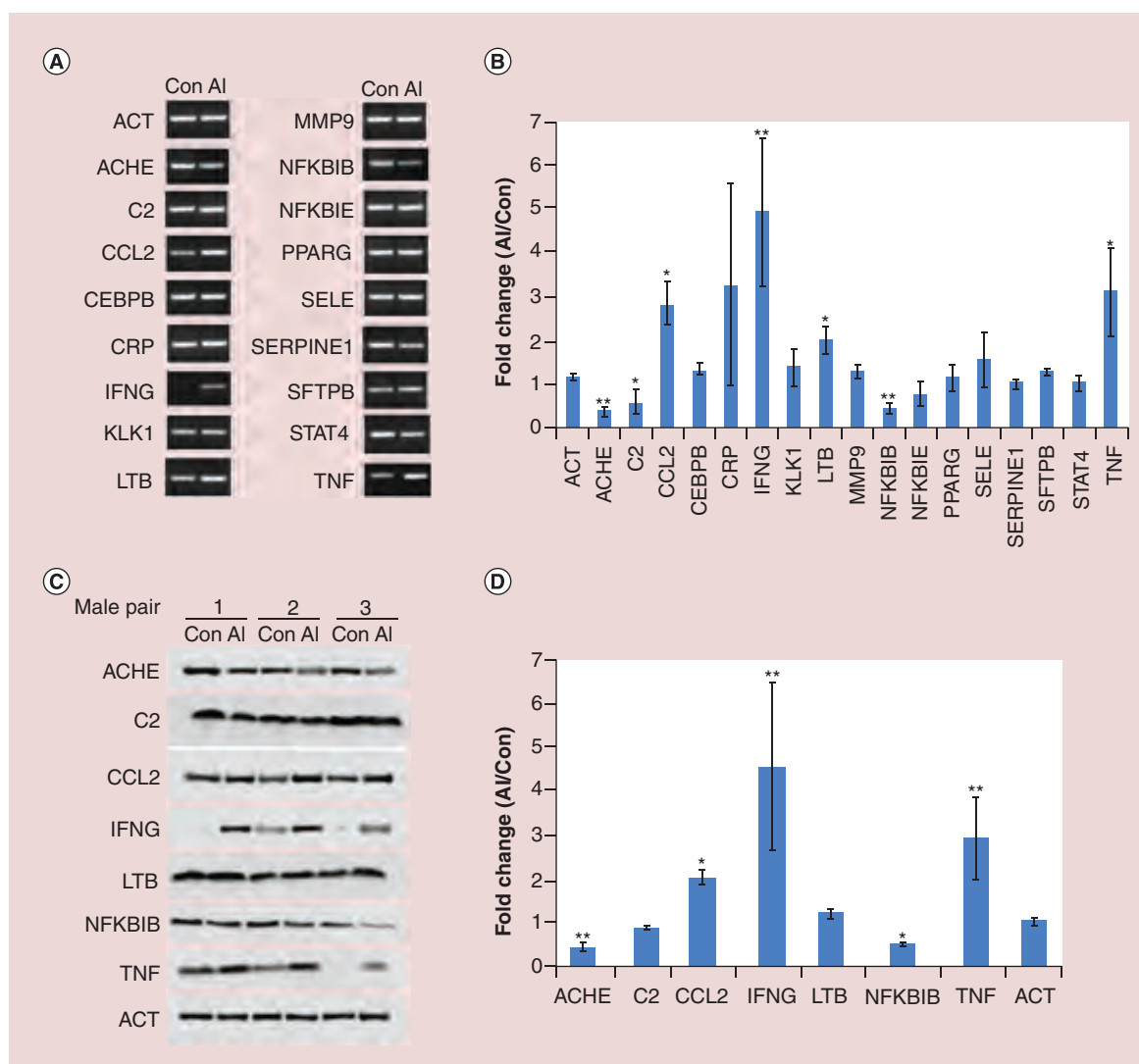
Although it is still premature to make definitive conclusions given the relatively small sample size, these results suggest that an immunoinflammatory response was activated and neural activity decreased by Al injection. Moreover, our results are in agreement with Lukiw *et al.* who demonstrated upregulation of NF- $\kappa$ B responsive and proinflammatory genes by nanomolar Al treatment [58].

Altogether, the gene-expression studies following Al treatment point to a greater complexity than perhaps previously anticipated. Not only can Al evoke direct neural damage and trigger activation of adverse immune-mediated signals, but it can also directly influence gene expression, thus triggering more complex interactions between genes and toxins. Insofar as the latter may be correct, it will be highly important in the future to determine where in the human lifespan can Al impact gene expression and how long such changes might last.

### Key aspects of Al chemistry in relation to biological molecules

Owing to its 3<sup>+</sup> charge, Al attracts negatively charged ions and electrons, but because it cannot transition to other oxidation states besides 3<sup>+</sup>, Al is not a direct component in any redox reactions, but may participate indirectly in Fenton reactions [61,62].

Moreover, the small ionic radius and the high charge of Al<sup>3+</sup> are its important properties by which this metal can exert its toxic activity. The Al ion (0.054 nm) is roughly the same size as the ferric (Fe) ion (0.065 nm) and much smaller than magnesium (Mg; 0.072 nm)



**Figure 1. Aluminum administered to mice in vaccine relevant dosages alters the expression of genes involved in immunoinflammatory response and neural function.** (A) Aluminum-induced gene-expression alterations in the brains of male CD-1 mice. The expression levels of seven neural and innate immunity-related genes were significantly altered in aluminum-injected compared to control male mice as determined by semiquantitative RT-PCR analyses.  $\beta$ -actin was used as the internal standard. (B) Quantification of the expression change shown in (A). Data are presented as fold difference as compared with controls. Histograms report the mean  $\pm$  standard error of the mean of three independent experiments determined by densitometry. \* $p < 0.05$ ; \*\* $p < 0.01$ . (C) The protein levels of the seven genes with altered expression levels after aluminum injection were verified by western blots.  $\beta$ -actin was used as internal standard. (D) Quantification of the protein level change shown in (C). Data are shown as mean signal intensity  $\pm$  standard error of the mean of three independent experiments. \* $p < 0.05$ ; \*\* $p < 0.01$ . Al: Aluminum-injected male brains; Con: Saline control males.

and calcium (Ca) ions (0.100 nm). Thus in biological systems, Al can effectively replace these essential bio-metals in many enzymatic reactions [63–68]. For example, Al binds the extracellular iron carrier transferrin [69–71] which in turn, may facilitate its own transport across the blood–brain barrier [17]. Furthermore, owing to its greater affinity for anionic groups, Al potently interferes with reactions that depend on reversible dissociation. Processes involving rapid  $\text{Ca}^{2+}$  exchange are inhibited by Al substitution [63,67,68,72]. Similarly, at

nanomolar concentrations, Al inhibits many  $\text{Mg}^{2+}$  and ATP-dependent enzymes, including tubulin GTPase [66]  $\text{Na}^+ \text{K}^+$  ATP-ase [73], hexokinase [69,74], RNA polymerase [51,55,75], choline acetyltransferase [76–78], ferroxidase (ceruloplasmin [79]) and calmodulin-dependent ATPase [65,67,72], as it binds ATP in a complex that is several orders of magnitude more stable than that with Mg (the association constant for  $\text{Al}^{3+}$  is  $10^7$ -times that of  $\text{Mg}^{2+}$  [66]). Al also binds other nucleotides (GTP and CTP) [80] as well as phosphate headgroups of lipid moi-

eties in membrane systems. Apart from altering membrane properties [81], Al has the potential to interfere with any reaction that requires phosphoryl transfer and ATP/GTP hydrolysis [63,64,72].

Given the ubiquity of enzymatic systems and signaling cascades that depend on G-protein signaling, phosphorylation, ATP, GTP, calcium, magnesium and iron, the spectrum of physiological processes that can be adversely affected by Al is extremely vast. In spite of this, in the absence of chronic renal failure, the toxic effects of Al (especially at low doses) appear to be primarily manifested in the brain [4,15,32,33,69,70,72,81–95]), although in vulnerable populations such as infants, prolonged exposure to both high and low doses of Al may also lead to metabolic bone disease [26,96]. Furthermore, Al neurotoxicity appears to be compartmentalized as highly sensitive imaging techniques, as well as methods for quantifying focal accumulations of Al, repeatedly show that Al associates with specific brain regions and cellular compartments, primarily those associated with memory processing and cognitive function [32–34,82,85,97–103].

There are estimated to be 20,000–25,000 protein coding genes in the human genome [104] and even more variant proteins, up to 100,000, that seem to be possible through post-translational modifications. Given this, there are many macromolecules with which  $\text{Al}^{3+}$  species can interact. For example, eukaryotic proteins are polypeptides of various combinations and lengths composed of an array of 23 amino acids joined by peptide bonds. Each of the 23 amino acids has a unique side chain consisting of various organic substituents. Al can interact with the side chains [105], some of which – serine, threonine and tyrosine – are phosphorylated, enabling phosphoregulation of enzyme activity and binding with other proteins. Al can disrupt all of these side chains and the processes dependent on them [106].

In summary, for all the above reasons, Al cannot be considered as ‘inert’, nor biologically harmless.

### Al, vaccines & vaccine adjuvants

While the bulk of human exposure to Al typically comes from diet, a less obvious but nonetheless not negligible source may be from Al adjuvants used in vaccines. These may include vaccines against diphtheria, tetanus, pertussis, hepatitis B, anthrax, *Haemophilus influenzae* and human papillomavirus (HPV), among others [12,107,108]. In western countries, a typical child may be injected with as much as 4.225 mg of elemental Al by the age of 12 months [109]. Our review of currently licensed vaccine package inserts in the US is consistent with this figure. For example, according to the standard US vaccination schedule, every vaccinated child will receive a total of 5–6 mg of Al by the

age of 2 years, or up to 1.475 mg of Al during a single visit to the pediatrician [17].

Mitkus *et al.* [109] reported that this dosage is within the US Agency for Toxic Substances and Disease Registry’s minimum risk levels for infants, extrapolating data from a volunteer study of adults using a radioactive Al tracer [110] and a toxicokinetic study performed on rabbits [111]. The authors used the creatinine clearance differential between children and adults to estimate total Al body burden for infants following vaccination [109]. This estimation appears to have been based upon an assumption that Al excretion parallels creatinine clearance, an assumption that is simply incorrect both on theoretical and experimental grounds. Indeed, creatinine clearance in urine is used as a marker for water clearance and it is extremely unlikely that Al excretion follows water. Moreover, rapid excretion of Al would nullify the very basis of having it as an adjuvant in the first place. In particular, although the half-life of enterally or parenterally absorbed Al from the body is short (approximately 24 h), the same cannot be assumed for Al adjuvants as in vaccines. Indeed, experiments in adult rabbits demonstrate that Al hydroxide, the most commonly used adjuvant and immunotherapy Al salt, is poorly excreted. The cumulative amount of Al hydroxide excreted in the urine of adult rabbits as long as 28 days post intramuscular injection was less than 6% as measured by accelerator mass spectrometry [112].

Further research studies show that other than with antigens, Al can form unexpected complexes with other vaccine excipients. Recently, Lee explored the melting profiles of the residual HPV L1 gene DNA contaminant which was detected in the quadrivalent HPV vaccine Gardasil [113]. This quadrivalent vaccine contains genotype-specific L1 capsid proteins of four HPV strains (HPV-16, -18, -6 and -11) in the form of virus-like particles as active ingredients in addition to the Al adjuvant. Because viral DNA fragments if present in the vaccine may bind to the insoluble Al adjuvant (as well as free  $\text{Al}^{3+}$ ), Lee [113] developed a PCR-based test for HPV L1 gene DNA detection in the final products of Gardasil. The results showed that all samples tested (a total of 16 Gardasil vials) contained residues of the synthetic HPV-11 L1 gene DNA and/or HPV-18 L1 gene DNA. At least seven of the 16 samples also contained HPV-16 L1 gene DNA which was amplified by a pair of modified nondegenerate primers [113]. Notably, the specific melting profile of the HPV-16 L1 gene DNA detected in Gardasil vials was similar to that of the HPV-16 L1 gene DNA recently discovered in the post-mortem blood and spleen of a young woman who suffered a sudden unexpected death 6 months following Gardasil vaccination [114,115]. Collectively, the findings by Lee suggest that the insoluble Al–HPV DNA



complexes may persist in the bodies of vaccine recipients long-term after injection (i.e., up to 6 months), thus perhaps increasing the risk for adverse immune responses [115].

In summary, one of the reasons for the long retention of Al adjuvants in bodily compartments, including systemic circulation, may be due to its tight association with the vaccine antigen or other vaccine excipients (i.e., contaminant DNA). Even dietary Al has been shown to accumulate in the CNS over time, producing Alzheimer's disease type outcomes in experimental animals fed equivalent amounts of Al to what humans consume through a typical western diet [83,116].

### Macrophagic myofasciitis: the Al adjuvant syndrome

The long retention of Al adjuvants was first identified and thereafter extensively studied in macrophagic myofasciitis (MMF) patients. MMF is a condition characterized by highly specific myopathological alterations at deltoid muscle biopsy, first recognized in 1998, and subsequently shown to be due to long-term persistence of vaccine-derived Al compounds within macrophages at the site of previous vaccination – up to 8 to 10 years post injection [6,7,117,118]. Patients diagnosed with MMF tend to be female (70%) and middle-aged at the time of biopsy (median 45 years), and having received one to 17 intramuscular Al-containing vaccine administrations (mean 5.3) in the 10 years before MMF detection [8].

Clinical manifestations in MMF patients include diffuse myalgia, arthralgia, chronic fatigue, muscle weakness and cognitive dysfunction. In particular, up to 93% of patients suffer from chronic fatigue (over 6 months in duration [119]) and up to 89% from chronic diffuse myalgias (over 6 months in duration) with or without arthralgias [8]. Fatigue is disabling in 87% and affects patient's physical and mental functioning in 53% of cases [119]. Overt cognitive alterations affecting memory and attention are manifested in 51% of cases [8]. In addition to chronic fatigue syndrome, 15–20% of patients with MMF concurrently develop an autoimmune disease, the most frequent being multiple sclerosis-like demyelinating disorders, Hashimoto's thyroiditis and diffuse dysimmune neuromuscular diseases, such as dermatomyositis, necrotizing autoimmune myopathy, myasthenia gravis and inclusion body myositis [8]. Even in the absence of overt autoimmune disease, low titers of various autoantibodies, increased inflammatory biomarkers and abnormal iron status are commonly detected [120].

The pathological significance of the MMF lesion has long been poorly understood because of the lack of an obvious link between the persistence of Al agglom-

erates in macrophages at sites of previous vaccination and delayed onset of systemic and neurological manifestations. Nonetheless, that the MMF lesion is linked to a systemic illness was strongly suggested by the fact that a statistically significant association was found between chronic myalgias and fatigue, and the presence of MMF lesions at muscle biopsy in patients. In particular, using electron microscopy, Gherardi *et al.* [118] detected intracytoplasmic crystalline inclusions typical of the MMF lesion in 40 out of 40 MMF cases and 0 out of 80 controls who suffered from other, MMF-unrelated multisystemic chronic diseases (dermatomyositis or muscle dystrophy). Diffuse myalgias were more frequent in patients with MMF lesions than those without ( $p < 0.0001$ ).

Medical histories of these cases showed that 50 out of 50 (100%) MMF patients received 1–9 (median 4) doses of Al-containing vaccines within 10 years prior to biopsy. Delay from the last vaccination to biopsy ranged from 3 months to 8 years (median 36 months). Myalgia onset was subsequent to the vaccination (median 11 months) in 94% of patients. Al-containing vaccine administration was carried out prior to onset (44 patients) or worsening (two patients) of myalgias (46 out of 47, 98%). A total of 30% of patients developed myalgias within 3 months after vaccination, 61% within 1 year and 80% within 2 years. A total of 34% of MMF patients also had a concurrent autoimmune disease [118].

Additionally, Gherardi *et al.* reported the MMF rate of detection in vaccinated patients [118]. In 113 patients with various neuromuscular disorders and previous vaccination with Al-containing vaccines who underwent a deltoid muscle biopsy, 97 (87%) had no detectable MMF lesions, and 16 (13%) had. The delay from immunization to biopsy could be established on the basis of the vaccination booklet in the 16 MMF+ patients and in 81 MMF– patients. The status MMF+ or MMF– could not be attributed to a difference in the delay from immunization to biopsy, this delay being strictly similar in both groups (MMF+ range: 12–96 months, median: 42 months; MMF– range: 3–96, median: 42; MMF+ vs MMF–  $p =$  not significant). Out of 16 prospectively detected MMF+ patients, 15 (94%) had typical arthromyalgias and chronic fatigue.

Taken together, these data make a merely coincidental association of MMF with chronic myalgias very unlikely. Moreover, in the series of cases investigated by Gherardi *et al.*, MMF lesions constantly included a lymphoid component ranging from lymphoplasmacytic infiltrates to organized tertiary lymphoid tissue, assessed in an ongoing immunological process at time of biopsy [118]. A persistent systemic immune activation that fails to 'switch off' has been regarded as the possi-

ble cause of chronic fatigue and arthromyalgias [121,122], through a sustained release of inflammatory cytokines and production of autotoxic T cells and autoantibodies [123–125]. Consistent with this interpretation, Gherardi *et al.* [118] noted that MMF patients have B-cell hyperlymphocytosis and higher IL-6 circulatory levels than healthy vaccinated controls as well as detectable circulating antinuclear and anti-phospholipid autoantibodies (50%). These data suggest that MMF may be associated with a shift of immune responses towards a Th-2 profile, which is typically induced by Al hydroxide [126], and which probably contributes to the emergence of chronic fatigue and associated manifestations [127].

In summary, these experimental observations cited above demonstrate that not all subjects vaccinated with Al containing-vaccines develop MMF lesions. However, these studies also show that MMF pathology constitutes a systemic illness (with myalgias, arthralgias, chronic fatigue and autoimmune manifestations), rather than a mere local injection-site reaction. Consistent with this hypothesis are the findings of a case–control study on MMF by Bonnefont-Rousselot *et al.*, aimed at determining the presence of oxidative stress in MMF patients [128]. A total of 30 MMF cases (nine males, 21 females; aged  $42 \pm 14$  years), whose diagnosis was confirmed by deltoid biopsy, have been included and compared with 38 sex- and age-matched healthy controls (ten males, 28 females; aged  $43 \pm 8$  years). The blood oxidative stress status was evaluated by assaying six parameters: plasma lipid peroxidation products (thiobarbituric acid-reactive substances) and antioxidant defense systems (plasma vitamin E and glutathione peroxidase (GSH-Px) activity, erythrocyte GSH-Px and SOD activities). The results showed significantly lower levels of plasma GSH-Px activity, selenium and vitamin E concentration in the MMF group compared with the controls ( $p = 0.004$ ,  $p = 0.003$  and  $p = 0.009$ , respectively), with a positive correlation in MMF patients between plasma GSH-Px activity and selenium concentration ( $p = 0.0001$ ). Given that Al is a well-known pro-oxidant [89], it should not be surprising to find evidence of oxidative stress in MMF. In summary, the case–control studies by Gherardi *et al.* [118] and Bonnefont-Rousselot *et al.* [128] both show that MMF constitutes a systemic pathology rather than simply a presence of a benign localized Al-rich muscle lesion as often incorrectly asserted.

### MMF-associated cognitive dysfunction

As mentioned above, 51% of MMF patients suffer from cognitive alterations [8]. Notably, the MMF-associated cognitive dysfunction (MACD) is a unique MMF-specific phenomenon that provides further evidence for the multisystemic nature of MMF. In particular, unlike other chronic pain syndromes where neuropsychological

impairment results from the nonspecific combination of pain, fatigue and depression, MACD seems to reflect a more specific condition, not correlated with pain, fatigue or depression, and independent of symptom duration [6]. This point is of special importance since physicians frequently ascribe cognitive impairment in MMF patients to depression. Thus, although frequently disabling, MACD is often underestimated and underdiagnosed by routine examinations. A comprehensive battery of neuropsychological tests in MMF patients without multiple sclerosis showed alterations in all individuals, consistent with mild cognitive impairment but including at least one test reaching the dementia threshold in 96% of cases [7]. Compared with arthritis controls matched for pain severity and duration, depression and educational level, MMF patients displayed distinctive impairment of visual memory, working memory and dichotic listening, a pattern suggestive of cortico-subcortical organic damage involving fronto-parieto-thalamo-striatal areas, with deep white matter alterations [7].

Although MMF patients do not fulfill the Diagnostic and Statistical Manual of Mental Disorders, Fourth Edition (DSM-IV) criteria for dementia, they do present with a notable cognitive complaint. In particular, their neuropsychological evaluation reveals abnormal cognitive performance by age in some domains, consistent with the diagnosis of mild cognitive impairment (MCI) [6]. MCI is a diagnostic entity that was initially set up to identify patients with Alzheimer's disease at a very early point in the cognitive decline [129]. The term MCI has more generally been used to refer to cognitive dysfunction of insufficient severity to constitute dementia [130]. In applying recent diagnostic scheme for MCI to a cohort of MMF patients, Passeri *et al.* found that the majority of them fulfilled the criteria for MCI of non-amnesic type, most often of a multidomain nature [6]. However, in most MMF patients, cognitive deficits were sufficiently severe that the term 'mild' seemed rather inappropriate, thus leading to introduction of a new MCI subtype, referred to as 'severe MCI', in order to characterize patients with lower cognitive deficits [6]. In MMF patients, cognitive dysfunction caused major disability, both in professional skills and daily life [6,7]. This feature is reminiscent of recent observations in very mild Alzheimer's disease, where the dysexecutive phenotype was associated with more problem solving difficulties than the predominant amnesic phenotype [131].

### Biodistribution of poorly soluble Al-adjuvant nanoparticles across the blood–brain barrier: evidence for understanding the systemic nature of MMF

Until recently, the cognitive dysfunction in MMF patients has been largely ignored or downplayed by



the medical community despite the fact that MMF remains the most thoroughly investigated post-vaccination condition in which a mechanistic link with Al adjuvants has now been described. In particular, recent experiments in animal models have revealed that a proportion of injected vaccine-derived Al compounds does not stay localized at the site of injection but rather, escapes the muscle mainly within immune cells, thus gaining access to regional lymph nodes. Thereafter, Al-loaded cells exit the lymphatic system, reach the bloodstream (presumably through the thoracic duct) and eventually travel to distant organs including the spleen, liver and the brain, where Al deposits are detected up to 1 year following injection [11]. The neurodelivery of Al adjuvants as well as surrogate compounds (nanoparticle fluorescent surrogates) to the mouse brain was found to be dependent on the monocyte chemoattractant protein 1 (MCP-1/CCL2) as intramuscular injection of murine rCCL2 strongly increased particle incorporation into intact brain while CCL2-deficient mice had decreased neurodelivery.

Regarding the latter finding, the most recent publication by Cadusseau *et al.* shows that selective elevation of the MCP-1/CCL2 chemokine may represent a biological marker relevant to the pathophysiology of MMF. This outcome again points to a systemic nature of MMF pathology. In particular, Cadusseau *et al.* performed extensive cytokine screening on the sera from 44 MMF patients and on the sera of sex- and age-matched healthy controls as well as the sera of patients with various types of inflammatory neuromuscular diseases [132]. Thirty cytokines were quantified using a combination of Luminex® technology and ELISA. There was a significant mean increase of serum levels of MCP-1/CCL2 in MMF patients compared with healthy subjects. MMF patients showed no elevation of other cytokines, a result which contrasted with the findings in inflammatory disease patients in whom CCL2/MCP-1 serum levels were unchanged, whereas several other inflammatory cytokines were elevated (IL1 $\beta$ , IL5 and CCL3/MIP1 $\alpha$ ).

In addition to macrophage-mediated delivery described above, there is a growing body of data to suggest that adjuvant Al is biosequestered by albumin, transferrin and within macrophages of the reticulo-endothelial system after intramuscular injection. According to Ganrot [133], insoluble metal hydroxides are thought mainly to be taken up by the reticulo-endothelial cells, while soluble salts of trivalent ions are mainly bound to the skeleton or excreted in the urine. Ubiquitous heparan sulfate proteoglycans, which decorate the glycocalyxes of our cell membranes, are likely to act as multidentate chelators or biosequestrants of Al [134].

A study on rabbits by Flarend *et al.* [111] showed that absorption following intramuscular Al particulate injections into the blood was not instantaneous, as only some of the Al was absorbed from the injection depot over the first 28 days. These data are supported by the Khan *et al.* [11] study suggesting that the initial trajectory for Al hydroxide from the muscle is into the lymphatic system carried by circulating macrophages. Such findings refute the notion that adjuvant nanoparticles remain localized and exert their immunostimulation through a 'depot effect'. On the contrary, Al from vaccine adjuvants can cross the blood-brain and blood-cerebrospinal fluid barriers and incite immunoinflammatory responses in neural tissues [4,135–137].

These outcomes led Khan *et al.* to suggest that repeated doses of Al hydroxide are 'insidiously unsafe', especially in closely spaced immune challenges presented to an infant or a person with damaged or immature blood-brain or blood-cerebrospinal fluid barriers [11]. Given macrophages acting as highly mobile 'Trojan horses' [8], the warning by Khan *et al.* suggests that cumulative Al from repeated doses in vaccines may produce the cognitive deficits associated with long-term encephalopathies and degenerative dementias in humans [11].

In keeping with the above studies on Al adjuvants and their impact in the CNS, Lujan *et al.*, described a severe neurodegenerative syndrome in commercial sheep linked to the repetitive inoculation with Al-containing vaccines [137]. In particular, the 'sheep adjuvant syndrome' mimics in many aspects human neurological diseases linked to Al adjuvants. Moreover, the outcomes in sheep were first identified following a mass-vaccination campaign against blue tongue and have now been successfully reproduced under experimental conditions following administration of Al-containing vaccines. Notably, the adverse chronic phase of this syndrome affects 50–70% of the treated flocks and up to 100% of the animals within a given flock. The condition is made worse by cold weather conditions, suggesting synergy with other stress producing factors. The sheep syndrome is characterized by severe neuro-behavioral outcomes – restlessness, compulsive wool biting, generalized weakness, muscle tremors, loss of response to stimuli, ataxia, tetraplegia, stupor, inflammatory lesions in the brain and the presence of Al in the CNS tissues, coma and death [137]. These findings extend those of Khan *et al.* who demonstrated the ability of Al adjuvants to cross the blood-brain barrier [11], and they further show that Al in the brain can trigger severe long-term neurological damage.

Other animal models show that subcutaneous injections of Al hydroxide induced apoptotic neuronal death

and decreased motor function in mice [4,136]. In newborn mice they were associated with weight increases, behavioral changes and increased anxiety [10].

Cumulatively, the above data may also explain how and why the vast majority of reported adverse reactions following vaccinations are neurological and neuropsychiatric [6–9].

### Relation of Al adjuvants to autism spectrum disorders?

Recently, we conducted a study to compare the Centers for Disease Control and Prevention (CDC) recommended vaccine schedules for children's vaccines in the US (1991–2008) to changes in autism rates during this same period according to data sourced from the US Department of Education (original references in [46]). The data sets, graphed against each other, showed a pronounced and statistically highly significant correlation between the number vaccines with Al and the changes in autism rates. Further data showed that a significant correlation exists between the amounts of Al given to preschool children and the current rates of autism in seven western countries. Those countries with the highest level of Al-adjuvanted vaccines had the highest autism rates. The observed correlation between the number of Al-adjuvanted vaccines and autism was further tested using Hill's criteria for causality [46] and met eight of nine of these indicating that vaccines containing Al are highly likely to be at least partially causal for autism.

The analyses of the US Vaccine Adverse Events Reporting System (VAERS) database by Seneff *et al.* likewise appears to support the notion that Al in vaccines is one of the environmental risk factors implicated in autism [45]. In this study, the authors noted that reports of autism in VAERS increased steadily at the end of the last century, during a period when mercury (Hg) was being phased out from vaccines, while the Al adjuvant burden was being increased. Using standard log-likelihood ratio techniques, Seneff *et al.* have further identified several signs and symptoms that were significantly more prevalent in vaccine reports after the year 2000 (when removal of Hg from vaccines commenced), including cellulitis, seizure, depression, fatigue, pain and death, which are also significantly associated with Al-containing vaccines [45]. That high Al burden might be an etiological factor in autism is further supported by two other recent studies [43,44]. Melendez *et al.* have shown an elevation of several metals including chromium, arsenic and particularly Al in the blood of autistic children in comparison to the reference values of normal children [43]. Melendez *et al.* have further identified two important factors regarding exposure to toxic metals: in 80% of cases the autistic

children used controlled drugs, and 90% of them had received all recommended vaccines [43]. In addition, 70% of mothers took vaccines and 80% of them ate canned food and fish during pregnancy. Hence the results by Melendez *et al.* suggest that cumulative exposure to Al from dietary and pharmaceutical sources (i.e., Al-containing drugs and vaccines) in early periods of developmental vulnerability (both pre- and post-natal) may contribute to the development of autism spectrum disorders [43].

Finally, Yasuda and Tsutsui recently summarized the results of a metallomics study in which scalp hair concentrations of 26 trace elements were examined for 1967 autistic children (1553 males and 414 females aged 0–15 years old) [44]. In total, 584 (29.7%), 347 (17.6%) and 114 (5.8%) of children were found to be deficient in zinc (Zn), Mg and Ca, respectively. Both Mg and Ca can be displaced by Al in biochemical reactions as discussed above [17]. In addition, there is data suggesting that Al can also displace Zn [138]. Consistent with these observations, a significant proportion of study children were found to suffer from toxic metal overload, chiefly, Al. In particular, 339 (17.2%), 168 (8.5%) and 94 (4.8%) individuals were found with high burdens of Al, cadmium (Cd) and lead (Pb), respectively, and 2.8% or less from Hg and arsenic (As). Notably, high toxic metal burdens were more frequently observed in infants aged 0–3 years old, whose incidence rates were 20.6%, 12.1%, 7.5%, 3.2% and 2.3% for Al, Cd, Pb, As and Hg, respectively. Yasuda and Tsutsui made an important observation regarding the function of Zn and Zn-finger proteins in transcriptional regulation [44]. Namely Zn-finger proteins influence several candidate genes reported to be associated with the development of autism, such as MTF1, metallothionein, ZnT5, COMMD1, ERK1, TrkB and ProSAP/Shank that themselves are involved in Zn signaling and homeostasis. It is thus plausible that Zn deficiency observed in the autistic subjects might induce critical epigenetic alterations that would further interfere with neuronal maturation during early development.

Altogether, the above findings indicate that Al is yet another environmental agent that can now be added to the list of xenobiotics associated with developmental immunotoxicity (as defined by Dietert and Dietert [139]) and thus an important and yet underappreciated risk factor in disorders of the autism spectrum.

Given all of the above, it appears paradoxical that while there has been a concerted effort to reduce the Al burden in parenteral feedings to premature infants (owing to the observation that 4–5 µg/kg per day of Al can be associated with nervous system and bone toxicity), there has been no concern for the increas-

ing load of Al administered to infants through vaccinations [140]. As mentioned above, preterm infants exposed for >10 days to standard parenteral solutions had impaired neurologic development at 18 months of age [25]. At 13–15 years, those randomized to standard parenterals had lower lumbar spine bone mass, and in nonrandomized analyses, those with neonatal Al intake above the median had lower hip bone mass [20]. In spite of this, there seems to be less concern about the potential risks of injected vaccine-derived Al whose clearance from the CNS may be virtually impossible owing to lack of recirculation and progressive accumulation [11].

### Al adjuvants & ASIA syndrome

Shoenfeld *et al.* reviewed the large body of evidence that implicates adjuvant administration preceding the onset of immune-mediated diseases including siliconosis, Gulf War syndrome and MMF syndrome [141]. Collectively, these illnesses present similar clinical features which are now designated as being part of a new syndrome called ‘autoimmune/autoinflammatory syndrome induced by adjuvants’ (ASIA) [9,142,143]. Many of these appear to arise owing to the use of Al adjuvants [8,144]. Outcomes fitting the ASIA criteria have been reported in sheep also following Al adjuvant exposure from vaccines as cited above from the work of Lujan and colleagues [137].

Compelling evidence for a causal role of vaccine adjuvants in triggering serious autoimmune disorders have been presented by Quiroz-Rothe *et al.* who described a case of post vaccination polyneuropathy resembling Guillain-Barré syndrome in a dog [145]. In this case, there was an apparent cause–effect relationship between vaccination and onset of clinical signs associated with the presence of antibodies against myelin. The authors noted that the vaccines used were obtained by cultures in renal cells and did not contain nervous tissue antigens. Thus either viral or other vaccine antigens, or the adjuvants included in the vaccines, might have triggered the formation of antimyelin antibodies by over stimulation of the dog’s immune system. However, the fact that two different vaccines from two different manufacturers were involved strongly suggests a polyclonal activation induced by the vaccine adjuvants without the participation of myelin as the more probable pathogenesis.

Other controlled studies in dogs vaccinated with commercially available rabies and canine distemper vaccines showed a significant increase in the titres of IgG antibodies reactive with ten autoantigens, an effect not observed in unvaccinated dogs [146]. Although molecular mimicry or a ‘bystander activation’ of self-reactive lymphocytes, could be the cause

for these autoimmune manifestations, the relatively large number and variety of auto-antigens observed (as in the cases of autistic children), points to a polyclonal activation or adjuvant reaction. Moreover, this adjuvant effect, associated with the development of a wide range of autoantibodies has been typically associated with vaccines containing higher levels of adjuvants [147].

There are several plausible mechanisms that support the role of Al adjuvants in induction of autoimmunity. Particularly notable in this regard is the well-established research on Al’s crucial role in activating the NLRP3 inflammasome signaling (and its downstream mediators caspase-1 and IL-18) [148–150], which is responsible for the immune adjuvant stimulatory properties of Al. Unfortunately, activation of the NLRP3 inflammasome pathway is also critically involved in the development of chronic autoimmune and inflammatory diseases including Type 2 diabetes, demyelinating diseases of the CNS, inflammatory bowel disease, colitis and atherosclerosis [151–155]. Activation of the inflammasome and its downstream components, proinflammatory cytokines IL-1 $\beta$  and IL-18, is also strongly implicated in promotion of other CNS disorders, including Alzheimer’s disease, Parkinson’s disease and multiple sclerosis [151,152], all of which have independently been linked to environmental Al exposure [17,28,47,156].

Other vaccine adjuvants may be capable of inducing autoimmune reactions in humans as well. Nohynek *et al.* [157] and Partinen *et al.* [158] provided evidence of a significant increase in adolescent narcolepsy in Finland following vaccination with a lipid-based adjuvant in the Pandemrix H1N1 influenza vaccine. These data have now been reproduced in other European countries [159–161]. Whether these outcomes truly reflect negative impacts of the particular adjuvant on the CNS or whether other components of the vaccine alone or in combination with the adjuvant were responsible remains uncertain [159].

### Implications for immunotherapy

The demonstrated impact of Al vaccine adjuvants on both the central nervous and immune systems as cited above make it reasonable to question whether the relatively widespread use of Al salts as general immune stimulants in allergy ‘immunotherapy’ might not also be problematic in the same manner. As recently examined by Exley [3], many of the same considerations apply: Al is neither inert nor harmless in biological systems, it is clearly neurotoxic [4,17–19,162–164] and can readily enter the CNS transported by immune cells [11]. In addition, as Exley points out, Al not only serves to boost antigenic responses, it is

also antigenic on its own [165]. Thus, this dual activity raises questions about how the human body reacts to any future exposures to Al [165]. For example, there is evidence that Al in adjuvants is also acting as an antigen as a significant proportion of vaccine recipients retain a 'memory' of their exposure to Al, showing delayed hypersensitivity to subsequent exposures to Al [166,167]. Thus, vaccination as well as allergen therapies that incorporate Al-based adjuvants may sensitize recipients to adverse outcomes from future exposures to Al.

Exley *et al.* further note that the sensitization to Al may simply be one manifestation of the physiological response to biologically available Al [165]. The biological availability of Al, as defined by its propensity to induce a biochemical response in an affected system, is known to depend upon the establishment over time of a threshold concentration, or burden, of Al [168]. The system (i.e., cell or tissue), copes with the burgeoning burden of Al up until a threshold concentration is reached. The immunological memory of early exposures to biologically available Al may vary widely within the recipients such that thereafter there could be many different biochemical responses to a future exposure to Al. In the case of future Al-adjuvant containing vaccinations, the threshold may be achieved instantaneously in individuals who had retained a memory of their earlier exposure to Al and could instigate severe immune responses with wide ranging health implications [165].

The wider cascade of effects may involve the recruitment of Al antigens in other parts of the body or it may be mediated through other antigens that have been sensitized through their previous coadministration with Al adjuvant. An example of this is the sensitization to food allergens following their coadministration with Al salts. Notably, the immunostimulatory properties of Al have been routinely exploited for inducing mast cell-dependent allergic sensitization to food proteins, which ultimately results in intestinal inflammation and diarrhea [169,170]. Mast cells play key roles in a wide range of inflammatory gastrointestinal pathologies in which they compromise mucosal immunity and increase intestinal permeability [169–171]. Particularly relevant in the context of this review is the fact that gastrointestinal dysfunction and food allergies appear to be the most common non-neurological comorbidities in both ASIA and disorders of the autism spectrum [9,171]. These observations provide further compelling evidence supporting the role of Al adjuvant over-exposure in both of these syndromes [43,45,46,141,144].

In summary, an individual susceptibility to an adverse reaction from Al may be dependent upon

the combination of a previous sensitization to Al, for example, via childhood vaccination, and an ongoing Al overload from all sources [165]. While the body may cope robustly with a mild but persistent exposure to Al, the coping mechanism will be suddenly and dramatically overwhelmed by a new exposure to Al adjuvant. The latter, will not only enhance the antigenicity itself, but it will raise the level of the immune response against all significant body stores of Al. Under these conditions an individual's everyday exposure to Al will continue to fuel the response and many symptoms of associated autoimmunity will occur. The individual will now respond adversely to Al exposures, which previously were not sufficient to elicit a biological response [165]. When we take into account that as many as 1% of recipients of Al-containing adjuvants may be sensitized to future exposures to Al [167], then a note of caution could be made regarding future mass overexposure to this form of adjuvant via excessive vaccinations or other forms of immunostimulation (i.e., allergen therapy).

## Conclusion

The use of Al salts in vaccines or in immunotherapies may not be as safe as commonly considered owing to Al's known toxic actions in the nervous system. Furthermore, Al as an antigenic compound can trigger autoimmune reactions. The combination of both actions may render the overuse of Al for such applications 'insidiously' unsafe for human health.

## Future perspective

Our rapidly growing knowledge of Al actions in the nervous system stands in marked contrast to the increasing use of Al salts for vaccines and general immune stimulation. Based on the current and emerging literature, it seems unlikely that in the future Al will be considered safe for human use in any of the current medicinal applications. If this view is correct, the medical community would be well advised to seek other truly safe adjuvant formulations for vaccines and find other means to stimulate general immune responses.

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## Executive summary

- Aluminium (Al) is a known neurotoxin with the demonstrated potential to damage the nervous system in animals and humans.
- Al can also trigger adverse immunoinflammatory reactions.
- The combination of both neuro- and immuno-toxic actions of Al in human medicinal applications (vaccinations and immunotherapy) may render the overuse of Al 'insidiously' unsafe for humans.
- Efforts should be pursued by the pharmaceutical industry and regulatory agencies to develop safer adjuvants for human use.

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# Highly delayed systemic translocation of aluminum-based adjuvant in CD1 mice following intramuscular injections



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## ABSTRACT

Concerns regarding vaccine safety have emerged following reports of potential adverse events in both humans and animals. In the present study, alum, alum-containing vaccine and alum adjuvant tagged with fluorescent nanodiamonds were used to evaluate i) the persistence time at the injection site, ii) the translocation of alum from the injection site to lymphoid organs, and iii) the behavior of adult CD1 mice following intramuscular injection of alum (400 µg Al/kg). Results showed for the first time a strikingly delayed systemic translocation of adjuvant particles. Alum-induced granuloma remained for a very long time in the injected muscle despite progressive shrinkage from day 45 to day 270. Concomitantly, a markedly delayed translocation of alum to the draining lymph nodes, major at day 270 endpoint, was observed. Translocation to the spleen was similarly delayed (highest number of particles at day 270). In contrast to C57BL/6J mice, no brain translocation of alum was observed by day 270 in CD1 mice. Consistently neither increase of Al cerebral content, nor behavioral changes were observed. On the basis of previous reports showing alum neurotoxic effects in CD1 mice, an additional experiment was done, and showed early brain translocation at day 45 of alum injected subcutaneously at 200 µg Al/kg. This study confirms the striking biopersistence of alum. It points out an unexpectedly delayed diffusion of the adjuvant in lymph nodes and spleen of CD1 mice, and suggests the importance of mouse strain, route of administration, and doses, for future studies focusing on the potential toxic effects of aluminum-based adjuvants.

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## 1. Introduction

Aluminum (Al) is the third most abundant element in the Earth's crust and it is ubiquitously present in our everyday life in a great variety of objects (cooking utensils, food packaging, housing materials, pharmaceutical products, cosmetics, etc.). Al is found in all body fluids (blood, cerebral spinal fluid, interstitial fluid of the brain, lymph, sweat, seminal fluids and urine) [1]. Despite the widespread use of Al in our environment leading to this increase of its bioavailability, Al has no known biological role [2].

Furthermore, it is widely accepted that Al and Al compounds are neurotoxic for animals and humans [3,4]. For instance, Al exposure has been implicated in the pathology of several neurodegenerative diseases associated with cognitive impairments, as Alzheimer's disease [5–7]. The molecular mechanisms by which it causes neuronal damage

are not fully understood [8], but it is generally accepted that the nervous system is particularly sensitive to oxidant-mediated damage [9], and that the neurotoxicity of Al is caused by its ability to increase oxidative damage in the brain [10].

Finally, the bioavailability of Al, its ability to cross the blood–brain barrier, and the relatively slow rate of elimination from the brain contribute to progressive accumulation of Al into the brain [11–13], and enhance neurotoxicological risk [14].

Many severe infectious diseases can be prevented by vaccine and some of them have been eradicated. Furthermore novel vaccine strategies are now being developed as promising therapies to overcome diseases such as cancer. However, though vaccines are commonly and safely used, and are generally well tolerated by most people, they occasionally cause adverse effects, such as ill-defined conditions usually manifesting as symptoms such as myalgia, arthralgia, chronic fatigue and development of autoantibodies [15]. No consensus exists so far on a cause-to-effect relationship, but vaccine adjuvants have been suspected to be associated with several inflammatory/neurodegenerative or autoimmune conditions impacting the central nervous system

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such as multiple sclerosis [16], amyotrophic lateral sclerosis [17] and autism [6]. A new syndrome has thus been identified by Shoenfeld in 2011, the autoimmune/auto-inflammatory syndrome induced by adjuvants (ASIA) [18].

Several papers from the literature suggest that vaccines containing aluminum adjuvants may be insidiously unsafe over the long-term. This is in line with the role of environmental aluminum that is continuously suspected to represent a possible co-factor of several chronic diseases [19–21,1].

Among unusual reactions to aluminum hydroxide (alum) containing vaccines, macrophagic myofasciitis (MMF) is an inflammatory lesion described in 1998 [22], and recognized as a “distinctive histopathological entity that may be caused by intramuscular injection of Al-containing vaccines” [23].

MMF affects mainly women (>70% of total known cases), and is characterized by highly specific myopathological alterations observed in patients suffering from a combination of diffuse myalgias, arthralgia, chronic fatigue and cognitive impairment such as alterations affecting working memory and attention [22,24–27].

Alum-adjuvanted vaccines are usually administered in France through intramuscular injection into the deltoid muscle in adults [28]. In MMF patients deltoid muscle biopsies showed crystalline cytoplasmic inclusions in macrophages corresponding to alum agglomerates of vaccine origin [29]. The constant detection of these agglomerates in MMF assesses the unusually long persistence time of alum in affected individuals [30].

Both Al oxyhydroxide and Al hydroxyphosphate are used as vaccine adjuvants [31,32]. Indeed, Al has been added to vaccines since the early part of the twentieth century to enhance the primary immunization [33]. The role of Al adjuvants was believed to prolong the retention of adsorbed antigens at the injection site, thus reducing the amount of antigen needed per dose and the number of required doses [34,35]. However, the “depot” theory has been challenged by early ablation of the injected site [36] and mechanisms of alum immunopotential only begin to be progressively understood [31].

Al containing vaccines are commonly used, such as vaccines against tetanus, hepatitis A, hepatitis B, human papillomavirus, haemophilus influenzae B, pneumococcal and meningococcal infections, and anthrax [37]. FDA regulations limit the Al content of an individual vaccinal dose to 0.85 mg of elemental Al [38].

Previous results have shown that Al particles, as other poorly degradable particles, do not stay localized in the injected muscle tissue, but can rather disseminate within phagocyte cells to lymph nodes and distant sites including the spleen and brain [39]. A previous study of our group looked at aluminum translocation after intramuscular injection of alum-containing vaccine in C57BL/6J mice. Aluminum was detected in the injected muscle, but also in distant organs such as the spleen, a few days after injection, and then in the brain where it was still detected one year later. Using surrogate labeled particles containing precipitated alum, a rapid phagocytosis of injected particles by muscle monocyte lineage cells and their translocation via lymph and blood vessels were confirmed. Particles reached the brain as soon as 3 weeks post-injection and were shown to accumulate albeit very slowly and in small numbers [39]. Recently, we developed a new tool allowing tracing of  $\text{Al}(\text{OH})_3$  particles in the tissues at very low levels and over the long-term [40]. This method consists of tagging Al adjuvant itself (Alhydrogel®) with fluorescent nanodiamonds (fNDs) functionalized with hyperbranched polyglycerol (HPG). The complex alum-nanodiamonds (AluDia) had physico-chemical properties similar to HBV vaccine [40]. When injected in the *tibialis anterior* (TA) muscle of C57BL/6J mice, it allowed the monitoring of lymphatic and systemic biodistribution of AluDia particles and their presence in the brain tissue, 3 weeks after the intramuscular injection.

The potential impact of aluminum adjuvant on the nervous system has been studied in mouse models. Aluminum adjuvant, dosed at 100  $\mu\text{g}$  Al/kg and subcutaneously injected in CD1 mice, induced motor

deficits and anxiety increases associated with motor neuron death and astrogliosis [17]. Although no motoneuron death was observed when the dose was increased 3-fold, Shaw and Petrik [41] observed a microglial and astrocytic reactivity in the spinal cord of CD1 mice that present with an increase in anxiety, significant impairments in a number of motor functions and diminished spatial memory capacity. A neuroinflammatory syndrome has been described in sheep after the repetitive administration of Al-containing vaccines [42]. Recently, impairment of neurocognitive functions and brain gliosis was reported in a murine model of systemic lupus erythematosus-like disease following intramuscular injection of Al hydroxide or vaccine against the hepatitis B virus (HBV) (200  $\mu\text{g}$ /mouse) [43].

Although progressive shrinkage of the local granuloma [44,45] and rapid translocation of alum from the injected site to draining lymph nodes (dLNs) and spleen have been repeatedly demonstrated [39,40], long-term biodistribution of alum particles trapped in the local granuloma remains unexplored. To examine this point we designed a longitudinal study in which alum, alum-containing vaccine and alum tagged with fluorescent nanodiamonds were used in adult CD1 mice to evaluate i) the persistence time at the injection site, ii) the long-term translocation of alum from the injection site to the lymphoid organs, and iii) the behavior and motricity of animals following intramuscular injection of alum.

## 2. Materials and methods

### 2.1. Dose of exposure

The dose of 400  $\mu\text{g}$  Al/kg was chosen to model a plurivaccination with the HBV ENGERIX® vaccine. Medical histories of MMF patients revealed that 100% (50/50 patients) of them received 1–9 (median 4) doses of an Al-containing vaccine within 10 years prior to their diagnosis [29]. A 60-kg woman injected with 1 dose of HBV ENGERIX® vaccine receives 500  $\mu\text{g}$  of Al, i.e. 8.3  $\mu\text{g}$  Al/kg of body weight. The allometric conversion from human to mouse (FDA guidance 5541) gives a final amount of approximately 100  $\mu\text{g}$  Al/kg. 400  $\mu\text{g}$  Al/kg was used to mimic a cumulative effect induced by 4 shots.

### 2.2. Animals

155 female CD1 mice, weighing 25–30 g (7 weeks old), were obtained from Charles Rivers Laboratories (France). Upon arrival, the females were housed at 5 per cage. Animals were maintained under a 12:12 light cycle, at a constant temperature ( $22 \pm 2^\circ\text{C}$ ) and a relative humidity of  $55 \pm 10\%$ . Mice were protected from Al-containing materials and were given free access to food and water. After a 1-week period for acclimatization, mice were separated in two experimental series.

All these experiments on animals were performed with respect to the guidelines provided by the European Union (Directive 2010/63/EU) [46].

#### 2.2.1. AluDia translocation series

After the acclimatization period, 35 8-week old females were separated into 7 experimental groups of 5 animals each receiving 3 intramuscular (im) injections in the left *tibialis anterior* muscle or 3 subcutaneous (sc) injections in the neck, each of 20  $\mu\text{L}$  with a 4-day interval between each injection. The 7 groups received AluDia: 200  $\mu\text{g}$  Al/kg, im; 400  $\mu\text{g}$  Al/kg, im; 200  $\mu\text{g}$  Al/kg, sc; and 400  $\mu\text{g}$  Al/kg, sc. The AluDia complex used was identical to the one prepared by Eidi et al. [40]. Briefly, the functionalized fluorescent nanodiamonds (fNDs) were prepared by milling synthetic HPHT (High Pressure High Temperature) micron powder holding nitrogen-vacancy centers (at the origin of permanent fluorescence) created by electronic irradiation and annealing [47]. Afterwards, the fNDs are functionalized with hyperbranched polyglycerol (HPG) synthesized from glycidol (Sigma Aldrich, Saint Quentin Fallavier, France) [48] which ensures the colloidal stability of the



suspension in buffer and the formation of the complex with aluminum particles. The AluDia complex was prepared by mixing fND-HPG (1.3 g/L) and Alhydrogel® (10 g/L) suspensions at a ratio of 1:17 v/v and followed by a thorough agitation and a few minutes sonication. AluDia suspension was then diluted to reach the appropriate concentration in PBS. In the physiological conditions we used, AluDia particle size and zeta potential were very similar to those of Alhydrogel® alone or HBV vaccine [40].

### 2.2.2. Adjuvant/vaccine series

After the acclimatization period, 120 8-week old females were separated into 3 experimental subgroups of 40 animals each receiving 3 intra-muscular injections of 20 µL in TA, with a 4-day interval between each injection.

The 3 groups were: Alhydrogel® group (400 µg Al/kg) (InvivoGen, Toulouse, France); Vaccine HBV ENGERIX® group (400 µg Al/kg) (Glaxo, Rixensart, Belgium) and a PBS control group (InvivoGen, Toulouse, France).

### 2.2.3. Behavioral tests and endpoint for sacrifice

Animals were enrolled in a battery of 8 complementary tests two weeks before the endpoint. At the end of the behavioral tests (45, 135, 180, 270 days post-injection), animals were sacrificed with an overdose of pentobarbital (100–150 mg/kg, intraperitoneal injection) and samples (TA muscles, dLNs, spleen, and brain) were removed and quickly frozen in isopentane, then stored at  $-80^{\circ}\text{C}$  until use. Precautions were taken to avoid external environmental aluminum contamination of the samples.

Muscle samples of 3 animals from each group were dedicated to the analyses of the granuloma size in the injected muscle whereas brain samples of 5 animals were dedicated to the measurement of Al concentration.

### 2.3. Muscle granuloma size at the injection site

The granuloma size was semi-quantitatively assessed on muscle sections stained with hematoxylin–eosin in treatment groups that received either the adjuvant Alhydrogel® or the HBV vaccine ( $n = 3$  muscles per group). Sections were observed with  $20\times$  objectives and granuloma was assessed according to its size. Four granuloma groups were determined: without (0), small (+), medium (++) and large (+++) granulomas. Then, the percentage of each size group was calculated at each time point.

### 2.4. AluDia translocation

AluDia translocation from injection site to target organs (dLNs, spleen, and brain) was assessed as previously described by Eidi et al. [40] for 7 AluDia groups: 400 µg Al/kg, im 45, 135, 180 or 270 days following injection; 200 µg Al/kg, im; 200 µg Al/kg, sc, and 400 µg Al/kg, sc 45 days post-injection.

### 2.4.1. Tissue preparation and particle counting

Serial cryosections of the muscle and spleen (20 µm thick), inguinal lymph node (12 µm thick) and brain (coronal plane, 40 µm thick) were cut and stored at  $-20^{\circ}\text{C}$  until particle counting or treatment. Tissue sections were successively deposited on 10 different SuperFrost®-plus slides in order to obtain 10 identical series. The total number of particles per organ was assessed by multiplying by 10 the number of particles found in a single series.

### 2.4.2. Epifluorescence microscopy and microspectrometry

For fND detection, a DPSSL 532 nm (200 mW) laser beam was used as the illumination source and was guided to the microscope by a fiber optic. A long pass 600 nm emission filter was used to collect only wavelengths higher than 600 nm. Fluorescence images were obtained with a Princeton Instruments EMCCD Camera Rolera EM-C<sup>2</sup>, with typical exposure times. Spectra of the fluorescent spots were acquired by focusing the fluorescent object emission from the microscope onto an Acton SP2150i spectrometer (Princeton instruments), and detected with a PIXIS-100B-eXcelon CCD camera (Princeton Instruments).

### 2.5. Brain Al concentration

Analyses were carried out on 5 brains per group (groups PBS, Alhydrogel® (400 µg Al/kg) and HBV vaccine (400 µg Al/kg), 45, 135, 180 or 270 days following injection) according to the published method of House et al. [49]. Significant precautions were taken throughout the study to minimize contamination. These included storage of all plastic-based laboratory-ware in 5% v/v conc. HCl and, before use, rinsing of all such apparatus in several volumes of ultrapure water (cond.  $<0.067\text{ mS cm}^{-1}$ ). Where required, the rinsed apparatus was air-dried in a dedicated incubator at  $37^{\circ}\text{C}$ . Al concentrations were determined by TH GFAAS in half brains dried to a constant weight at  $37^{\circ}\text{C}$  and digested in a microwave (MARS Xpress CEM Microwave Technology Ltd.) in a mixture of 1 mL 15.8 M HNO<sub>3</sub> (Fischer Analytical Grade) and 1 mL of 30% w/v H<sub>2</sub>O<sub>2</sub> (BDH Aristar Grade). Digests were clear and colorless or light yellow with no visible precipitate or fatty residue. Upon cooling each digest was diluted to a total volume of 5 mL with ultrapure water.

Total Al was measured immediately post-digestion using an AAnalyst 600 atomic absorption spectrometer with a transversely heated graphite atomizer (THGA) and longitudinal Zeeman-effect background corrector and an AS-800 autosampler with WinLab32 software (Perkin Elmer, UK). Standard THGA pyrolytically-coated graphite tubes with integrated L'Vov platform (Perkin Elmer, UK) were used. The Zeeman background corrected peak area of the atomic absorption signal was used for the determinations.

Results were expressed as µg Al/g tissue dry weight. Each determination was the arithmetic mean of three injections with a relative standard deviation  $<10\%$ .

**Table 1**

A semi-quantitative study of the progressive decrease of granuloma size in the injected muscle with Alhydrogel or HBV vaccine.

Group	Days	No granuloma (0)	Small granuloma (+)	Medium granuloma (++)	Large granuloma (+++)	Total granuloma
Alhydrogel® 400 µg Al/kg, im	D45	7%	14%	46%	32%	93%
	D135	35%	21%	18%	26%	65%
	D180	24%	28%	43%	5%	76%
	D270	65%	18%	10%	6%	35%
	D45	32%	42%	22%	4%	67%
HBV vaccine® 400 µg Al/kg, im	D135	21%	35%	31%	13%	79%
	D180	35%	41%	25%	0%	65%
	D270	69%	25%	6%	0%	31%

According to their size, the observed granulomas were divided to four types: without (0), small (+), medium (++) and large (+++) granulomas. Then, percentage of each size in the observed muscles was calculated, for  $n = 3$  animals per group.

**Table 2**

A quantitative study of the translocation of AluDia particles following intramuscular injections at the dose of 400 µg Al/kg, 45, 135, 180 or 270 days after injections.

AluDia	Particle counts		
	Ing DLNs	Spleen	Brain
	Mean ± SD	Mean ± SD	Mean
D45	1145 ± 87	15 ± 3	0
D135	3820 ± 123	55 ± 12	0
D180	7372 ± 194	177 ± 32	0
D270	115,478 ± 377	785 ± 61	0

Results are expressed as mean ± SD of n = 3 mice/group per organ and per time point. Ing dLNs, inguinal draining lymph nodes.

## 2.6. Behavioral and motor testing

A battery of 8 behavioral or physical tests was performed at 45, 135, 180 or 270 days after the third injection in groups PBS, Alhydrogel® (400 µg Al/kg) and HBV vaccine (400 µg Al/kg). Tests were chosen in order to assess locomotor activity in the open-field [50], level of anxiety in the o-maze [51,52], short-term memory in the novel object recognition test [53–56], muscular strength in the wire mesh hang [57], grip strength test [58], locomotor coordination in the rotarod test [59], depression in the tail suspension test [60], and pain sensitivity in the hot plate test [61]. Detailed procedures can be found in the Supplementary data.

## 2.7. Statistical analysis

Tissue Al data were analyzed using a non-parametric Kruskal–Wallis test and a Mann–Whitney procedure for multiple comparisons. Data from behavioral tests were analyzed using a one-way analysis of variance (one-way ANOVA). Post hoc comparisons have been performed using the Bonferroni's test when ANOVA was significant.

Significance was set at  $p < 0.05$ . All statistical analyses were carried out using SPSS 16.0 software (SPSS Inc., Chicago, IL, USA).

## 3. Results

### 3.1. Muscle granuloma size at the injection site

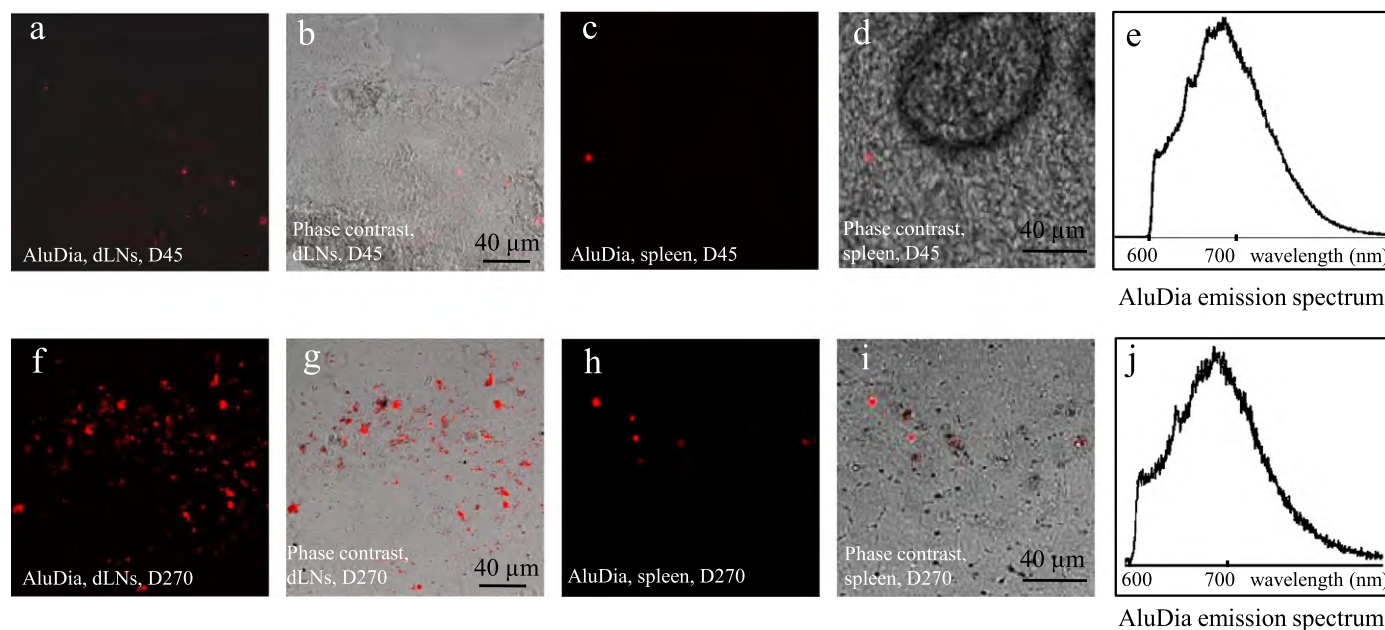
Serial sections of the injected muscle 45, 135, 180 and 270 days after Alhydrogel® (400 µg Al/kg) or HBV vaccine (400 µg Al/kg) injection showed progressive shrinkage of muscle granuloma (Table 1). At D45 all animals had granuloma with a majority of sections showing a granuloma (93% for Alhydrogel®, 67% for HBV vaccine). At D270, in contrast to previous time points, one animal was free of granuloma and a majority of overall muscle sections showed no granuloma (65% for Alhydrogel®, 69% for HBV vaccine) (Table 1).

### 3.2. AluDia translocation to dLNs and spleen

The study of translocation of AluDia particles (400 µg Al/kg) from the muscle to distant organs showed progressive increase of AluDia particles in inguinal dLNs from D45 to D270 after injection (Table 2). Indeed, 1145 and 115,478 AluDia particles were counted in inguinal dLNs at D45 and D270, respectively (Fig. 1). At D270, this 100 fold increase appeared as striking accumulation of AluDia in the interfollicular areas of dLNs (Table 2 and Fig. 1). In the same way, AluDia particles increased by 52 fold in the spleen (15 to 785 particles) between D45 and D270 (Table 2 and Fig. 1). Of note, particle concentrations were still increasing at the D270 endpoint in both dLNs and spleen.

### 3.3. Brain translocation of AluDia and behavioral/motricity tests

Surprisingly, no particles were observed in the brains at any analyzed times after im injection of AluDia (Table 2). Consistently, as assessed by furnace atomic absorption spectrometry, animals receiving im injection of Alhydrogel® (400 µg Al/kg) or HBV vaccine (400 µg Al/kg) showed no increase of cerebral  $Al^{3+}$  level compared to control animals injected with PBS (Table 3). Similarly behavioral and motor tests yielded no salient changes in elevated o-maze, open field, novel object recognition test, wire mesh hang test, grip strength test, rotarod test, tail suspension test, and hot plate test (Supplementary data).



**Fig. 1.** AluDia accumulation in inguinal dLNs (a, b, f, g) and spleen (c, d, h, i) following AluDia im injection in the *tibialis anterior* muscle (400 µg Al/kg) at D45 (a–e) and at D270 (f–j). a, c, f, h: The red specific fluorescence of AluDia excited by a 532 nm laser source. b, d, g, i: Phase contrast. e and j: AluDia luminescence spectrum with a specific peak at 700 nm.

**Table 3**Aluminum cerebral concentration measured by furnace atomic absorption spectrometry ( $\mu\text{g/g}$  of dry weight).

Cerebral Al concentration	Control	Alhydrogel® group 400 $\mu\text{g}$ Al/kg, im	HBV vaccine group 400 $\mu\text{g}$ Al/kg, im	Kruskal–Wallis test
D45	0.54095 (0.3250–1.4837)	0.57335 (0.0234–8.8778)	0.90625 (0.6104–1.3623)	n.s.
D135	0.02485 (0.0179–0.1877)	0.4317 (0.0200–33.3432)	0.6843 (0.1214–1.2061)	n.s.
D180	0.0956 (0.0174–0.8776)	0.0143 (0.0133–0.3540)	0.0451 (0.0158–0.6317)	n.s.
D270	1.0534 (0.3975–2.8053)	0.01495* (0.0123–0.1859)	0.0141* (0.0122–0.0206)	$p < 0.05$

Results are expressed as median and quartiles (in brackets) of  $n = 5$  brains/group. Non-parametric Kruskal–Wallis test followed by a Mann–Whitney procedure was used for multiple comparisons.

\*  $p < 0.05$ , statistical significant difference from controls.

Taking into account that neurotoxic effects were previously reported in CD1 mice after sc injection of Alhydrogel® at 100  $\mu\text{g}$  Al/kg [17] and 300  $\mu\text{g}$  Al/kg [41], we examined whether the route of administration or the dose could influence brain translocation of AluDia. We observed that 3 out of 4 CD1 mice injected by the sc route with 200  $\mu\text{g}$  Al/kg showed particle incorporation into the brain 45 days after injection (Table 4 and Fig. 2). Notably, this was not observed at higher dose (400  $\mu\text{g}$  Al/kg) for the sc route, and at any dose for the im route.

#### 4. Discussion

This longitudinal study showed that alum (Alhydrogel® or HBV vaccine) injected into the muscle constantly induces a granuloma similar to MMF that shrinks with time with marked clearance of granulomatous lesions observed from D180 to D270. This is similar to what was previously observed with the AluDia complex [40]. Granuloma shrinkage in the muscle was associated with concurrent replenishment of inguinal dLNs (100 fold increase of AluDia particles from D45 to D270). Similar translocation of alum from the muscle to dLNs was previously observed at much earlier time points in C57BL/6J mice [39]. We assume that two waves of lymphatic translocation may occur after im injection of alum: an early one peaking at D4 [39] and a markedly delayed one associated with muscle granuloma shrinkage observed in the present study thanks to a long-term evaluation not performed in previous studies. We assume that this delayed lymphatic draining flux is the normal way of clearance for alum trapped in the post-vaccinal granuloma. Similarly to translocation to dLNs, we observed markedly delayed AluDia translocation to the spleen, with a maximum number of particles being detected in this organ at D270. Alum translocation from the muscle to spleen was previously shown to assess particle exit from lymphatic pathways to the blood stream [39]. Since the spleen was previously shown to incorporate a first peak of particles at D7 post-im injection in C57BL/6J mice [40], the present study suggests a delayed second wave of adjuvant translocation to the spleen in line with that observed in dLNs.

The present study confirms that alum is extremely biopersistent [29, 37] and that alum biopersistence can be observed in both the injected muscle and distant organs, including dLNs and spleen. Regarding the strong immunostimulatory effects of alum and the unrequired depot formation for its adjuvant activity [36], long-term biopersistence of

alum in lymphoid organs is clearly undesirable, and may cast doubts on the exact level of long-term safety of alum-adjuvanted vaccines [37].

The lack of brain translocation alum after im injection of 400  $\mu\text{g}$  Al/kg was puzzling. Notably, neither elevated Al concentration in the brain nor neurobehavioral changes were observed in these experimental conditions, ruling out significant translocation of soluble Al to the brain in the absence of physical incorporation of alum particles, and the induction of neurobehavioral effects by chronic peripheral immune activation linked to persistence of alum within the immune cells [35].

It is not excluded that the observed difference in the biodisposition of alum in C57BL/6J and CD1 mice, including diffusion kinetics and the occurrence of brain translocation, may in part reflect differences in the genetic background of the two strains [62]. We previously demonstrated that the size of the alum-induced granuloma in rats is dramatically influenced by their genetic background, the granuloma being much smaller in Lewis rats with Th1 biased immune responses compared to Sprague–Dawley rats with balanced Th1/Th2 immunity [45]. The C57BL/6 mouse strain is known to exhibit a Th1-prone, pro-inflammatory type response to injury [63,64]. To our knowledge, the T helper immunity status of CD1 mice is not known.

Interestingly, C57BL/6 mice produce more MCP-1/CCL2 than other strains [64], and this major inflammatory monocyte chemoattractant is crucially involved in both systemic biodistribution and neurodelivery of Al particles captured by monocyte-lineage cells [39]. Notably, increased circulating MCP-1/CCL2 is the sole identified biomarker in myalgic encephalomyelitis patients with MMF [65]. Moreover, human MMF is mainly observed in middle aged or elderly individuals, a time when MCP-1/CCL2 production increases and immuno-senescence occurs [66]. Clarification of the influence of mouse strains Th1 and Th2-biased immune responses in AluDia brain translocation clearly deserves future studies.

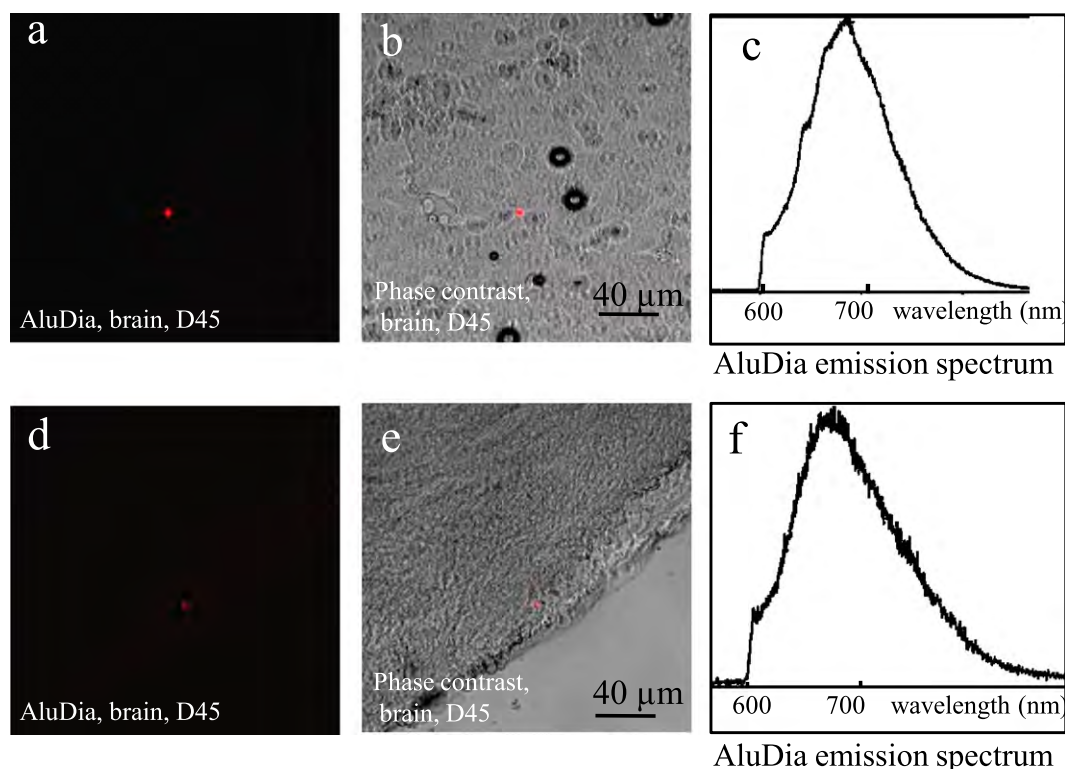
In previously published studies, motor and behavioral impairments were observed following sc (behind the neck) Alhydrogel® injection to CD1 mice with doses of 100 and 300  $\mu\text{g}$  Al/kg [17,41]. These effects were associated with Al deposits in the central nervous system (spinal cord) assessed by Morin stain. To examine if the route of exposure may represent an important factor for alum toxicity, a nested study was conducted herein, showing that alum particles may penetrate the brain at D45 after the sc (and not im) injection, performed at the dose of 200  $\mu\text{g}$  Al/kg (and not at the dose of 400  $\mu\text{g}$  Al/kg). A higher rate of brain translocation after sc injection may be explained by a much higher density of dendritic cells with high migrating properties, in the skin compared to the muscle. The fact that half dose resulted in brain translocation, which was not observed at higher dose, is reminiscent of the non-monotonic dose/response curves previously observed with environmental toxins, including particulate compounds [67]. In another study, we similarly observed neurobehavioral changes at 200 but not 400  $\mu\text{g}$  Al/kg (Crépeaux et al., manuscript in preparation). The exact significance of such observations is unknown, but one may speculate that huge quantities of alum injected in the tissue may

**Table 4**A qualitative study of the translocation of AluDia particles following intramuscular or subcutaneous injections at the doses of 200 or 400  $\mu\text{g/kg}$ , 45 days after injections.

AluDia	Particle counts			
	im 200 $\mu\text{g}$ Al/kg	im 400 $\mu\text{g}$ Al/kg	sc 200 $\mu\text{g}$ Al/kg	sc 400 $\mu\text{g}$ Al/kg
Brain	0	0	15 $\pm$ 7	0

Results are expressed as mean  $\pm$  SD of  $n = 4$  mice/group per organ and per time point. im, intramuscular; sc, subcutaneous.





**Fig. 2.** AluDia in the brain (animal 1: a, b, c; animal 2: d, e, f) following AluDia sc injection (200 μg Al/kg) at D45. a and d: The red specific fluorescence of AluDia excited by a 532 nm laser source. b and e: Phase contrast. c and f: AluDia luminescence spectrum with a specific peak at 700 nm.

induce blockade of critical macrophage functions such as migration and xeno/autophagic disposition of particles, as previously reported for infectious particles [37].

## 5. Conclusion

We observed a strikingly delayed, previously unknown, systemic translocation of alum particles injected into the muscle, with conspicuous alum accumulations in the lymphatic system and spleen 9 months after injection. In addition to the crucial “t” factor, our results strongly suggest the influence of the mouse strain, the dose and the route of administration on alum biodisposition. All these parameters should be taken into account in the design of future alum toxicological studies.

## List of abbreviations

AluDia	complex alum-nanodiamonds
ASIA	autoimmune/auto-inflammatory syndrome induced by adjuvants
dLNs	draining lymph nodes
FDA	Food and Drug Administration
HBV	hepatitis B virus
HPG	hyperbranched polyglycerol
HPHT	High Pressure High Temperature
im	intramuscular
fNDs	fluorescent nanodiamonds
MMF	macrophagic myofasciitis
MCP-1/CCL2	monocyte chemoattractant protein 1/chemokine ligand 2
PBS	phosphate buffer saline
TA	tibialis anterior
THGA	spectrometer with a transversely heated graphite atomizer
TH GFAAS	graphite furnace atomic absorption
Th1 & Th2	T helper 1 & T helper 2
sc	subcutaneous

## Conflicts of interest

The authors declare that there are no conflicts of interest.

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## Appendix A. Supplementary data

Supplementary data to this article can be found online at <http://dx.doi.org/10.1016/j.jinorgbio.2015.07.004>.

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# Behavioral abnormalities in female mice following administration of aluminum adjuvants and the human papillomavirus (HPV) vaccine Gardasil

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**Abstract** Vaccine adjuvants and vaccines may induce autoimmune and inflammatory manifestations in susceptible individuals. To date most human vaccine trials utilize aluminum (Al) adjuvants as placebos despite much evidence showing that Al in vaccine-relevant exposures can be toxic to humans and animals. We sought to evaluate the effects of Al adjuvant and the HPV vaccine Gardasil versus the true placebo on behavioral and inflammatory parameters in female mice. Six-week-old C57BL/6 female mice were injected with either, Gardasil, Gardasil + pertussis toxin (Pt), Al hydroxide, or, vehicle control in amounts equivalent to human exposure. At 7.5 months of age, Gardasil and Al-injected mice spent significantly more time floating in the forced swimming test (FST) in comparison with vehicle-injected mice (Al,  $p = 0.009$ ; Gardasil,  $p = 0.025$ ; Gardasil + Pt,  $p = 0.005$ ). The increase in floating time was already highly significant at 4.5 months of age for the Gardasil and Gardasil + Pt group ( $p \leq 0.0001$ ). No significant differences were observed in the number of stairs climbed in the staircase test which measures locomotor activity. These results indicate that differences observed in the FST were unlikely due to locomotor dysfunction, but rather due to depression. Moreover, anti-HPV antibodies from the sera of Gardasil and Gardasil + Pt-injected mice showed cross-reactivity with the mouse brain protein extract. Immunohistochemistry analysis revealed microglial activation in the CA1 area of the hippocampus of Gardasil-injected mice. It appears that Gardasil via its Al adjuvant and HPV antigens has the ability to trigger neuroinflammation and autoimmune reactions, further leading to behavioral changes.

**Keywords** Gardasil · Aluminum · ASIA syndrome · Autoantibodies · Autoimmunity · Neuroinflammation

## Abbreviations

Al Aluminum  
ASIA Autoimmune/autoinflammatory syndrome induced by adjuvants

$\beta$ 2-GPI  $\beta$ 2-Glycoprotein I  
FST Forced swimming test  
HPV Human papilloma virus  
Pt Pertussis toxin  
U. S. FDA United States Food and Drug Administration

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## Introduction

Like other drugs, vaccines can cause adverse events, but unlike conventional medicines, which are prescribed to people who are ill, vaccines are administered to healthy individuals. Hence, there is an added concern regarding risks associated with vaccinations. While most reported side effects from vaccines are mild and transient, serious adverse events do occur and can even be fatal [1, 2].

There are currently major stumbling blocks in our understanding of the exact mechanisms by which such events can be triggered. The main reason for this is the poor methodological quality of many clinical studies that evaluate vaccine safety and the lack of in-depth research into adverse phenomena [3]. In addition, adverse events may not fit into a well-defined category of an autoimmune disease but rather, present themselves as a constellation of non-specific symptoms (i.e., arthralgia, myalgia, fatigue, nausea, weakness, paresthesia, depression, mild cognitive disturbances) [2]. Another complicating factor in researching vaccine-related adverse events is that the latency period between vaccination and the development of an overt and diagnosable autoimmune and/or neurological disease can range from days to many months [4–6], likely depending on individuals' genetic predispositions and other susceptibility factors (i.e., previous history of autoimmune disease or previous history of adverse reactions to vaccines).

From the above, it is clear that establishing a definite causal link between vaccinations and disease manifestations in humans remains a complex task. Thus, the potential risks from vaccines remain currently ill-understood and controversial. A further obfuscation to our understanding of potential risks from vaccinations stems from the persistent use of aluminum (Al) adjuvants-containing placebos in vaccine trials [7]. Indeed, contrary to popular *assumptions* of inherent safety of Al in vaccines, there is now compelling data from both human and animal studies which implicates this most widely used adjuvant in the pathogenesis of disabling neuroimmuno-inflammatory conditions [8–11].

Due to their capability of enhancing the immune response to foreign antigens, substances with adjuvant properties have been used for decades to enhance the immunogenicity of human and animal vaccines [12]. Because of their immune-potentiating capacity, adjuvants enable the usage of smaller amount of antigens in vaccine preparations and are thus attractive from a commercial standpoint. Nonetheless, enhanced immunogenicity also implies enhanced reactogenicity. Indeed, although Al acts as an effective vehicle for the presentation of antigens, this process is not always benign since the adjuvant itself is

intrinsically capable of stimulating pathological immune and neuro-inflammatory responses [9–11, 13–16]. In spite of these data, it is currently maintained by both the pharmaceutical industry and drug-regulating agencies that the concentrations at which Al is used in vaccines does not represent a health hazard [17].

Apart from potential hazards associated with adjuvant use, other ingredients in vaccines also have the capacity of provoking undesirable adverse events. Indeed, since the mechanisms by which the host's immune system responds to vaccination resemble the ones involved in the response to infectious agents, a recombinant or a live attenuated infectious antigen used for vaccination, may inflict a range of immune and autoimmune responses similar to its parallel infectious agent [18, 19].

The HPV vaccine Gardasil is one of many vaccines currently on the market that is adjuvanted with Al. Since the licensure by the US Food and Drug Administration (FDA) and subsequent introduction on the market in 2009, the HPV vaccine has been linked to a variety of serious neurological and autoimmune manifestations. Notably, out of 152 total cases identified via PubMed 129 (85 %) are related to neuro-ophthalmologic disorders (Table 1). It should be noted that the pattern of adverse manifestations emerging from HPV vaccine case reports, matches that reported through various vaccine safety surveillance systems worldwide, with nervous system and autoimmune disorders being the most frequently reported [20].

Like most other vaccine safety trials, the trials for the HPV Gardasil vaccine utilized an Al-containing placebo [21, 22] and hence the safety profile of the vaccine remains obscured by the use of a potentially toxic placebo [7]. Thus, in order to investigate better, the safety profile of Gardasil, as well as the Al adjuvant, in the current study, we evaluated and compared the effects of Al and whole HPV vaccine formulation versus that of a true placebo on behavioral, neurohistological and autoimmune parameters in young female C57BL/6 mice.

## Materials and methods

### Mice husbandry

Six-week-old C57BL/6 female mice were obtained from Harlan Laboratories (Jerusalem, Israel) and were housed in the animal facility at Sheba Medical Center. The mice were raised under standard conditions,  $23 \pm 1$  °C, 12-light cycle (6:30 am–6:30 pm) with ad libitum access to food and water. The Sheba Medical Center Animal Welfare Committee approved all procedures.

**Table 1** Summary of cases of autoimmune and inflammatory manifestations following HPV vaccination reported in the peer-reviewed medical literature

Number of case reports	Age	Symptoms/main clinical features	Final diagnosis	References
2	17	Visual impairments	ADEM	[52]
	20	Headache, nausea, vomiting, diplopia		[53]
5	16	Upper limb pseudoathetosis	CIS/MS/	[54]
	16	Acute hemiparesis	Clinically definite MS	
	21	Incomplete TM, left optic neuritis		
	25	Headache, incomplete TM		
	26	Incomplete TM, brainstem syndrome		
2	19	Leg numbness, mid-thoracic back pain	Demyelinating disease unspecified	[55]
	18	Blurriness, paresthesia, optic neuritis		
1	11	Mood swings, abnormal eye movements, dizziness, leg weakness, myoclonic jerks	Opsoclonus myoclonus	[56]
4	17	Back pain, progressing spastic paraparesis, right arm weakness, left eye visual loss	Neuromyelitis optica	[57]
	14	Back pain, right thigh dysesthesias, left optic neuritis		
	13	TM with flaccid paraplegia		
	18	Back pain and leg weakness, complete loss of monocular vision		
2	16	Visual loss, headaches, left hemiparesis	Optic neuritis	[58]
	17	Visual disturbances, demyelinating lesions		[59]
2	27	Paresthesia, demyelinating lesions	TM fitting the criteria for MS	[59]
	26	Progressive paresthesia, demyelinating lesions		
1	15	Facial paralysis	Bell's palsy	[59]
1	12	Nausea, vertigo, severe limb and truncal ataxia, and persistent nystagmus	Cerebellar ataxia	[60]
1	19	Chronic (3 months) disabling shoulder pain	Brachial neuritis	[61]
53	12–39	Orthostatic intolerance, severe non-migraine-like headache, excessive fatigue, cognitive dysfunction, gastrointestinal discomfort, widespread neuropathic pain	Dysautonomia, POTS, orthostatic intolerance and CRPS	[62]
40	11–17	Headaches, general fatigue, coldness of the legs, limb pain and weakness, orthostatic intolerance, tremors, persistent asthenia		[63]
6	20	Weight loss, dizziness, fatigue, exercise intolerance		[64]
	22	Diarrhea, weight loss, fatigue, dizziness, syncope		
	12	Syncope, pre-syncope, dizziness, small fiber neuropathy		
	15	Dizziness, headache, pre-syncope, syncope		
		Paresthesia, tachycardia, fatigue, headache,		
	14	diarrhea, weight loss		
	18	Paresthesia, leg pain, orthostatic intolerance, Fatigue, dizziness		
4	16	Paresthesia, numbness, limb paralysis, pain		[65]
	13	Allodynia, numbness, severe pain		
	15	Paresthesia, numbness, severe pain		
	12	Paresthesia, muscle weakness, pain		
1	14	Headaches, dizziness, recurrent syncope, orthostatic intolerance, fatigue, myalgias, tachycardia, dyspnea, visual disturbances, phonophobia, cognitive impairment, insomnia, gastrointestinal disturbances, weight loss		[66]
2	11	Widespread neuropathic pain, paresthesia, insomnia, profound fatigue	Fibromyalgia	[67]
	14	Widespread neuropathic pain and paresthesia		
1	32	Paresthesia, muscle twitching, myalgia, fatigue, hyperhidrosis, and tachycardia, exercise intolerance	Autoimmune myotonia	[68]

**Table 1** continued

Number of case reports	Age	Symptoms/main clinical features	Final diagnosis	References
3	14	Skin rash, fever, nausea, stomach aches, headache, insomnia, night sweats, arthralgia, anxiety, depression, amenorrhea, elevated serum levels of follicle-stimulating hormone (FSH) and luteinizing hormone (LH) and low levels of estradiol	POF	[69]
	13	Depression, sleep disturbance, light-headedness, tremulousness, anxiety, cognitive dysfunction, amenorrhea, high serum levels of FSH and LH with undetectable estradiol		[70]
	21	A menorrhea preceded by oligomenorrhea, high serum levels of FSH and LH and low estradiol		
3	16	5 months amenorrhea preceded by 12 months oligomenorrhea, hot flashes, low serum levels of estradiol and Anti-Müllerian hormone		
	18	6 months amenorrhea, low serum levels of estradiol and Anti-Müllerian hormone		
	15	3 months amenorrhea preceded by 9 months oligomenorrhea, hot flashes, low serum levels of estradiol and undetectable Anti-Müllerian hormone		
2	15	Vasculitic rash, soft tissue swellings of ankles and forearms, arthralgia, lethargy, epistaxis	Vasculitis	[71]
	15	Severe flare of cutaneous vasculitis		
1	16	Fatigue associated with prolonged menorrhagia, antiplatelet autoantibodies	Thrombocytopenic purpura	[72]
1	11	Jaundice, hepatosplenomegaly elevated serum aminotransferases	Autoimmune hepatitis	[73]
1	26	Severe constant epigastric pain, vomiting, fever	Pancreatitis	[74]
3	17	Arthralgias, pruritic rashes on lower extremities, bipedal edema, livedo reticularis, proteinuria, positive ANA and anti-dsDNA antibodies	SLE	[75]
	45	Intermittent fever, generalized weakness, oral ulcers, alopecia, malar rash, photosensitivity, arthritis, intestinal pseudo-obstruction, ascites, positive ANA, anti-dsDNA, anti-Ro/SSA and anti- La/SSB antibodies		
	58	Malar and scalp rashes, fever, easy fatigability, cervical lymph nodes, gross hematuria and pallor, severe anemia and thrombocytopenia, active nephritis, patient expired a day after hospital admission		
6	32	Fatigue, severe myalgia, polyarthralgia, anorexia, severe skin rash, malar rash, aphthous stomatitis, pharyngodynia, cervical lymphadenopathy, alopecia, severe weight loss, anemia, positive ANA and anti-dsDNA antibodies		[76]
	29	Weakness, diarrhea, malar rash, photosensitivity, arthritis, alopecia, severe weight loss, proteinuria, positive ANA and anti-dsDNA antibodies		
	16	High-grade fever, generalized asthenia, diffuse polyarthralgia, multiple erythematous annular cutaneous lesions on the face, trunk, and lower limbs, positive ANA and lupus anticoagulant		
	16	Fever, pharyngodynia, erythematous skin lesions of elbows and knees, generalized asthenia, anorexia, polyarthralgia, anti-cardiolipin and lupus anticoagulant		
	19	Mild arthralgia, dyspnea, cervical lymphadenopathy, skin rash, positive ANA and anti-dsDNA antibodies		
	13	Erythematous facial rash, fever, periorbital edema, weight loss, malaise, fatigue, alopecia, cervical, axillary and inguinal lymphadenopathy, anemia, thrombocytopenia, positive ANA, anti-RNP, anti-Smith and anti-RO/SSA antibodies		
1	19	Myalgia, arthralgia, generalized weakness, oral ulcers, Raynaud's phenomenon, alopecia, headache, dyspnea, tachycardia, positive ANA, anti-Sm, anti-Ro, anti-RNP, anti-dsDNA, leukopenia, and complement consumption		[77]
1	20	Myalgias, arthralgias, livedo reticularis, Raynaud's phenomenon, headache, tinnitus, positive ANA, lupus anticoagulant and anti-CCP	Rheumatoid arthritis	[77]

**Table 1** continued

Number of case reports	Age	Symptoms/main clinical features	Final diagnosis	References
1	16	Knee joint swelling, low back, buttock and chest wall pain, elevated leukocyte count in the synovial fluid, elevated C-reactive protein	Juvenile spondyloarthritis	[77]

Out of 152 reported cases, 129 (85 %) relate to neuro-ophthalmic disorders

ANA antinuclear antibodies; ADEM acute disseminated encephalomyelitis; CIS clinically isolated syndrome; CRPS complex regional pain syndrome; MS multiple sclerosis; POF primary ovarian failure; POTS postural orthostatic tachycardia syndrome (disorder of the autonomic nervous system); SLE systemic lupus erythematosus; TM transverse myelitis

### Injection procedures and experimental design

Six-week-old C57BL/6 female mice received three injections (spaced 1 day apart) of either (a) quadrivalent HPV vaccine Gardasil, (b) Gardasil + pertussis toxin (Pt), (c) Al hydroxide or (d) vehicle control (19.12 mg/mL NaCl, 1.56 mg/mL L-histidine). The number of injected animals was 19 per experimental group. Gardasil, Al and vehicle were injected intramuscularly (i.m.), while the Pt was given intraperitoneally (ip). The amount of injected Al and the HPV vaccine was the equivalent of human exposure. In particular, each mouse in the Gardasil and Gardasil + Pt group received 0.25 µl of Gardasil (dissolved in 20 µl of vehicle solution). 0.25 µl of Gardasil is the equivalent of a human dose since the average weight of a six-week-old mice is approximately 20 g. Gardasil is given as a 0.5-mL dose to teenage girls of cca 40 kg. Thus, a 20-g mouse receives cca 2000 × less of the vaccine suspension than a human. Similarly, each mouse in the Al adjuvant group received 5.6 µg/kg body weights Al hydroxide dissolved in 20 µl vehicle solution. A single Gardasil dose contains 225 µg of Al and is given to a cca 40-kg female. This equates to 5.6 µg Al hydroxide/kg body weight. The mice in the Pt group received 250 ng of Pt with each injection of Gardasil. Pt was added to this group for the purpose of damaging the blood–brain barrier. Since the actual adjuvant form used in Gardasil, amorphous Al hydroxyphosphate sulfate (AAHS), is a proprietary brand of the vaccine manufacturer and is not commercially available, we used Alhydrogel as a substitute.

Five out of 19 animals from each of the four experimental groups were used for sera collection purposes. These animals were not subjected to behavioral testing as sera were collected via retro-orbital bleeding which is a stressful procedure that in addition often leads to vision deficits. The behavior of mice was evaluated at three and 6 months post-immunization for (1) locomotor function and depression by the forced swimming test (FST), (2) locomotor and explorative activity by the staircase test and (3) cognitive functions by the novel object recognition test. Following the first round of behavioral testing at

4.5 months of age, five mice from each of the four experimental groups were killed and brain tissues were collected and processed for histological examinations. Blood specimens were also collected at this time for serological analysis.

### Behavioral tests

#### Forced swimming test

The FST is the most widely used model of depression in rodents. It is commonly used for evaluation of antidepressant drugs, and experiments aimed at inducing and examining depressive-like states in basic and pre-clinical research [23, 24]. Nonetheless, it should be noted that increased floating time in the FST apart from being indicative of depressive behavior can also indicate locomotor dysfunction. For the purpose of this test, mice were placed in individual glass beakers (height 39 cm, diameter 21.7 cm) with water 15 cm deep at 25 °C. On the first day, mice were placed in the cylinder for a pretest session of 10 min, and later were removed from the cylinder, and then returned to their home cages. Twenty-four hours later (day 2), the mice were subjected to a test session for 6 min. The behavioral measure scored was the duration (in seconds) of immobility or floating, defined as the absence of escape-oriented behaviors, such as swimming, jumping, rearing, sniffing or diving, recorded during the 6-min test.

#### Staircase test

Locomotor, explorative activity and anxiety were evaluated by the staircase test, as described previously by Katzav et al. [25]. In this test, stair-climbing and rearing frequency are recorded as measures of general locomotor function, exploratory activity and anxiety/attention. The staircase maze consisted of a polyvinyl chloride enclosure with five identical steps, 2.5 × 10 × 7.5 cm. The inner height of the walls was constant (12.5 cm) along the whole length of the staircase. The box was placed in a room with constant lighting and isolated from external noise. Each

mouse was tested individually. The animal was placed on the floor of the staircase with its back to the staircase. The number of stairs climbed and the number of rears were recorded during a 3-min period. Climbing was defined as each stair on which the mouse placed all four paws; rearing was defined as each instance the mouse rose on hind legs (to sniff the air), either on the stair or against the wall. The number of stairs descended was not taken into account. Before each test, the animal was removed and the box cleaned with a diluted alcohol solution to eliminate smells.

#### *Novel object recognition test*

This is a visual recognition memory test based on a method described by Tordera et al. [24]. The apparatus, an open-field box (50 × 50 × 20 cm), was constructed from plywood painted white. Three phases (habituation, training and retention) were conducted on three separate test days. Before the training session, the mice were individually habituated by allowing them to explore the box for 10 min (day 1). No data were collected at this phase. During training sessions (day 2), two identical objects were placed into the box in the northwest and southeast corners (approximately 5 cm from the walls), 20 cm away from each other (symmetrically) and then the individual animal was allowed to explore them for 5 min. Exploration of an object was defined as directing the nose to the object at a distance of  $\leq 1$  cm and/or touching it with the nose and rearing at the object; turning around or sitting near the object was not considered as exploratory behavior. The time spent in exploring each object was recorded as well as the number of interactions with both objects. The animals were returned to their home cages immediately after training. During the retention test (day 3), one of the familiar objects used during the training session was replaced by a novel object. Then, the animals were placed back into the box and allowed to explore the objects for 5 min. The same parameters were measured as during the training session, namely the time spent in exploring each of the two objects and the number of interactions with them. All objects were balanced in terms of physical complexity and were emotionally neutral. The box and the objects were thoroughly cleaned by 70 % alcohol before each session to avoid possible instinctive odorant cues. A preference index, a ratio of the amount of time spent exploring any one of the two items (old and new in the retention session) over the total time spent exploring both objects, was used to measure recognition memory.

#### *Statistical analysis*

Results are expressed as the mean  $\pm$  SEM. The differences in mean for average immobility time in the FST, the

staircase test parameters (number of rearing and stair-climbing events) and novel object recognition were evaluated by ANOVA and Tuckey for multiple comparisons in the post hoc analysis. Significant results were determined as  $p < 0.05$ .

#### **Brain perfusion and fixation**

The mice were anesthetized by an i.p. injection of ketamine (100 mg/kg) and xylazine (20 mg/kg) and killed by transcardiac perfusion with phosphate-buffered saline (PBS) followed by perfusion with 4 % paraformaldehyde (PFA, Sigma-Aldrich Israel Ltd., Rehovot Israel) in phosphate buffer (PO<sub>4</sub>, pH 7.4). After perfusion, the brain was quickly removed and fixed overnight in 4 % PFA (in PO<sub>4</sub>, pH 7.4) at 4 °C. On the following day, the brain was cryoprotected by immersion in 30 % sucrose in 0.1 M PO<sub>4</sub> (pH 7.4) for 24–48 h at 4 °C before brain cutting. Frozen coronal Sects. (30–50  $\mu$ m) were cut on a sliding microtome (Leica Microsystems GmbH, Wetzlar, Germany), collected serially and kept in a cryoprotectant at –20 °C until staining.

#### **Detection of autoantibodies in the sera**

The levels of autoantibodies in the mice sera were tested by a homemade ELISA 1 month post-injection. Briefly, ELISA plates (M9410, Sigma-Aldrich) were coated separately with 20  $\mu$ g/well of different antigens: Gardasil which contains the HPV L1 major capsid protein of HPV types 6, 11, 16 and 18, mouse brain protein extract, mouse brain phospholipid extract, Al hydroxide, dsDNA and  $\beta$ 2glycoprotein-I ( $\beta$ 2GPI). The plates were incubated overnight at 4 °C, washed and blocked with 3 %BSA in PBS 1 h at 37 °C. Sera were added at dilution of 1:200 for 2 h at room temperature. The binding was probed with goat anti-mouse IgG conjugated to alkaline phosphatase at concentration of 1:5000 for 1 h at 37°C. Following appropriate substrate, the data were read by ELISA reader at 405 nm.

#### **Inhibition assay**

Brain protein extracts were prepared by lysis of brains from five healthy C57BL/6 mice, using ice-cold lysis buffer containing 50 mM Tris (pH 7.5), 150 mM NaCl, 10 % glycerol, 1 % Triton X-100, 1 mM EDTA, 1 mM PMSF, 1 mM sodium vanadate, 0.1 % protease inhibitor mixture (Sigma-Aldrich L-4391 St Louis, MO, USA) for 30 min on ice and centrifuged at 13,000 rpm for 20 min. The lysate was dialyzed against PBS. Protein concentration was determined by BCA Protein Assay Kit (Pierce, Thermo scientific, Rockford, IL, USA).



ELISA plates were coated with the HPV vaccine Gardasil which contains the HPV L1 major capsid protein of HPV types 6, 11, 16 and 18. Following blocking with 5 % skim milk powder, sera from the immunized mice, at different dilutions 1:200–1:10,000, were added to the plates in order to define 50 % binding of the sera to the HPV. Next, dilutions of sera which showed 50 % binding to HPV were incubated overnight at 4 °C with different concentrations of mouse brain protein extract (10–50 µg/ml) as the inhibitor. The following day, the mixtures were subjected to ELISA plates coated with HPV for 2 h at room temperature. The binding of the antibodies which did not create complex with the brain protein extract was probed with anti-mouse IgG conjugated to alkaline phosphatase, followed by the appropriate substrate. The percentage of inhibition was calculated as follows: % inhibition =  $100 - [(OD \text{ of tested sample without inhibitor} - OD \text{ of tested sample with inhibitor}) / (OD \text{ of tested sample without inhibitor})] \times 100$ .

### Brain tissue immunostaining

Brain sections were stained free-floating, incubated with the first antibodies overnight at 4 °C. The slices were then washed in PBS + 0.1 % Triton X-100 and incubated at room temperature for 1 h with the corresponding fluorescent chromogens-conjugated secondary antibody. Sections were stained for specific antigens with antibodies against activated microglia (anti-Iba-1, polyclonal, Abcam, Cambridge, UK) and astrocytes (anti-GFAP monoclonal, Dako, Carpinteria, CA, USA). Counter staining was performed with Hoechst (Sigma-Aldrich Israel Ltd., Rehovot Israel).

### Image acquisition, quantification and statistical analyses

Iba-1 and GFAP immunostaining was visualized using  $\times 4/0.1$  NA,  $\times 10/0.25$  NA and  $\times 40/0.65$  NA objective lenses on a Nikon eclipse 50i fluorescence microscope equipped with a Nikon DS Fi1 camera. In order to minimize bleaching of the fluorescence, images were obtained by serially moving the slide with no fluorescence and then acquiring the images in a standard manner. All sections were then studied quantitatively for differences in immunostaining density among the groups, using Image J software (NIH, USA). Region of interests (ROIs) was drawn manually using the 'Polygon selection' tool. Brain regions were identified using a mouse brain atlas. ROIs were chosen to represent anatomical regions previously shown to be involved in cognition and/or to exhibit variable sensitivity to neuroinflammation in other models. The mean intensity of the specific ROIs ( $\times 10$  magnification) was recorded for each individual animal recorded

(Analyze >> Measure), and data were analyzed using SPSS statistical software (version 15.0). Univariate analysis was conducted for each ROI/Antibody separately using 'group' as a fixed factor and 'experiment' as a Covariate. Post hoc analysis, one-way ANOVA, Student's *t* test, simple regression or correlation analysis was used when appropriate, according to the experimental design. Significance level was determined in one-tailed and two-tailed tests. The level of statistical significance of differences is  $p < 0.05$ .

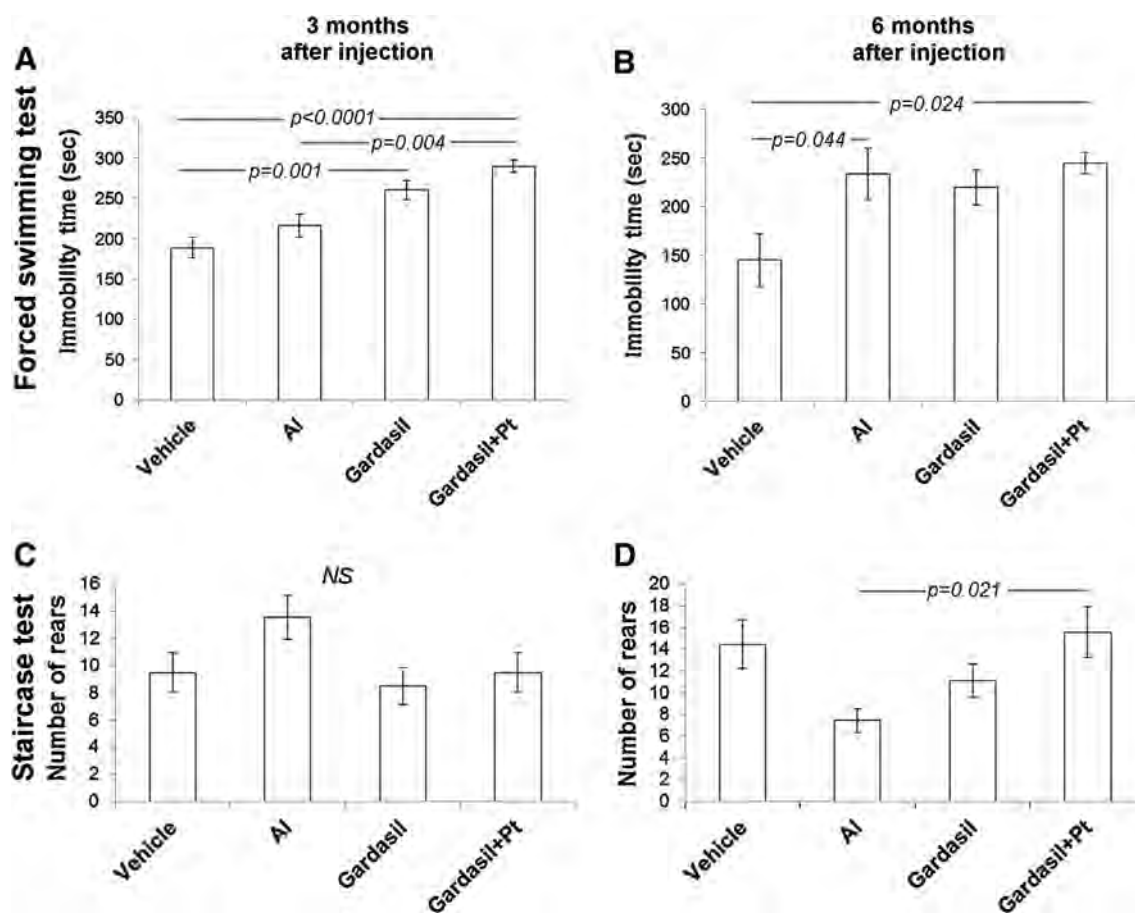
## Results

### Behavioral tests

The ANOVA analysis showed significant differences in the performance of the mice in the forced swimming and the staircase tests 3 months after injection (Fig. 1). The specific differences were detected by the post hoc test which showed that the two groups injected with the Gardasil vaccine spent significantly more time floating compared to control mice and Al-injected mice (Fig. 1a). No significant differences were found between the groups in the overall memory skills (measured by the novel object recognition test), locomotor function, exploratory activity and anxiety which were measured in the staircase apparatus (Fig. 1c).

The analysis after the behavioral testing at 6 months post-injection demonstrated that the alterations in the FST performance were sustained in the group injected with Gardasil + Pt compared to control mice ( $p = 0.024$ ; Fig. 1b), indicating that the effect of Gardasil + Pt exposure was long-lasting. Moreover, at 6 months post-injection, the Al-injected group likewise spent significantly more time floating compared to the control group ( $p = 0.044$ , Fig. 1b). Although the Gardasil group showed increased floating time compared to the vehicle-injected control group, the observed difference was not statistically significant. Given that after the first round of testing at 3 months post-injection, we killed five animals from each of the four experimental groups; it is possible that our experiment was insufficiently powered to detect milder adverse effects arising from the different treatments. Significant differences were also observed in the rearing frequency in the staircase test. Namely, the Al-injected mice showed a significantly lower frequency of rearing compared to the group injected with Gardasil + Pt in the staircase test ( $p = 0.021$ ; Fig. 1d). A lower frequency of rearing is an indication of a reduced exploratory response to a novel environment, and, it can also indicate a non-selective attention deficit. There was no statistically significant difference in the number of stairs climbed in the staircase test between the groups (not shown). In the FST,





**Fig. 1** Effects of AI, Gardasil and Gardasil + Pt toxin injections on behavioral tests. **a** and **b** show the floating time in C57BL/6 female mice as evaluated by the forced swimming test (FST). Results are presented as duration in seconds (mean  $\pm$  SEM) of immobility, defined as the absence of escape-oriented behaviors, such as swimming, jumping, rearing, sniffing or diving, recorded during the 6-min test. **a** Three months post-injection ( $n = 14$  per treatment

group); **b** Six months post-injection ( $n = 9$  per treatment group). **b**, **c** show the reduced exploratory activity in C57BL/6 female mice as evaluated by the rearing frequency in the staircase test. Results are presented as the number of rears (mean  $\pm$  SEM) during a 3-min testing period. **a** Three months post-injection ( $n = 14$  per treatment group); **b** Six months post-injection ( $n = 9$  per treatment group)

however, the changes were still significant despite the lower number of animals. No significant differences in behavior were observed in the novel object recognition test.

### Autoantibody profile and inhibition assay

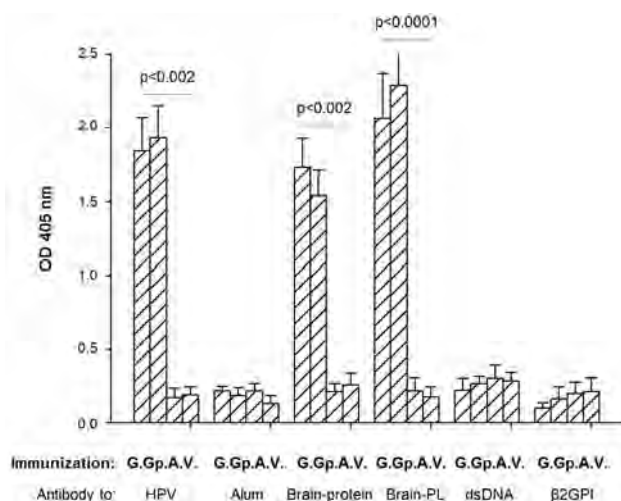
One month post-injection of either AI, Gardasil and Gardasil + Pt, the profile of serum antibodies was analyzed at dilution of 1:200. Elevated levels of antibodies recognizing the HPV L1 capsid protein of HPV types 6, 11, 16 and 18 ( $p < 0.002$ ), as well as anti-brain protein extract ( $p < 0.002$ ) and anti-brain phospholipid extract antibodies ( $p < 0.001$ ) were observed in the two groups of mice that received the HPV vaccine (Fig. 2). The titers of anti-HPV antibodies, anti-brain protein extract and anti-brain phospholipid extract antibodies were reduced after 2 months (data not shown). No elevation in the titers of anti-AI-

hydroxide, anti-dsDNA and anti- $\beta$ 2GPI antibodies, was detected in the sera of any of the four treatment groups of mice (Fig. 2).

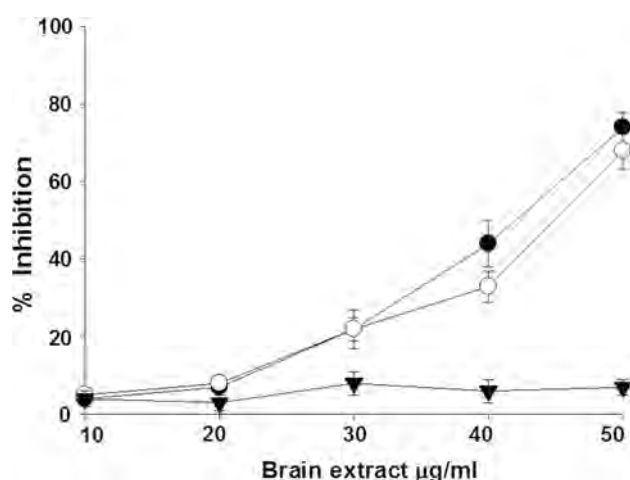
The binding of anti-HPV antibodies from the sera from the two treatment groups immunized with Gardasil to HPV L1 antigens was significantly inhibited by the mouse brain protein extract in a dose-dependent manner in comparison with AI-injected mice whose sera were negative for anti-HPV antibodies (Fig. 3).

### Brain tissue immunostaining

Following the behavioral tests at 4.5 months of age, five animals were killed from each of the four experimental groups and used for brain immunostaining procedures. With this relatively small group size, there were no clear changes between the groups in both astrocyte and

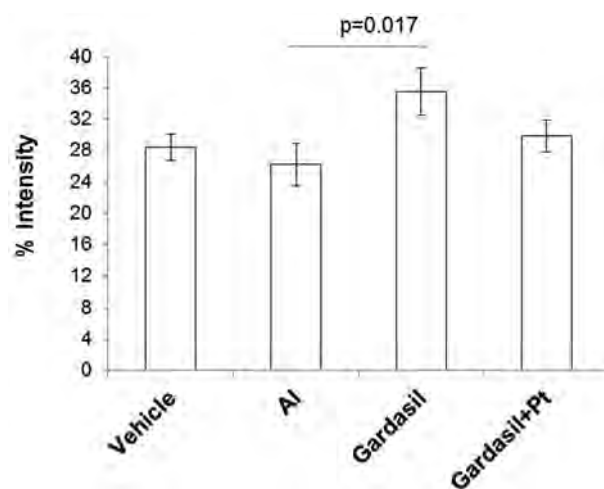


**Fig. 2** Titers of serum antibodies 1 month post-injection with either AI (A), Gardasil (G), Gardasil + Pt toxin (Gp) and vehicle (V). A homemade ELISA was used to detect the levels of anti-HPV, anti-AI hydroxide (Alum), anti-mouse brain protein extract, anti-mouse brain phospholipid (PL) extract, anti-dsDNA and anti-β2glycoprotein-I (β2GPI) antibodies in the sera of immunized mice. Pools of sera ( $n = 5$  per treatment group) were used as samples. All sera samples were assayed in triplicate. Data are presented as mean OD 405  $\pm$  SEM



**Fig. 3** Inhibition of the binding of antibodies from the sera of Gardasil-injected mice to components of the vaccine (presumably the HPV antigens) by the mouse protein extract. Pools of sera ( $n = 5$  per treatment group) were used as samples. All sera samples were assayed by duplicates in independent experiments. Data are presented as mean (% Inhibition)  $\pm$  SEM where % inhibition =  $100 - [(OD \text{ of tested sample without inhibitor} - OD \text{ of tested sample with inhibitor}) / (OD \text{ of tested sample without inhibitor})] \times 100$  (inverted triangle) AI, (filled black circle) Gardasil, (open circle) Gardasil + Pt

microglia staining in any of the regions of interests we investigated (CA1, CA3, dentate gyrus and the striatum). Nonetheless, there was a significant difference between the groups in the density of Iba-1 immunostaining using one-tailed analysis ( $p = 0.046$ ). Further post hoc analysis revealed significant increase in Iba-1 density in the CA1 of



**Fig. 4** Iba-1 immunostaining in the CA1 area of the hippocampus of C57BL/6 female mice injected with AI, Gardasil and Gardasil + Pt toxin. Brain sections from five animals out of each group were examined quantitatively for differences in immunostaining density using Image J software (NIH, USA) as described in “Materials and methods”. The data are presented as % mean (% Intensity)  $\pm$  SEM

Gardasil-immunized mice compared to AI-injected mice ( $p = 0.017$ ; Fig. 4). These results suggest that the CA1 might be vulnerable to small changes in neuroinflammation as a result of Gardasil immunization.

## Discussion

The present results show alteration of behavioral responses and neuro-inflammatory changes in mice as a result of AI and Gardasil vaccine injection in exposure doses which are equivalent to those in vaccinated human subjects. In particular, mice injected with AI and Gardasil spent significantly more time floating in the FST test (measure indicative either of locomotor dysfunction or depressive behavior), compared to control animals (Fig. 1a, b). In contrast, no significant differences were observed in the number of stairs climbed in the staircase test which is a measure of locomotor activity.

In addition, the AI-injected group showed abnormal responses to a novel environment, which was manifested in reduced rearing frequency in the staircase test, which indicates a reduction in exploratory behavior (Fig. 1d). The number of stairs and rears in this test is normally used to provide measures of general physical motor abilities and level of interest in the novelty of the environment. Rearing in response to environmental change (i.e., removing a mouse from the home cage and placing the animal in an open box or a staircase apparatus) is also considered an index of non-selective attention in rodents, while rearing

during object investigation likely reflects selective attention [26].

We further observed significant increase in levels of anti-HPV antibodies, and antibodies targeting the brain protein and the brain phospholipid extract components in the two groups of mice that received the Gardasil injection (Fig. 2). Moreover, the recognition of vaccine components (presumably the HPV L1 capsid protein species) by the antibodies from the sera of Gardasil-immunized mice was inhibited in a dose-dependent manner by the mouse brain protein extract (Fig. 3). On the basis of these results, it would appear that the anti-HPV antibodies from Gardasil-vaccinated mice have the capacity to target not only the HPV antigens but also brain antigen(s), either directly or via negatively charged phospholipids. Finally, we observed significant inflammatory changes in the Gardasil-injected mice, namely the presence of activated microglia in the CA1 area of the hippocampus (Fig. 4).

### Possible mechanisms of vaccine-induced injury

#### *The role of adjuvants*

It is interesting to note that, in our hands, the extent of adverse neurological manifestations was similar in the three treatment groups whose only common denominator was the Al compound. As we noted above, the clinical trials for both HPV vaccines, Gardasil and Cervarix, used an Al-containing placebo and the safety of the vaccines was thus presumed on the finding that there was an equal number of adverse events in the vaccine and the alleged placebo group [21, 22, 27–31]. The HPV vaccines, like many other vaccines, are adjuvanted with Al in spite of well-documented evidence that Al can be both neuro- and immuno-toxic [10, 11, 13, 32–35] and hence does not constitute an appropriate placebo choice.

The appearance of diverse adverse neurological and immuno-inflammatory manifestations following routine vaccinations is well documented in the medical literature (Table 1). Although the classical explanations for these phenomena have largely centered on vaccine antigens, in recent years attention has shifted to Al adjuvants. Consequently, in the last decade, studies on animal models and humans have indicated that Al adjuvants have an intrinsic ability to inflict adverse immune and neuro-inflammatory responses [9–11, 13, 14, 33, 35–37]. This research culminated in delineation of ASIA-‘autoimmune/inflammatory syndrome induced by adjuvants’, which encompasses the wide spectrum of adjuvant-triggered medical conditions characterized by a misregulated immune response [2]. Notably, the vast majority of adverse manifestations experimentally triggered by Al in animal models and those

associated with administration of adjuvanted vaccines in humans are neurological and neuropsychiatric [2]. These observations should not be particularly surprising given Al’s well-established neurotoxic properties [38, 39]. What has, however, been argued is that the concentrations at which Al is used in vaccines are not sufficient to cause neurotoxicity [17, 40]. This argument, however, is not supported by recent evidence.

It should be noted that the long-term biodistribution of nanomaterials used in medicine is largely unknown. This is likewise the case with the Al vaccine adjuvant, which is a nanocrystalline compound spontaneously forming micron/submicron-sized agglomerates. It has been recently demonstrated that Al adjuvant compounds from vaccines, as well as Al-surrogate fluorescent nanomaterials, have a unique capacity to cross the blood–brain and blood–cerebrospinal fluid barriers and incite deleterious immuno-inflammatory responses in neural tissues [10, 13, 41]. Thus, a proportion of Al particles escapes the injected muscle, mainly within immune cells, travels to regional draining lymph nodes, then exits the lymphatic system to reach the bloodstream eventually gaining access to distant organs, including the spleen and the brain. Moreover, the Trojan horse mechanism by which Al loaded in macrophages enters the brain, results in the slow accumulation of this metal, due to lack of recirculation [10, 41]. The sustained presence of Al in central nervous system tissues is likely responsible for the myriad of cognitive deficits associated with administration of Al-containing vaccines in patients suffering from post-vaccination chronic systemic disease syndromes including macrophagic myofasciitis (MMF) [9, 11, 35].

Thus, contrary to prevalent assumptions, Al in the adjuvant form is not rapidly excreted but rather, tends to persist in the body long-term. As demonstrated by Khan et al. [41], intramuscular injection of Al-containing vaccine in mice is associated with the appearance of Al deposits in distant organs, such as spleen and brain, which were still detected 1 year after injection. Similarly, Al-particle fluorescent surrogate nanomaterials injected into muscle were found to translocate to draining lymph nodes and thereafter were detected associated with phagocytes in blood and spleen. Particles linearly accumulated in the brain up to the 6-month end point. They were first found in perivascular CD11b + cells and then in microglia and other neural cells. The ablation of draining lymph nodes dramatically reduced the biodistribution of injected Al-fluorescent surrogate nanocompounds. In addition, the nanoparticle delivery into the brain was found to be critically dependent on the major monocyte chemoattractant protein MCP-1/CCL2 as intramuscular injection of murine rCCL2 strongly increased particle incorporation into intact brain while CCL2-deficient mice had decreased neurodelivery [41].

In the ASIA syndrome, there could be a the prolonged hyperactivation of the immune system and chronic inflammation triggered by repeated exposure and unexpectedly long persistence of Al adjuvants in the human body (up to years post-vaccination) [6, 42]. It is probable that one of the reasons why Al adjuvants are retained long-term in bodily compartments including systemic circulation is due to their tight association with vaccine antigens or other vaccine excipients [43]. Even dietary Al has been shown to accumulate in the central nervous system over-time, producing Alzheimer's disease type outcomes in experimental animals given dietary equivalent amounts of Al to what humans consume through a typical Western diet [44].

The ability of Al adjuvant nanoparticles to cross the blood–brain barrier via a macrophage-dependent Trojan horse mechanism may explain in part why some vaccines have a predilection to affect the central nervous system [8, 10, 33, 35, 39]. Another explanation comes from the fact that Al nanomaterials can on their own damage the blood–brain barrier and induce neurovascular injury [16, 45]. Collectively, these studies [16, 41, 45] show that nano-Al can accumulate in brain cells, inducing nerve and blood vessel damage and protein degradation in the brain. Persistent accumulation of nano-Al compounds regardless the source (i.e., vaccines, dietary) in the central nervous system may thus increase the likelihood of the development of acute and/or chronic neurological disorders.

With respect to the particular Al compounds used in HPV vaccines, AAHS in Gardasil and ASO4 (3-0-desacyl-4'-monophosphoryl lipid A (MPL) adsorbed onto Al hydroxide) in Cervarix, it should be noted that these new adjuvants induce a much stronger immune response than conventional Al adjuvants used in other vaccines (i.e., Al hydroxide and Al phosphate) [46]. Stronger immunogenicity of an adjuvant formulation also implies by default stronger reactogenicity and risk of adverse reactions. Because of the differences in immune-stimulating properties between different Al adjuvant compounds, safety of a particular adjuvant formulation cannot be a priori assumed on the basis of the allegedly good historical track record of other formulations. Rather, they need to be thoroughly evaluated case by case.

According to the US FDA, a placebo is, '*an inactive pill, liquid, or powder that has no treatment value*' [47]. From the literature cited above as well as the present study, it is obvious that Al in adjuvant form is neither inactive nor harmless and hence cannot constitute as a valid placebo. Commenting on the routine practice of using Al-based adjuvants as placebos in vaccine trials Exley recently stated that it is necessary to make a very strong scientific case for using a placebo which is itself known to result in side effects and that no scientific vindication for such practice is

found in the relevant human vaccination literature [7]. Conceivably, there is even less justification for using a novel and more potent Al formulation than those that have been in standard use (Al phosphate and hydroxide). The only aim that this practice achieves is to give potentially misleading data on vaccine safety. Moreover, it is unethical to give a placebo to healthy clinical trial subjects that has no benefit but rather, may cause harm.

#### *The role of vaccine-induced antigens: immune cross-reaction*

As noted above, we observed significant elevation of antibodies recognizing Gardasil components, most likely the HPV L1 capsid protein of HPV types 6, 11, 16 and 18 ( $p < 0.002$ ) and of antibodies targeting the mouse brain protein ( $p < 0.002$ ) and phospholipid extracts ( $p < 0.001$ ) in the sera of Gardasil-immunized mice (Fig. 2). The binding of anti-HPV antibodies from the sera of mice injected with Gardasil to components of the HPV vaccine, presumably the HPV L1 antigens, was inhibited in a dose-dependent manner by using mouse brain protein extract as the inhibitor (Fig. 3). Taken together, these results suggest that antibodies from Gardasil-vaccinated mice have the capacity to target not only the HPV L1 antigens but also brain antigen(s), either directly or via negatively charged phospholipids.

This interpretation is consistent with the findings of Kanduc [48] who showed that antigen present in both HPV vaccines Gardasil and Cervarix (the major capsid L1 protein of HPV-16) shares amino acid sequence similarity with numerous human proteins, including cardiac and neuronal antigens, human cell-adhesion molecules, enzymes and transcription factors. Moreover, such contention is also supported by a case of severe acute cerebellar ataxia (ACA) following HPV vaccination where combined immunosuppressive therapy with methylprednisolone pulse and intravenous immunoglobulin (IVIG) therapies as well as immunoadsorption plasmapheresis resulted in complete recovery of the patient. In this particular case, the patient (12-year-old girl) developed symptoms of ACA, including nausea, vertigo, severe limb and truncal ataxia, and bilateral spontaneous continuous horizontal nystagmus with irregular rhythm, 12 days after administration of the HPV vaccine. Severe ACA symptoms did not improve after methylprednisolone pulse and IVIG therapies, but the patient recovered completely after immunoadsorption plasmapheresis [49]. Although no significant antibodies were detected in this patient, the remarkable effectiveness of immunoadsorption plasmapheresis strongly suggested that some unidentified antibodies were involved in the pathophysiology of ACA [49]. Citing the work of Kanduc [50], the authors of this case



have stated that further research on molecular mimicry between human proteins and HPV16 L1-derived peptide is needed to determine the exact pathologic mechanism of ACA [49]. Altogether, these observations suggest that possible immune cross-reactions derived from utilization of HPV L1 antigens in current HPV vaccines might be a risk for cardiovascular and neurological autoimmune abnormalities [48, 50]. Our observation that nearly 85 % (129/152) of HPV vaccine adverse case reports in the current scientific literature relate to neuro-ophthalmic abnormalities may lend further support for this conclusion (Table 1).

## Conclusions

In summary, both AI and Gardasil vaccine injections resulted in behavioral abnormalities in mice (Figs. 1, 2, 3). Furthermore, immunostaining analysis showed an increase in the Iba-1 density in the CA1 area of the hippocampus in Gardasil-immunized mice in comparison with AI-injected mice, thus suggesting that CA1 might be vulnerable to neuroinflammation as a result of Gardasil immunization (Fig. 4).

In addition, we observed that the brain protein extract significantly inhibited in a dose-dependent manner, the binding of total IgG isolated from the sera of Gardasil-immunized mice to components of the vaccine, most likely, the HPV L1 capsid antigenic component (Fig. 3). Therefore, it is likely that mice immunized with the HPV vaccine developed cross-reactive anti-HPV antibodies which in addition to binding to the HPV L1 capsid protein may also bind to brain auto-antigens. The putative target antigen(s) should be further identified by immunoprecipitation and proteomics analyses.

In light of these findings, this study highlights the necessity of proceeding with caution with respect to further mass-immunization practices with a vaccine of yet unproven long-term clinical benefit in cervical cancer prevention [20, 51] and which in the other hand is capable of inducing immune-mediated cross-reactions with neural antigens of the human host. This note of caution becomes even more relevant when considering the continually increasing number of serious disabling neurological adverse events linked to HPV vaccination reported in the current medical literature (Table 1) and in vaccine surveillance databases [20].

Finally, in light of the data presented in this manuscript, new guidelines should be requested on the use of appropriate placebos in vaccine safety trials [7].

## Compliance with ethical standards

**Conflict of interest** Yehuda Shoenfeld has acted as a consultant for the no-fault US National Vaccine Injury Compensation Program. L.T. has served as an expert witness in cases involving adverse reactions following qHPV vaccine administration. The other co-authors declare no competing interests.

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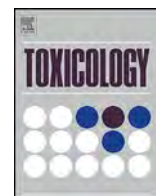
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# Non-linear dose-response of aluminium hydroxide adjuvant particles: Selective low dose neurotoxicity



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## ABSTRACT

Aluminium (Al) oxyhydroxide (Alhydrogel<sup>®</sup>), the main adjuvant licensed for human and animal vaccines, consists of primary nanoparticles that spontaneously agglomerate. Concerns about its safety emerged following recognition of its unexpectedly long-lasting biopersistence within immune cells in some individuals, and reports of chronic fatigue syndrome, cognitive dysfunction, myalgia, dysautonomia and autoimmune/inflammatory features temporally linked to multiple Al-containing vaccine administrations. Mouse experiments have documented its capture and slow transportation by monocyte-lineage cells from the injected muscle to lymphoid organs and eventually the brain. The present study aimed at evaluating mouse brain function and Al concentration 180 days after injection of various doses of Alhydrogel<sup>®</sup> (200, 400 and 800 µg Al/kg of body weight) in the *tibialis anterior* muscle in adult female CD1 mice. Cognitive and motor performances were assessed by 8 validated tests, microglial activation by Iba-1 immunohistochemistry, and Al level by graphite furnace atomic absorption spectroscopy.

An unusual neuro-toxicological pattern limited to a low dose of Alhydrogel<sup>®</sup> was observed. Neurobehavioural changes, including decreased activity levels and altered anxiety-like behaviour, were observed compared to controls in animals exposed to 200 µg Al/kg but not at 400 and 800 µg Al/kg. Consistently, microglial number appeared increased in the ventral forebrain of the 200 µg Al/kg group. Cerebral Al levels were selectively increased in animals exposed to the lowest dose, while muscle granulomas had almost completely disappeared at 6 months in these animals.

We conclude that Alhydrogel<sup>®</sup> injected at low dose in mouse muscle may selectively induce long-term Al cerebral accumulation and neurotoxic effects. To explain this unexpected result, an avenue that could be explored in the future relates to the adjuvant size since the injected suspensions corresponding to the lowest dose, but not to the highest doses, exclusively contained small agglomerates in the bacteria-size range known to favour capture and, presumably, transportation by monocyte-lineage cells. In any event, the view that Alhydrogel<sup>®</sup> neurotoxicity obeys “the dose makes the poison” rule of classical chemical toxicity appears overly simplistic.

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**Abbreviations:** Al, aluminium; dLNs, draining lymph nodes; im, intra-muscular; MMF, macrophagic myofasciitis; NOR, novel object recognition test; PFA, paraformaldehyde.

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## 1. Introduction

Many severe infectious diseases can be prevented and some of them have been eradicated by vaccines. Commonly used vaccines are generally well tolerated and considered safe by regulatory agencies. However, as other effective medical compounds, vaccines may occasionally cause adverse effects. In particular, a condition

manifesting by the combination of myalgia, arthralgia, chronic fatigue, cognitive dysfunction, dysautonomia and autoimmunity has been temporally linked to aluminium adjuvant-containing vaccine administration, called Macrophagic Myofasciitis (MMF) (Gherardi and Authier, 2003; Authier et al., 2003; Exley et al., 2009; Rosenblum et al., 2011; Santiago et al., 2014; Brinthe et al., 2015; Palmieri et al., 2016).

Although no consensus has been reached so far on a cause-to-effect relationship, environmental aluminium has long been suspected to act as a co-factor of several chronic neurological diseases (Van Rensburg et al., 2001; De Sole et al., 2013; Exley 2013, 2014) and the idea has emerged that aluminium adjuvants may be insidiously unsafe over the long-term in some predisposed individuals (reviewed in Tomljenovic and Shaw, 2011; Gherardi et al., 2015). Among aluminium salts used in vaccines, crystalline Al hydroxide or oxyhydroxide (Alhydrogel®) is the more widely used and is found in vaccines against tetanus, hepatitis A, hepatitis B, *Haemophilus influenzae* B, pneumococcal and meningococcal infections, and anthrax (Gherardi et al., 2015). This adjuvant consists of primary particles in the nano-sized range spontaneously forming micron-sized agglomerates (Eidi et al., 2015).

Although aluminium salts have been added to vaccines since 1926 (Glenny et al., 1926), exact mechanisms underlying their immuno-potentiating effects remain incompletely understood (Exley et al., 2010). Previous studies from our laboratory have shown that alum particles, as other poorly degradable particles, may not stay entirely localized in the injected tissue in mice, but can disseminate within phagocytic cells to regional lymph nodes and then to more distant sites and to the brain (Khan et al., 2013; Crépeaux et al., 2015; Eidi et al., 2015). In contrast to a previous belief, alum is characterized by striking biopersistence within immune cells in both the injected muscle, and the draining lymph nodes (dLNs) and spleen, where it may be found in conspicuous quantities 9 months after injection (Crépeaux et al., 2015). In humans, long term biopersistence of aluminium hydroxide within innate immune cells causes a specific lesion at site of previous immunization, called MMF, that may be detected up to >12 years after the last vaccine injection (Gherardi et al., 2001) in patients with a clinical condition now designated as ASIA 'Autoimmune/inflammatory syndrome induced by adjuvants' (Shoenfeld and Agmon-Levin, 2011).

The potential impact of aluminium adjuvant on the nervous system has been studied in mouse models. Alhydrogel® adjuvant, dosed at 100 µg Al/kg and subcutaneously injected in CD1 mice induced motor deficits and cognitive alterations associated with motor neuron death and a significant increase (350%) of reactive astrocytes indicative of an inflammatory process (Petrik et al., 2007). Although no motor neuron death was observed at the dose of 300 µg Al/kg, both microglial and astroglial reactions were observed in the spinal cord and were associated with altered motor and cognitive functions in CD1 mice (Shaw and Petrik, 2009).

In the same way, a neuro-inflammatory/degenerative syndrome has been described in sheep after repeated administrations of alum-containing vaccines (Luján et al., 2013), and impairment of neurocognitive functions and brain gliosis were reported in a murine model of systemic lupus erythematosus-like disease following intramuscular injection of Al hydroxide or vaccine against the hepatitis B virus (Agmon-Levin et al., 2014).

Previous in vivo aluminium adjuvant neurotoxicological studies did not include dose-response analyses. However, several reports studying neurotoxicity of soluble aluminium compounds administered by the oral route (Al chloride, Al nitrate, Al ammonium sulfate) to rodents showed a non-linear biphasic response on acetylcholinesterase activity (Kumar, 1998), dopamine turnover (Tsunoda and Sharma, 1999), nitric oxide synthase expression (Kim, 2003), and behavioural performances (Roig et al., 2006).

Poorly understood biphasic Al effects were also observed in vitro: cell cultures showing increased cell growth at low concentrations and diminished cell growth at high concentrations (Exley and Birchall, 1992). Similar unusual observations were made in studies of hippocampal long-term potentiation (Platt et al., 1995), and neuronal cell death in NSC-34 neuron-like cells (Eidi et al., 2015).

The present dose-response study was designed to evaluate long-term aluminium hydroxide neurotoxicity by assessing mouse behaviour, aluminium cerebral concentrations and microglial changes in CD1 mice 180 days after intramuscular injections of Alhydrogel®. Strikingly, the lower dose selectively induced neurobehavioural changes, cerebral aluminium level increases and microglial activation.

## 2. Materials and methods

### 2.1. Alhydrogel® doses

Animals were injected with Alhydrogel® adjuvant (InvivoGen), the characteristics of which have been previously determined in terms of size and positive zeta potential (Eidi et al., 2015). Doses were calculated by reference to medical histories of MMF patients who received a median of 4 doses of an Al-containing vaccine within the 10 years prior to their diagnosis (Gherardi et al., 2001). A 60-kg woman (MMF affects mainly women) injected with 1 dose of HBV ENGERIX® vaccine (GSK laboratories, France) receives 500 µg of Al, i.e. 8.3 µg Al/kg of body weight. Extrapolating mouse to human dosage is a challenging issue. Although a firm scientific basis for allometric conversion is still lacking, we used an allometry calculation based on body surface area that reflects the metabolic rate to determine the human equivalent dose per Kg. This  $\times 12.3$  allometric conversion factor from human to mouse (Sharma and McNeill, 2009) is easy to apply, and has been recommended to us by toxicologists of the French drug agency (AFFSAPS). Conversion resulted in an approximate of 100 µg Al/kg mouse body weight for one human dose. Four groups were used: control group (phosphate buffered saline (PBS) vehicle: Phosphate 0.1 M; NaCl 0.9%; pH 7.4); Alhydrogel® groups at the doses of 200, 400 or 800 µg Al/kg, in 3 injections of Alhydrogel in 20 µL PBS with a four-day interval. The animals thus received the mouse equivalent of 2, 4 and 8 human doses of Al-containing vaccine.

### 2.2. Animals

40 female CD1 mice, weighing 25–30 g (7 week old), were obtained from Charles Rivers Laboratories (France). Upon arrival, the females were housed at 5 animals per cage. Animals were maintained under a 12 h light cycle (8.00: 20.00), at a constant temperature ( $22 \pm 2^\circ\text{C}$ ) and a relative humidity of  $55 \pm 10\%$ . Mice were given ad libitum access to food and water. After a 1-week period for acclimatization, 8-week old females were separated in 4 experimental groups of 10 animals, and 20 µL im injections were made in the left *tibialis anterior*, with a 4-day interval between each injection.

At the end of the behavioural tests, 5 animals per group were sacrificed with an overdose of pentobarbital and transcardially perfused with PBS followed by ice-cold 4% paraformaldehyde (PFA) in PBS. Brains were collected for histological examination, post-fixed in PFA for 4 h at  $4^\circ\text{C}$  and immersed overnight in a 30% sucrose/PBS solution, then frozen and stored at  $-80^\circ\text{C}$  until sectioning. Whole brains were serially cut into 40 µm-thick coronal cryosections stored at  $-20^\circ\text{C}$  until use.

The other 5 animals per group were sacrificed with an overdose of pentobarbital. Brains were retrieved, quickly frozen in isopentane and kept at  $-80^\circ\text{C}$  for subsequent determination of Al levels.

All the experiments on animals were performed in respect to the guidelines provided by the [European Union \(Directive 2010/63/EU\)](#).

### 2.3. Behavioural and motor testing

A battery of 8 behavioural or physical tests was performed in the 4 experimental groups ( $n = 10$  mice/group) 180 days after the third injection. Tests were chosen in order to assess locomotor activity in the open-field ([Walsh and Cummins, 1976](#)), level of anxiety in the O-maze ([Shepherd et al., 1994](#); [Coutellier et al., 2009](#)), short-term memory in the novel object recognition test ([Ennaceur and Delacour, 1988](#); [Dudchenko, 2004](#); [Ennaceur, 2010](#); [Moore et al., 2013](#)), muscular strength in the wire mesh hang ([Kondziela, 1964](#)) and the grip strength tests ([Maurissen et al., 2003](#)), locomotor coordination in the rotarod test ([Pratte et al., 2011](#)), depression in the tail suspension test ([Steru et al., 1985](#)), and pain sensitivity in the hot plate test ([Espejo and Mir, 1993](#)).

All the tests were performed under white light <100 Lux between 9 a.m. and 1 p.m. They were video-recorded and all the variables were analyzed by the same experimenter, using ViewPoint Life Sciences Inc software (Canada).

The animals were transferred to the behavioural testing room 30 min prior to beginning of test in order to let the animal adapt to the test room conditions. Between each animal, the apparatus was cleaned with a 30% ethanol solution. At the end of a whole testing session, mice were sacrificed and samples were retrieved.

#### 2.3.1. Open-field

The general locomotor activity was assessed by the open-field test ([Walsh and Cummins, 1976](#)). The apparatus was made of a square open-field arena (42 cm side  $\times$  25 cm high walls) with the floor divided into 3 distinct areas: the peripheral, the medium and the central areas. At the beginning of the test, the mouse was placed in the center of the central area, and was let free to explore for 5 min. During this period the total distance and the distance and time spent in each of the three areas and the number of rearing, were recorded.

#### 2.3.2. Elevated O-maze

The level of animal anxiety was assessed by the elevated O-maze test ([Shepherd et al., 1994](#)), with the advantage of the lack of the ambiguous central square compared to the traditional plus-maze ([Coutellier et al., 2009](#)). The maze was elevated to a 70 cm height, with 2 open (50  $\times$  10 cm) and 2 closed (50  $\times$  10  $\times$  40 cm) arms. Arms of the same type were opposite to each other. Each mouse was tested within a 5-min test session. At the beginning, a mouse was placed individually in one of the closed arms, and was allowed to freely explore the maze. The time spent in closed and open arms, latency time to exit the closed arm for the first time, and the number of head-dippings and rearings were recorded.

#### 2.3.3. Novel object recognition test

The novel object recognition test (NOR) was first proposed by [Ennaceur and Delacour in 1988](#). This test is based on the spontaneous behaviour of rodents to interact more with a novel object than with a familiar one because of their inherent preference for novelty. Thus, in this test, rodents must be able to remember the previously encountered familiar object to determine which object is “novel” during the test trial ([Moore et al., 2013](#)).

The NOR task can be configured to cover various aspects and types of memory, including working memory ([Dudchenko, 2004](#); [Ennaceur, 2010](#)).

The apparatus consisted of a square chamber (40  $\times$  40  $\times$  25 cm) and a digital camera was used to record behaviour videos. Videos

were analyzed and the time spent by mice exploring each object was measured. The test consisted of four sessions: habituation to the field (10 min, day 1), habituation to objects (5 min, day 1), familiarization phase with 2 identical objects (5 min, day 2), test 1 h later (5 min, day 2), with one familiar and one novel object. The novel objects were different in shape and colour but similar in size. The interaction of mouse with both objects (familiar and novel) was recorded for 5 min and percent discrimination index was calculated to determine memory performance as follow:

Discrimination index = exploration time with novel object / (exploration time with familiar object + novel object)  $\times$  100.

Exploration of an object is defined as the orientation of animal's snout toward the object, sniffing or touching with snout, while running around the object, sitting or climbing on it was not recorded as exploration ([Antunes and Biala, 2012](#)).

#### 2.3.4. Wire mesh hang test

The hang wire mesh test was designed to test muscle strength using all four limbs ([Kondziela, 1964](#)). The inverted screen is a 43 cm square of wire mesh consisting of 12 mm squares of 1 mm diameter wire. The time during which the animals were able to sustain their weight holding onto the metal rail suspended in midair above the surface of soft bedding material was recorded for a 5 min-maximum time. Each mouse was subjected to three trials and the best performance was retained. Mouse body weight was considered, because this variable can influence performance.

#### 2.3.5. Grip strength test

The rodent grip strength test was developed to measure muscular strength ([Maurissen et al., 2003](#)). The apparatus (Bio-GS3, Bioseb, France) consists of a grasping device or platform (i.e. grid and T-bar) that is connected to a load cell. The test measurement is conducted by allowing the animal to grasp the device and then having the experimenter pull it away until its grip is broken. The maximal force achieved by the animal was recorded for two types of measurements: forelimb measurement and forelimb and hindlimb measurement. Five such trials for the forelimbs and five others for the four limbs were performed and both best performances were kept.

#### 2.3.6. Accelerating rotarod

Motor coordination and balance were tested using an accelerating rotarod (LE8200, Bioseb, France) consisting of a 3 cm diameter drum (15 cm above the base), divided with flanges into five lanes ([Pratte et al., 2011](#)). The apparatus is electronically controlled and evenly increases the speed of the bar from 4 to 40 rpm over a 5-min session. The mice were placed on the rod body orientation opposite to beam movement in the longitudinal axis, so that forward locomotion was necessary to avoid a fall. The mice were acclimated and trained on a morning session, and then they were given five successive trials on the afternoon. The best trial (longest latency to fall) for each mouse was retained. Since body weight may affect performance, mouse weight was considered in the score determination.

#### 2.3.7. Tail suspension test

The method is based on the observation that a mouse suspended by the tail shows alternate periods of agitation characterized by intense motor activity and expense of energy, and waiting-behaviour with immobility and energy saving ([Steru et al., 1985](#)).

For these experiments, the mouse was hung on a hook by an adhesive tape placed 20 mm from the extremity of its tail. Mice were both acoustically and visually isolated. Each mouse was



suspended by its tail for 5 min, allowing the ventral surface and front and hind limbs to be video-recorded using a digital camera facing the test box. Total immobility time and latency time to be immobile were measured during the entire 5 min test period. Immobility was defined as the absence of initiated movements, and included passive waving of the body. Times were scored manually by observer watching the video. Each mouse was tested only once. Mouse body weight was considered in the score determination.

### 2.3.8. Hot plate test

The hot plate test is a behavioural model of nociception in which mice display several noxious-evoked patterns as well as exploratory and self-care responses (Espejo and Mir, 1993). The animals were individually placed on a preheated 50 °C hotplate (LE7406 Bioseb, France). An open-ended cylindrical Plexiglas tube with a 20 cm diameter and a 25 cm height was placed on top of the hot plate to prevent the mice from escaping but leaving their paws exposed to the hot plate. The time from placing the animals on the hot plate to the time of the first paw lick, the first rearing and the first jump were measured with a stopwatch. To prevent tissue damage, the mice were removed from the hot plate after 3 min regardless of their response. Mice were observed only once.

### 2.4. Microglia immunohistochemistry

Analyses were carried out on 3 brains per group. Brain sections were incubated with primary antibody Anti-Iba1 (goat ab5076, AbCam Paris, France, 1/2000 in PBS with 1% BSA) overnight at 4 °C. Then sections were incubated with secondary biotinylated rabbit anti-goat antibody (1/200, Vector Laboratories, Paris, France) for 2 h at room temperature. Labeling was determined using the chromogenic diaminobenzidine (DAB) method.

Microscopy: Brain sections were viewed with a Zeiss AxioPlan (Carl ZeissCanada Limited, Toronto, ON, Canada) microscope at 20× magnification. Images were captured using Zen2012 software. Microglia cell density and cell body area were measured in 4 regions mapped by reference to the Paxinos mouse brain atlas (Paxinos and Franklin, 2001): ventral forebrain, inferior colliculus and visual and motor cortex. Determinations were done on selected areas (mean area of 175,000  $\mu\text{m}^2$ ) in 3 animals per group, by at least 2 of us, blinded for the identity of the group.

### 2.5. Brain Al analysis

Analyses were carried out on 5 brains per group (groups PBS, Alhydrogel® 200, 400, 800  $\mu\text{g}$  Al/kg) 180 days following injection, according to the published method of House et al. (2012) and as described in our previous study (Crépeaux et al., 2015). Briefly, Al concentrations were determined by TH GFAAS in half brains dried to a constant weight at 37 °C and digested in a microwave (MARS

Xpress CEM Microwave Technology Ltd) in a mixture of 1 mL 15.8 M  $\text{HNO}_3$  (Fischer Analytical Grade) and 1 mL of 30% w/v  $\text{H}_2\text{O}_2$  (BDH Aristar Grade). Digests were clear and colourless or light yellow with no visible precipitate or fatty residue. Upon cooling each digest was diluted to a total volume of 5 mL with ultrapure water. Total Al was measured immediately post digestion using an AAnalyst 600 atomic absorption spectrometer with a transversely heated graphite atomizer (THGA) and longitudinal Zeeman-effect background corrector and an AS-800 autosampler with WinLab32 software (Perkin Elmer, UK). Standard THGA pyrolytically-coated graphite tubes with integrated L'Vovplatform (Perkin Elmer, UK) were used. The Zeeman background corrected peak area of the atomic absorption signal was used for the determinations.

Results were expressed as  $\mu\text{g}$  Al/g tissue dry weight. Each determination was the arithmetic mean of a triplicate analysis.

### 2.6. Muscle analysis

Analyses were carried out on 3 muscles per group (groups PBS, Alhydrogel® 200, 400, 800  $\mu\text{g}$  Al/kg) 180 days following injection. Serial muscle tissue sections of 10  $\mu\text{m}$  were successively deposited on 30 different Superfrost®-plus slides in order to obtain 30 identical series. For each animal one slide containing 20 representative longitudinal sections was used for haematoxylin-eosin staining, and two alternate slides were treated for Morin staining and CD11b immunostaining respectively.

- Immunostaining was done using commercial primary antibody routinely used in the lab, raised against CD11b (1/50, AbD Serotec, MCA711, Oxford, UK). The labeling was made with Cyanine 3 AffiniPure F(ab')<sub>2</sub> Fragment Donkey Anti-Rat (1/200, Jackson ImmunoResearch laboratory INC, Suffolk, UK).
- Al was stained with Morin (M4008-2 G, Sigma-Aldrich, Saint-Quentin-Fallavier, France) that was dissolved in a solution consisting of 0.5% acetic acid in 85% ethanol. Formation of a fluorescent complex with Al was detected under a 420 nm excitation wavelength as an intense green fluorescence with a characteristic 520 nm emission.
- Conventional microscopy was done using Carl Zeiss photonic and fluorescence microscopes.
- The presence of a muscle granuloma was semi-quantitatively assessed at magnification  $\times 20$ , and quoted as: 0 (no or virtually no inflammatory cell), + (1 to 3 small granulomas), ++ (>3 small granulomas), +++ (>3 large granulomas).

### 2.7. Statistical analysis

Normality distribution of data was first analyzed by Shapiro-Wilk test, and then parametric or non-parametric tests were decided according to p values of Shapiro-Wilk test, i.e. parametric

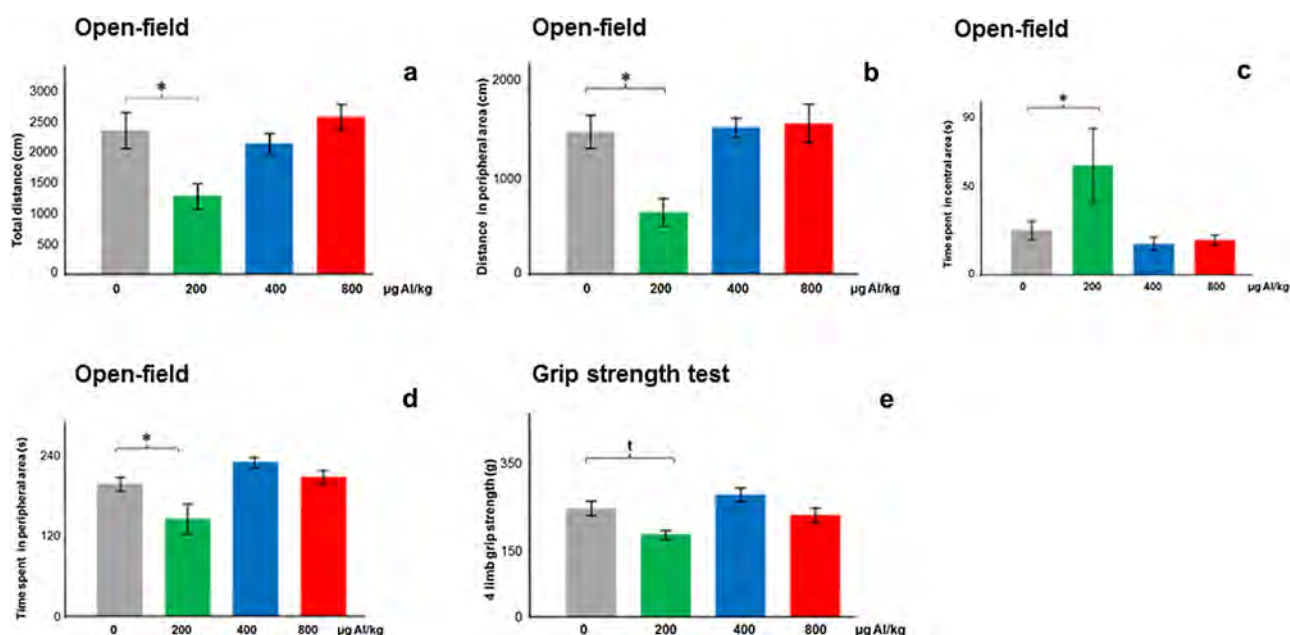
**Table 1**  
Effects of different doses of Alhydrogel® on motor activity and anxiety assessed in the open-field.

Open field	Control			Alhydrogel® 200 $\mu\text{g}/\text{kg}$			Alhydrogel® 400 $\mu\text{g}/\text{kg}$			Alhydrogel® 800 $\mu\text{g}/\text{kg}$			ANOVA	
	mean	$\pm$	sem	mean	$\pm$	sem	mean	$\pm$	sem	mean	$\pm$	sem	$F_{(3,39)}$	p
Total distance (cm)	2401.01	$\pm$	300.62	<b>1303.48</b>	$\pm$	<b>213.04</b> *	2181.90	$\pm$	166.76	2622.46	$\pm$	205.96	4.220	p < 0.05
Distance in central area (cm)	236.34	$\pm$	35.96	228.01	$\pm$	44.92	163.33	$\pm$	31.02	238.50	$\pm$	38.89	0.831	n.s.
Distance in intermediate area (cm)	677.41	$\pm$	108.29	431.85	$\pm$	86.21	459.20	$\pm$	64.02	811.68	$\pm$	76.32	3.205	p < 0.05
Distance in peripheral area (cm)	1487.26	$\pm$	173.89	<b>643.61</b>	$\pm$	<b>142.06</b> *	1530.20	$\pm$	106.15	1572.29	$\pm$	200.96	6.025	p < 0.01
Time spent in central area (s)	25.30	$\pm$	5.17	<b>62.34</b>	$\pm$	<b>21.22</b> *	17.67	$\pm$	3.678	19.89	$\pm$	2.92	4.157	p < 0.05
Time spent in intermediate area (s)	77.99	$\pm$	6.80	93.03	$\pm$	13.28	60.68	$\pm$	7.60	73.33	$\pm$	8.24	2.100	n.s.
Time spent in peripheral area (s)	196.68	$\pm$	9.94	<b>144.43</b>	$\pm$	<b>22.43</b> *	228.93	$\pm$	8.48	206.83	$\pm$	10.14	5.571	p < 0.01

Results are expressed as mean  $\pm$  S.E.M. of n = 10 mice/group. Bonferroni's t-test was used for multiple comparisons.

im, intra-muscular; n.s., not significant.

\* p < 0.05, statistical significant difference from controls.



**Fig. 1.** Effects of different doses of Alhydrogel<sup>®</sup> on mouse behaviour. Altered scores were selectively observed with low Alhydrogel<sup>®</sup> doses. (a) Total distance in the open field; (b) distance in the peripheral area in the open field; (c) time spent in the central area in the open field; (d) time spent in the peripheral area in the open field; (e) 4 limbs grip strength; 10 mice/group, results expressed as mean  $\pm$  S.E.M, ANOVA test with post-hoc Bonferroni's test. \*  $p < 0.05$ , statistical significant difference from controls; <sup>†</sup>  $p < 0.10$ , statistical tendency difference from controls.

tests (ANOVA or Student's *t*-test) can be used when normality distribution is assumed  $p > 0.05$ , whereas we used non-parametric test (Kruskal-Wallis test) when normality distribution is not assumed ( $p < 0.05$ ). Data from behavioural tests were analyzed using a one-way analysis of variance (one-way ANOVA). Post hoc comparisons have been performed using the Bonferroni's test when Anova was significant. Data from microglia IHC were analyzed using a Student's *t*-test. Data from Al concentration measurement were analyzed using a non-parametric Kruskal-Wallis test followed by a Mann-Whitney procedure modified for multiple comparisons when appropriate. Significance was set at  $p < 0.05$ . All statistical analyses were carried out using SPSS 16.0 software (SPSS INC., Chicago, IL, USA).

### 3. Results

#### 3.1. Body weight

The initial body weight was 30 g. Animals were weighed once a week during the whole procedure. No effects of treatment were observed on body weight (data not shown).

#### 3.2. Behavioural tests

##### 3.2.1. Open-field

In the open-field (Table 1), a one-way ANOVA showed a significant difference of the total distance walked ( $p = 0.012$ ), the distance in peripheral area ( $p = 0.002$ ), and time spent in both

central ( $p = 0.013$ ) and peripheral ( $p = 0.003$ ) areas (Fig. 1a–d). Bonferroni's post hoc analysis showed that mice from the group Alhydrogel<sup>®</sup> 200  $\mu\text{g Al/kg}$  crossed a significantly smaller total distance ( $p = 0.026$ ) and distance in the peripheral area ( $p = 0.005$ ) ( $1303.48 \pm 213.04$  cm and  $643.61 \pm 142.06$  cm respectively) than controls ( $2401.01 \pm 300.62$  cm). Furthermore, animals injected with Alhydrogel<sup>®</sup> 200  $\mu\text{g Al/kg}$  spent more time ( $p = 0.047$ ) in the central ( $62.34 \pm 21.22$  s) and less ( $p = 0.044$ ) in the peripheral areas ( $144.43 \pm 22.43$  s), as compared to controls (respectively  $25.30 \pm 5.17$  s and  $196.68 \pm 9.94$  s).

##### 3.2.2. Elevated o-maze

In the elevated O-maze (Table S1 in the Supplemental data section) no significant differences between groups were observed across all measured variables.

##### 3.2.3. Novel object recognition test

On the novel object recognition test (Table S2 in the Supplemental data section), one-way ANOVA did not reveal any statistical significant difference between groups across all studied variables.

##### 3.2.4. Grip strength test

In the grip strength test (Table 2), significant difference ( $p = 0.011$ ) between groups was observed for the 4-limb grip strength (Fig. 1e). Animals injected with Alhydrogel<sup>®</sup> at 200  $\mu\text{g Al/kg}$  tended ( $p = 0.076$ ) to have less strength ( $187.24 \pm 9.84$  g) compared to controls ( $246.76 \pm 16.46$  g).

**Table 2**

Effects of different doses of Alhydrogel<sup>®</sup> on muscular performances assessed in the grip strength test.

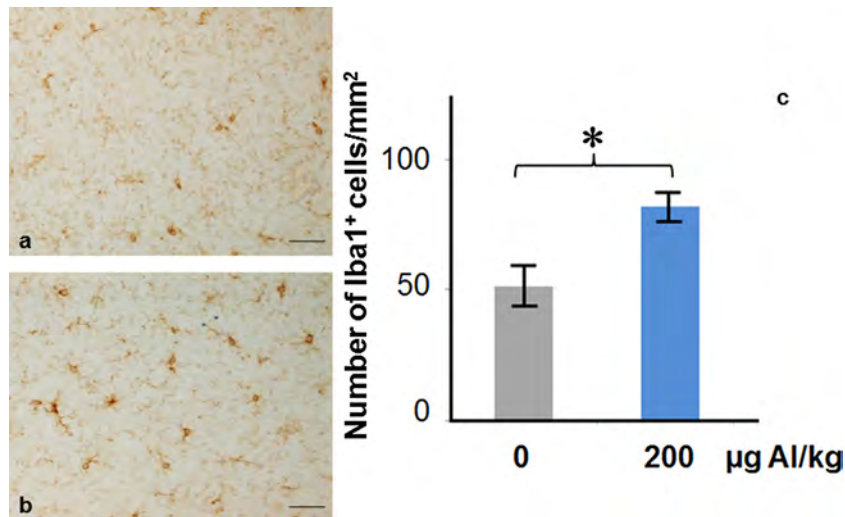
Grip strength test	Control			Alhydrogel <sup>®</sup> 200 $\mu\text{g/kg}$			Alhydrogel <sup>®</sup> 400 $\mu\text{g/kg}$			Alhydrogel <sup>®</sup> 800 $\mu\text{g/kg}$			ANOVA	
	mean	$\pm$	sem	mean	$\pm$	sem	mean	$\pm$	sem	mean	$\pm$	sem	$F_{(3,39)}$	$p$
Fore limbs (g)	171.69	$\pm$	6.36	162.85	$\pm$	10.68	157.33	$\pm$	7.14	160.20	$\pm$	6.61	0.788	n.s.
4 limbs (g)	246.76	$\pm$	16.46	<b>187.24</b>	$\pm$	<b>9.84<sup>†</sup></b>	278.59	$\pm$	15.95	231.97	$\pm$	17.32	4.188	$p < 0.05$

Results are expressed as mean  $\pm$  S.E.M. of  $n = 10$  mice/group. Bonferroni's *t*-test was used for multiple comparisons.

im, intra-muscular; n.s., not significant.

<sup>†</sup>  $p < 0.10$ , statistical tendency from controls.





**Fig. 2.** Iba1<sup>+</sup> microglial cell density in the ventral forebrain. Iba-1 immunostaining showed a slight increase of the microglial cell density in the group of mice injected with Alhydrogel® 200 µg Al/kg; (a) control mice injected with PBS; (b) mice injected with Alhydrogel® 200 µg Al/kg; (c) quantification of the microglial cell density. 3 mice/group; results expressed as means ± S.E.M, ANOVA test with post-hoc Bonferroni's test \*  $p < 0.05$ ; scale bars: 50 µm.

**Table 3**

Aluminum cerebral concentration measured by furnace atomic absorption spectrometry (µg/g of dry weight).

Cerebral Al concentration	Control	Alhydrogel® 200 µg/kg	Alhydrogel® 400 µg/kg	Alhydrogel® 800 µg/kg	Kruskal-Wallis
	0.0200 (0.0152–0.2088)	<b>1.0027</b> (0.3368–1.1493)	0.0143 (0.0127–0.0200)	0.0156 (0.0137–0.3970)	0.017

Results are expressed as median and quartiles (in brackets) of  $n = 5$  brains/group. Non parametric Kruskal-Wallis test followed by a Mann-Whitney procedure was used for multiple comparisons.

### 3.2.5. Wire-mesh hang test, accelerating rotarod, hot plate test and tail suspension test

No statistical differences were observed between the 4 experimental groups for these 4 tests (Tables S3–S6 in the Supplemental data section).

### 3.3. Microglia immunohistochemistry

As shown in Fig. 2, Alhydrogel® injections at doses of 200 µg Al/kg induced a significant increase ( $p = 0.033$ ) in the number of Iba-1<sup>+</sup> microglial cells in the ventral forebrain ( $81.90 \pm 5.30$  cells/mm<sup>2</sup>) compared to controls ( $51.43 \pm 7.87$  cells/mm<sup>2</sup>). Microglial density was similar to controls in visual and motor cortex and inferior colliculus in all groups. Microglial cell body size was similar in all groups (data not shown).

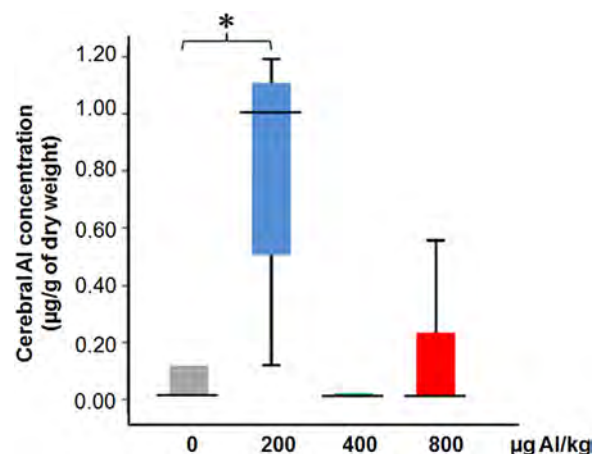
### 3.4. Cerebral Al level

The measurement of cerebral Al levels (Table 3) revealed a significantly ( $p = 0.011$ ) higher Al level in brains from animals injected with 200 µg Al/kg (median value 1.00 µg/g of dry weight) than in brains from control group (0.02 µg/g of dry weight). No significant increase was observed in animals injected with 400 or 800 µg Al/kg (Fig. 3).

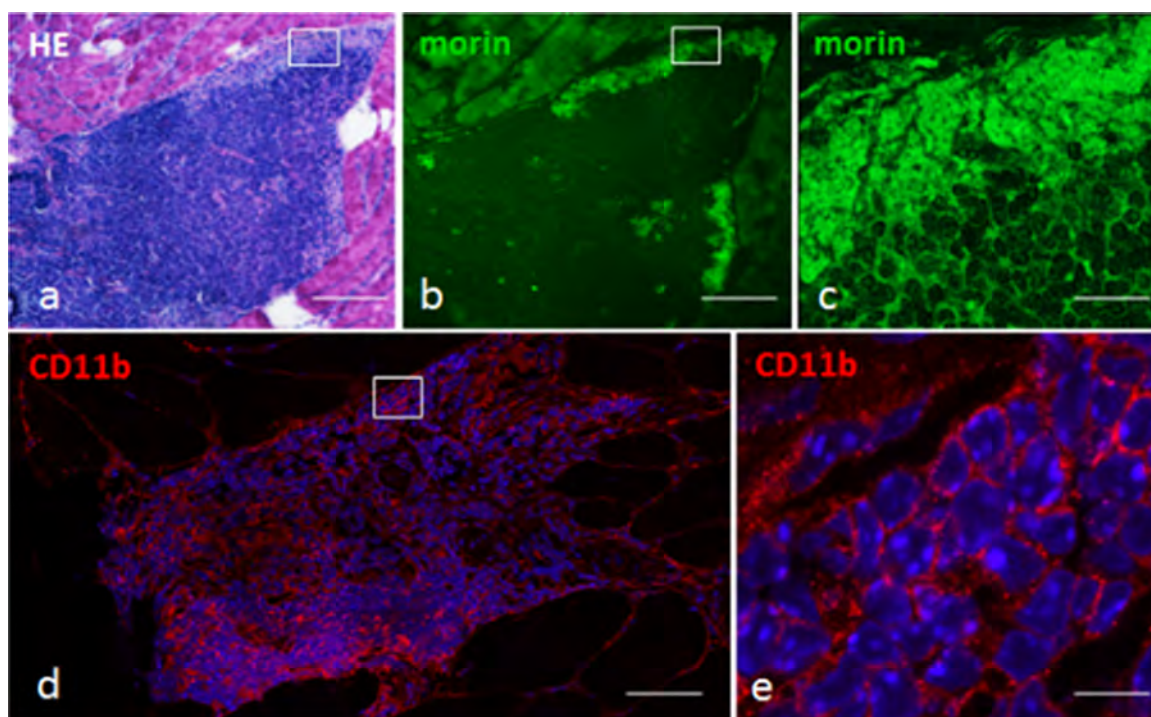
### 3.5. Muscle analysis

Granulomas with aluminium accumulations within macrophages were detected by Morin stain in the injected muscle of 6 animals (Fig. 4). As shown in Table 4, granulomas were found in 3/3 mice injected with 800 µg Al/kg, 3/3 mice injected with 400 µg Al/kg, and 0/3 mice injected with 200 µg Al/kg. The highest granuloma size was detected in mice injected with 800 µg Al/kg

(Fig. 4). An unusual aspect reminiscent of aluminium adjuvant-induced pseudo-lymphoma (Maubec et al., 2005) was observed in one case of the 800 µg Al/kg group and in another one of the 400 µg Al/kg group. The lesion appeared as a dense central area filled with monocyte-like and small lymphocytic cells and a rim of large macrophages with clear cytoplasm (Fig. 4a), in which aluminium was accumulated (Fig. 4b, c). Medium-sized CD11b-expressing monocyte lineage cells were found throughout the dense area of the lesion (Fig. 4d, e) often mixed with abundant nuclei of other mononuclear cell types as assessed by DAPI staining (Fig. 4d). Multinucleated giant cells were not found.



**Fig. 3.** Aluminium level determination in brain (µg/g of dry weight). Increased cerebral concentrations of aluminium were selectively observed with 200 µg/kg low Alhydrogel® dose. 5 mice/group; results expressed as median and range values, with quartiles boxes; non parametric Kruskal-Wallis test followed by Mann-Whitney test. \*  $p < 0.05$ .



**Fig. 4.** Muscle sections 6 months after Alhydrogel<sup>®</sup> injections (800 µg Al/kg).

(a) Pseudolymphomatous lesion including a dense central area filled with mononuclear cells and a rim of macrophages with clear cytoplasm (HE: haematoxyline eosin, scale bar: 40 µm); (b,c) the rim of macrophages is selectively associated with aluminium accumulation stained in green (Morin stain, scale bars: 40 µm and 100 µm respectively); (d) CD11b-expressing monocyte lineage cells are present throughout the dense area of the pseudolymphomatous lesion, mixed with abundant DAPI<sup>®</sup> nuclei of other mononuclear cell types (scale bar: 10 µm); (e) CD11b-expressing cells in an area prominently composed of medium-sized monocyte-lineage cells (scale bar: 20 µm).

**Table 4**

A semi-quantitative study of the granuloma size in injected muscle with Alhydrogel<sup>®</sup>.

Alhydrogel <sup>®</sup> group	No granuloma (0)	1 to 3 small granuloma (+)	>3 small granuloma (++)	>3 large granuloma (+++)
200 µg Al/kg	3	0	0	0
400 µg Al/kg	0	0	3	0
800 µg Al/kg	0	1	1	1

According to their size, observed granulomas were divided to four types: without granuloma (0), 1 to 3 small (+), >3 small (++) and >3 large (+++) granuloma. Then, number of animals of each criteria was determined, for n = 3 animals per group.

#### 4. Discussion

In the present study, 8 widely used behavioural tests performed 180 days after im injections of 200, 400, or 800 µg Al/kg in form of Alhydrogel<sup>®</sup>, in adult female CD1 mice, showed significant effects restricted to animals exposed to the lowest dose. Animals injected with 200 µg Al/kg showed decreased locomotor activity levels assessed by lower total distance crossed in the open-field, as reported previously after subcutaneous injection of 100 and 300 µg Al/kg of Alhydrogel<sup>®</sup> (Petrik et al., 2007; Shaw and Petrik, 2009), with concomitant decrease of the grip strength test suggestive of moderate motor weakness. In addition, increase of time spent in central area concomitantly with a decrease of both walked distance and time spent in peripheral area pointed to a behavioural change impacting the protective aversion of rodents for open spaces (Bourin et al., 2007), whereas other studies have reported increased anxiety levels (Petrik et al., 2007; Agmon-Levin et al., 2014). In sharp contrast, the highest doses of 400 and 800 µg Al/kg did not cause such changes. Consistently with the altered behavioural tests, microglial cell density appeared significantly increased in animals exposed to 200 µg Al/kg. This mild cerebral innate immune activation was selectively observed in ventral forebrain including the amygdaloid nuclei, which are implicated in

aversion/anxiety-like behaviours (LeDoux, 2007). Moreover, Al cerebral levels were significantly increased in animals injected with 200 µg Al/kg, but not in those injected with 400 and 800 µg Al/kg doses which showed neither neurobehavioural changes nor microglial reaction. The increased level of aluminium in brain was associated with an almost complete disappearance of aluminium-induced granuloma in mice injected with 200 µg Al/kg, while granulomas were constantly detected in the muscles injected with 400 or 800 µg Al/kg. In addition to conspicuous granuloma formation, 2/6 of these animals exhibited a pseudo-lymphomatous aspect suggesting an unusually strong local immune reaction to the foreign material.

In the present study we did not assess the concentration of Al in other tissues such as blood. Indeed, by using isotopic <sup>26</sup>Al, it was previously shown that the maximal increase in the plasma Al within 28 days after Al hydroxide im injection in the rabbit was about 2 ng/mL. Since the normal Al concentration was about 30 ng/mL in the animal, it was said that such a small increase would have been masked by the Al background if <sup>26</sup>Al-labelled adjuvants were not used (Flarend et al., 1997). Thus, Al plasma level determination on the long term, i.e. 6 months after im injection, cannot provide information in our mice. Furthermore in the present study the proposed method whereby Al is transported to organs and tissues

which are distant from the injection site does not actually involve the dissolution of the Al adjuvant into the muscle interstitial fluid and thereafter the blood but we are proposing that the transport of significant amounts of Al takes place in those cells which have infiltrated the injection site and taken up Al by endocytosis. Considering measurements of Al in muscle biopsies, we thought that they would not discriminate between extracellular and intracellular Al.

Evidence of a non-linear dose response curve of the neurotoxic effects of Alhydrogel<sup>®</sup>, with selective toxicity of the lowest dose used in the study challenges the classic toxicology paradigm “the dose makes the poison”. Non-monotonic dose-response curves have been previously reported in the field of aluminium toxicology. Non-monotonic biphasic neurotoxic effects have been observed both in vitro (see for review Exley and Birchall, 1992; Platt et al., 1995; Eidi et al., 2015) and in vivo (Kumar, 1998; Tsunoda and Sharma, 1999; Kim, 2003; Roig et al., 2006) after oral Al administration. However, the dose-response curve of the present study was not biphasic. Moreover, since cerebral aluminium level was not increased in mice injected with 400 or 800 µg Al/kg, the lack of neurotoxicity observed with these high doses was likely due to limited Al cerebral translocation, rather than to its paradoxical cytotoxic effects on neural cells. This puzzling result is challenging in the absence of solid knowledge on Alhydrogel<sup>®</sup> pharmacokinetics. We previously studied the fate of aluminium particles following im injections. Aluminium hydroxide is a highly hydrated crystalline compound composed of elementary nano-needles of approximately 2.2 nm × 4.5 nm × 10 nm (Mao et al., 2013) and displays a fibrous morphology at transmission electron microscopy (Shirodkar et al., 1990; Eidi et al., 2015). This compound spontaneously forms micron-sized agglomerates (Johnston et al., 2002), subjected to slight size variations after antigen adsorption (Eidi et al., 2015) and in vivo interactions with phosphate, organic acid and proteinaceous environments. A series of recent reports from our laboratory have shown that translocation of aluminium hydroxide may be specifically related to monocyte lineage cell uptake of this poorly biodegradable compound (Khan et al., 2013; Crépeaux et al., 2015; Eidi et al., 2015), likely resulting from phagocytosis or macropinocytosis (Mao et al., 2013).

Recent studies suggest that the adjuvant effect requires uptake by dendritic cells (Morefield et al., 2005) and combines i) local up-regulation of chemokines, including CCL2 (MCP-1) and CCL3 (MIP-1α), that increase the recruitment of immune cells into the injection site; ii) increase of antigen uptake by innate immune cells; iii) induction of monocyte differentiation into dendritic cells, and iv) facilitation of migration of dendritic cells towards the dLNs to prime adaptive immune responses (Seubert et al., 2008). Macrophages capture bacteria which are usually in the 1–4 µm size range (Kowalski et al., 1999). A previous report showed in vitro exposure of monocyte lineage THP1 cells to Alhydrogel<sup>®</sup> 200 µg Al/mL resulted in cellular incorporation of Alhydrogel<sup>®</sup> agglomerates, the size of which was 1.20 µm as measured by transmission electron microscopy after 24 h (Mold et al., 2014, 2016). Consistently, this size range was shown to be optimal for particle uptake by mouse peritoneal macrophages (1–2 µm) (Tabata and Ikada, 1988) and for particle attachment and subsequent internalization by mouse alveolar macrophages (2–3 µm), whereas internalization markedly drops when the size exceeds 4.2 µm (Champion et al., 2008).

Alhydrogel<sup>®</sup> biopersistence was confirmed in a variety of laboratory animal models up to 6–12 months post-injection, in both the injected muscle (Verdier et al., 2005; Authier et al., 2006; Khan et al., 2013; Eidi et al., 2015) and distant lymphoid organs (Crépeaux et al., 2015). Particles traffic from an injected tissue to the dLNs is size-dependent, smaller particles (20–200 nm) being able to drain in a free form whereas medium-sized particles (0.5–

2 µm) are exclusively subjected to cell transportation (Manolova et al., 2008). Although the point has not been precisely addressed in the literature for particles >2 µm, it seems possible that rapid cellular uptake of limited size particles is associated with quicker cell transportation to dLNs compared to large particles subjected to slow cell uptake, showing that, in this period of time, lower doses of adjuvant can diffuse in the body and reach the brain whereas higher ones do not, for a considered time point (Crépeaux et al., 2015).

On these grounds, we performed an exploratory evaluation of the size of agglomerates, a parameter that could be modified when concentration of the colloid suspension is increased to adjust doses (0.1, 0.2, 0.4 g Al/L in PBS 1X corresponding to 200, 400, and 800 µg Al/kg respectively). Dynamic light scattering showed that the colloid suspensions in PBS at pH 7.2 corresponding to the neurotoxic 200 µg Al/kg condition was exclusively composed of small bacteria-size agglomerates (mean = 1750 ± 100 nm), easily captured by innate immune cells. In contrast, suspensions corresponding to higher doses showed 2 size peaks, including one peak corresponding to very large agglomerates (about 35,000 nm) and another one corresponding to either small agglomerates (mean = 1500 ± 400 nm in the 400 µg Al/kg condition) or medium-sized agglomerates (mean = 4800 ± 500 nm in the 800 µg Al/kg condition).

Although further studies are clearly required to document the influence of Alhydrogel<sup>®</sup> agglomeration state on in vivo neurotoxic effects, such a finding would not be unprecedented in the field of particle toxicology since both cellular uptake and distribution in the body of other types of particles are influenced by the particle size (Buzea et al., 2007; Reddy et al., 2007; Landsiedel et al., 2012), and aggregation rate (Mühlfeld et al., 2008), two parameters that strongly determine particle toxicity (Bell et al., 2014; Leclerc et al., 2012; Nascarella and Calabrese, 2012; Mold et al., 2016).

In conclusion, the non-linear dose-response profile documented herein, in which the lowest dose but not the highest doses is neurotoxic in mice, is a novel insight in the field of aluminium adjuvant safety. It may suggest that Alhydrogel<sup>®</sup> toxicity obeys the specific rules of particle toxicology rather than any simplistic dose-response relationship. As a possible consequence, comparing vaccine adjuvant exposure to other non-relevant aluminium exposures, e.g. soluble aluminium and other routes of exposure, may not represent valid approaches. For example, aluminium retention rate observed after intravenous injections of traceable soluble aluminium citrate (Priest, 2004) has been used to set up the reassuring infant retention model of aluminium adjuvants (Mitkus et al., 2011). This model was based on the hypothesis that aluminium adjuvants are solubilized by citrate ions in muscle interstitial fluid (Flarend et al., 1997), without any consideration of quick adjuvant cellular uptake and systemic long term diffusion of adjuvant agglomerates (Khan et al., 2013; Eidi et al., 2015). In the context of massive development of vaccine-based strategies worldwide, the present study may suggest that aluminium adjuvant toxicokinetics and safety require reevaluation.

## Competing interests

The authors declare that there are no conflicts of interest.

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## Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at <http://dx.doi.org/10.1016/j.tox.2016.11.018>.

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# Is exposure to aluminium adjuvants associated with social impairments in mice? A pilot study

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## ABSTRACT

**Background:** Our group has shown that significant correlations exist between rates of Autism Spectrum Disorder (ASD) and total aluminum adjuvants given to children through vaccines in several Western countries. These correlations satisfied eight out of nine Hill criteria for causality. Experimental studies have demonstrated a range of behavioural abnormalities in young mice after postnatal exposure to aluminium. To build on our previous work, the current study will investigate the effect of aluminium adjuvants on social behaviour in mice. Anomalies in social interaction are a key characteristic of those with ASD.

**Methods:** Neonatal CD-1 mice pups were injected with either a total of 550 µg of aluminum hydroxide gel (experimental group) or saline (control) spread out during the first two weeks of postnatal life. The mice were then subjected to behavioural tests for social interest and social novelty at postnatal week 8, 17 and 29. p-Values were calculated using the Mann-Whitney and Kruskal Wallis tests.

**Results:** Aluminum injected mice showed diminished social interest compared to controls at week 8 ( $p = 0.016$ ) and 17 ( $p = 0.012$ ). They also demonstrated abnormal social novelty from controls at week 8 ( $p = 0.002$ ) and week 29 ( $p = 0.042$ ).

**Conclusion:** This is the first experimental study, to our knowledge, to demonstrate that aluminum adjuvants can impair social behaviour if applied in the early period of postnatal development. The study, however, is insufficient to make any assertive claims about the link between aluminium adjuvants and ASD in humans.

## 1. Introduction

Aluminium (Al) is the most abundant metal found in the Earth's crust, however, it has no known role in any biological processes and is thus considered to be non-essential for life [1]. Given the ubiquitous presence of aluminium in the modern environment, chronic exposure to aluminium is unavoidable.

Aluminium exposure commonly occurs through products such as deodorants, cosmetics, dyes, processed foods, antacids, medicinal pills, drinking water, and vaccine adjuvants [2] [3] [4]. Adjuvants are agents added to vaccines that act through various immune-stimulating mechanisms in order to increase the specific immune response or responses to infectious antigens [5].

Several studies have repeatedly confirmed that accumulation of aluminium from any source can produce neurotoxicity in the central nervous system (CNS) [6–16]. Aluminium has been etiologically linked with several diseases such as Alzheimer's disease, amyotrophic lateral sclerosis, dialysis encephalopathy, Parkinson's disease, Gulf-War syndrome, epilepsy and multiple sclerosis [18–20]. Aluminium adjuvants,

in particular, have been linked with a variety of neuromuscular and multiple organ system dysfunctions, including macrophagic myofasciitis (MMF), and autoimmune/inflammatory syndrome induced by adjuvants (ASIA) [21,22].

One of the factors that influences the toxic potential of aluminum is the route of administration [23]. For ingested aluminium, the poor solubility of aluminum compounds allows for its effective excretion by the kidneys; with only about 0.25% of the ionic aluminum getting absorbed into the blood for those with normal kidney function [24,25]. Sweat is another major route of aluminum excretion [26]. However, almost 100% of the intramuscularly injected aluminum (as in vaccine adjuvants) is absorbed into the systemic circulation and travels to different sites in the body such as the brain, joints and the spleen where it accumulates and is retained for years post-vaccination [8,9,25]. Moreover, although the half-life of enterally administered aluminum is short (approximately 24 h), adjuvanted aluminum takes much longer to be eliminated because of its exceptional affinity for the various antigens. The latter is the very feature that allows it to activate an elevated immune response and thus act as a desirable adjuvant. Two other key

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aspects to keep in mind while addressing the question of toxicity are: (1) the aluminum dose in a given duration; for instance, the dose of aluminum in the hepatitis B vaccine which contains the lowest content of aluminum (250 µg) is five times that absorbed through 6 months of breastfeeding (55 µg) [27], and (2) the stage of neurodevelopment of the person being vaccinated. For example, an infant in the United States, in its first two years, usually receives 27 vaccines as part of the routine pediatric vaccination schedule; many of which contain aluminum adjuvants. This is a crucial period for major neurodevelopmental processes in an infant's brain, including the onset of synaptogenesis and extensive pruning of excessive synapses, during which the brain is highly susceptible to neurotoxic insults.

Aluminum has many effects on both the immune and central nervous systems. Effects of aluminium's neuro- and immuno-toxicity include impairment of neurotransmission and synaptic activity, disruption of the blood-brain barrier, microglial activation and brain inflammation, impairment of brain-specific gene transcription, neurite damage, amyloidosis and impairment of genetic resistance towards autoimmunity in both adults and infants [20].

Many of the aforementioned characteristics associated with neurotoxicity have also been observed in those with autism spectrum disorder (ASD). ASD is a neurodevelopmental disorder with the most recent prevalence reported to be at 1:68 in the United States [28], about 2000 times that before 1980 when it was a 'rare' disorder with a low prevalence that was relatively stable [20]. A sudden exponential rise in the prevalence of ASD cannot be explained through genetics alone or even a change in diagnostic criteria as, in many ways, the diagnostic criteria have become more stringent [29]. Despite evidence of genetic predispositions, the pathogenesis of ASD is yet unknown. Several studies have investigated the possibility of an environmental trigger, interacting with a set of susceptible genes, leading to the phenotype of ASD [30].

There has been considerable speculation on the role of vaccines in the contribution of the rising prevalence of ASD. A study by our group has shown a strong correlation between the rising prevalence of ASD and an increased aluminium dose through vaccine adjuvants given during early postnatal life [31]. However, ecological studies are unable to establish causality and are primarily aimed at generating valid hypothesis that can be examined by further experiments.

Another study conducted by our group has shown anomalies in behavioural outcomes in mice injected with aluminium as per the US pediatric vaccination schedule [32]. The current study has been designed to build on previous work by testing for behavioural deficits specific to a core symptom of ASD, namely, deficits in social behaviour.

## 2. Methods and materials

### 2.1. Aluminium adjuvant

Alhydrogel®, an aluminium hydroxide (Al(OH)<sub>3</sub>) wet gel suspension, was used as a source of aluminium hydroxide. Alhydrogel™ 2% is a trademark of Brenntag Biosector and was purchased from INVIVOGEN.

### 2.2. Dosage and administration

The aluminium injection schedule in our study was intended to mimic the 2010 US pediatric vaccination schedule to maintain consistency with our previous work [31,32]. The approximate amount of aluminum in all those pediatric vaccines containing aluminium adjuvants (Table 1) at different ages in preschool children, was adapted from our previous study which found a strong correlation between prevalence of ASD and the exposure to aluminium from pediatric vaccination schedules.

As an extension of our previous work, the current study focused on the effects of aluminium on one key characterizing feature of ASD, namely anomalous social interaction. To investigate this, we have attempted to mimic the Al load from the US pediatric schedule as closely as practically possible, in CD-1 mice (Table 2) in a similar manner as done in our previous study [11]. For this purpose, new born mice pups were divided into two groups, Al injected ("Al") and saline controls ("Control"), consisting of 28 and 23 animals respectively. The litters after birth were equally and randomly divided into Al and control groups, both containing an equal number of males and females. The dosage of Al adjuvant injected in mice was approximately equivalent (µg/kg) to Al exposure through pediatric vaccines in children (Table 1).

Mice were weaned when they were sexually mature at 5–6 weeks of age. Since most pediatric vaccinations are given to children before the

**Table 1**

The following table displays the approximate total body burden of aluminum in preschool children from pediatric vaccines (in µg) at different ages as per the 2010 U.S. vaccination schedule [11]. The approximate equivalent amount of aluminum injected in CD-1 mice (according to the schedule in Table 2) is shown in bold text.

Vaccine	Birth	2 months	4 months	6 months	15 months	2 years	6 years
Hep B	250	250		250			
DPT <sup>a</sup>		375	375	375	375		375
Haemophilus influenza type b <sup>b</sup>		112.5	112.5	112.5	112.5		
Pneumococcal		125	125	125	125		
Hep A					250	250	
Total Al (µg)	250	862.5	612.5	862.5	862.5	250	375
Total Al (µg/kg bw)	73.5	172.5	107.5	113.5	78.4	19.8	19.3
Total Al (µg/kg bw) injected into neonatal CD-1 mouse	–	<b>170</b>	<b>150</b>	<b>110</b>	<b>80</b>	<b>20</b>	<b>20</b>

Note: Table 1 Adapted, with permission, from Shaw et. al [11]

<sup>a</sup> Mean value from three different brands of DTaP (Infanrix, Daptacel, Tripedia).

<sup>b</sup> Mean value from two different brands of Hib (PedVax and Hiberix).

**Table 2**

Dosage and schedule of aluminum hydroxide or saline injections in treated mice and control mice.

Treatment group	Amount of Al/saline injected each day (µg/kg bw)						Total Al or saline injected (µg/kg bw)
	PND 2	PND 3	PND 5	PND 9	PND 12	PND 16	
Al or saline	170	150	110	80	20	20	550

age of 6 years (Table 1), we carried out the schedule of injections in mice over their first three postnatal weeks (Table 2). The “AI” group received six injections of Al hydroxide (at 170, 150, 110, 80, 20 and 20 µg/kg body weight respectively), for a total of 550 µg/kg body weight. Although most pediatric vaccines are given intramuscularly (i.m.), our mice were administered subcutaneous injections (s.c.) into the “scruff”. The reason for this route of administration is to use a consistent methodology based on our initial studies [33,34]. Studies by other groups associated with our laboratory have now been conducted using i.m. injections [35–37]. Mice up to 12 days postnatal were injected with a micro-needle while older mice were injected with a standard 30 G needle. The total injection volume for each animal was 15 µl of either Al hydroxide (10.1 mg/ml) in saline or saline alone.

### 2.3. Animals and breeding

CD-1 mice were chosen because they are an outbred strain which allows for them to mimic the genetic diversity present in humans, and to maintain consistency in experimental procedures with our previous work [32,38]. Three male and four female CD-1 breeders were purchased from Charles River (Wilmington, MA) such that one of the males mated with two females. Females and males were housed separately in a room with an ambient temperature of 22 °C and a 12/12 h light/dark cycle. Purina mouse chow and water were available to the mice *ad libitum*.

During breeding, males and females were housed together in a total of three breeder cages. After impregnation, the males were housed separately from females and the females were monitored closely for the parturition date which was considered as day 0 of the postnatal day (PND). The first litter had 13 animals from which 7 pups were assigned to the “AI” group and 6 to the control group. The second litter, which was assigned to the “AI” group and the third litter, assigned to the control group consisted of 14 pups each. The fourth litter consisted of 10, of which 7 were assigned to the “AI” group and 3 to the control group. Since all four litters were not born on the same day, injections were carried out starting PND2 for each individual litter (Table 2).

Mice were weaned at PND35 after which females and males were housed separately in cages with no more than four mice per cage. The sociability test was conducted on mice at 8 weeks, 17 weeks, and 29 weeks of age. An olfactory test, which was traditionally conducted to test for olfactory communication, was conducted alongside the sociability test to ensure that the results from the social interaction tests were not due to an impaired olfaction as the social interest was operationalized in terms of sniffing time.

All experimental procedures on animals were approved by the University of British Columbia's (UBC) Animal Care Committee (protocol #A11-0042) and were in compliance with the Canadian Council on Animal Care regulations and guidelines.

### 2.4. Behavioural tests

#### 2.4.1. Social interaction

A subject mouse was habituated to the experimental setup for 5 min in the center chamber followed by another 5 min in all three chambers. The social interaction test consisted of two parts [39]. The first part, 10-minute long, tested for sociability by measuring time spent sniffing an object versus a ‘stranger’ mouse. The stranger mouse was a mouse that had never interacted with the subject mouse prior to this experiment. It was kept in a wired cage to prevent direct contact between the subject and experimental mice. The wired cage, however, allowed for tactile, auditory, visual and olfactory exchange between the subject and stranger mouse. A human observer recorded the amount of time the

subject mouse spent sniffing both wired cages (with stranger mouse and empty). The more time spent sniffing the stranger mouse as opposed to sniffing the empty cage determined the sociability of the mouse. An empty cage identical to that of the stranger's, was used as the object in the experiment.

The second part (10 minute long) was designed to test for social novelty and memory. Previous studies have shown that healthy mice showed preference for social novelty over familiarity in the same test, while mice strains with an ASD phenotype showed preference for familiarity [40,41]. Here, in our experiment, the subject mouse was now presented with a new stranger mouse and a familiar mouse (stranger mouse from test 1), and the time it spent sniffing both these mice was recorded using a stop-watch. The apparatus was wiped clean (with 70% ethanol) and dried between two trials to eliminate any residual odors from the previous trial. The test, including both part 1 and 2, was conducted at three time-points, at 8, 17 and 29 weeks. Protocols were adopted from previous studies on behavioural tests specific to ASD-like phenotype [39–46].

#### 2.4.2. Olfactory habituation/dishabituation

The olfactory test was conducted in this study for two purposes: 1) to see whether there were any significant differences in interest in social/non-social odors between the two groups of mice, and 2) to see whether both groups of mice had normally-functioning olfaction, which would in turn confirm that results from the social interaction test (Section 2.4.1) were indeed testing social interaction and not relying on impaired olfaction. The olfactory test relies on the principle of habituation/dishabituation for which 2 social and 2 non-social odors are introduced to mice one after another and the time spent sniffing is measured for each odor by the experimenter using a digital stop watch. The experiment was double-blinded as the experimenter keeping track of time was oblivious to the group of the mouse. It was carried out in a standard mouse cage using applicators for the introduction of odors. The non-social odors used in this study were banana and almond extract (1:100) due to their distinctness and proven effectiveness in previous studies. The protocol was followed exactly as described by Yang and Crawley [47]. This test was conducted at the following time-points—9 and 17 weeks for males, and 15 and 21 weeks for females.

### 2.5. Statistical analysis

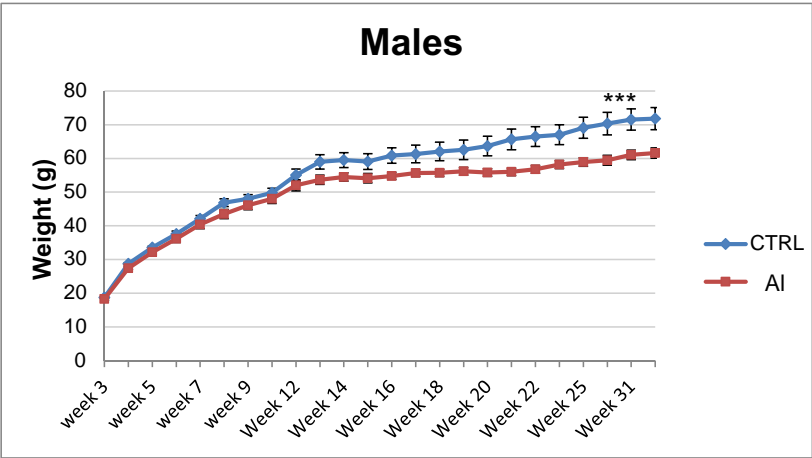
Values for each mouse on the individual tasks were used to calculate mean  $\pm$  S.E.M. for each group. The data was not normally distributed as determined using the Shapiro-Wilk test. Hence, the means between groups at each time point were compared using the Mann-Whitney test while the means between groups, across all time points were compared using Kruskal-Wallis test. Given that this was a pilot study, males and females were combined during analysis as power maximization was of utmost importance given the relatively limited sample size, and the highly noisy and variable nature of behavioural data. All data analysis was done using XLSTAT version 2014.3. Probability (p) levels < 0.05 were considered significant. Advice for this statistical analysis was provided by Steve Kalloger, a Clinical Research Consultant in the Department of Pathology at the University of British Columbia, Vancouver.

## 3. Results

### 3.1. Overall mouse development

No significant differences in food, water intake, or mortality were

A



B

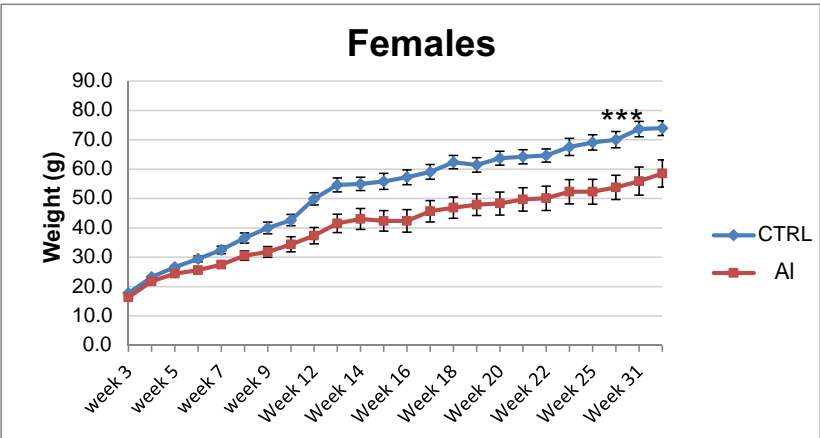


Fig. 1. Mean ( $\pm$  S.E.M.) weight of mice from 3 to 31 weeks of age for A) males B) females.

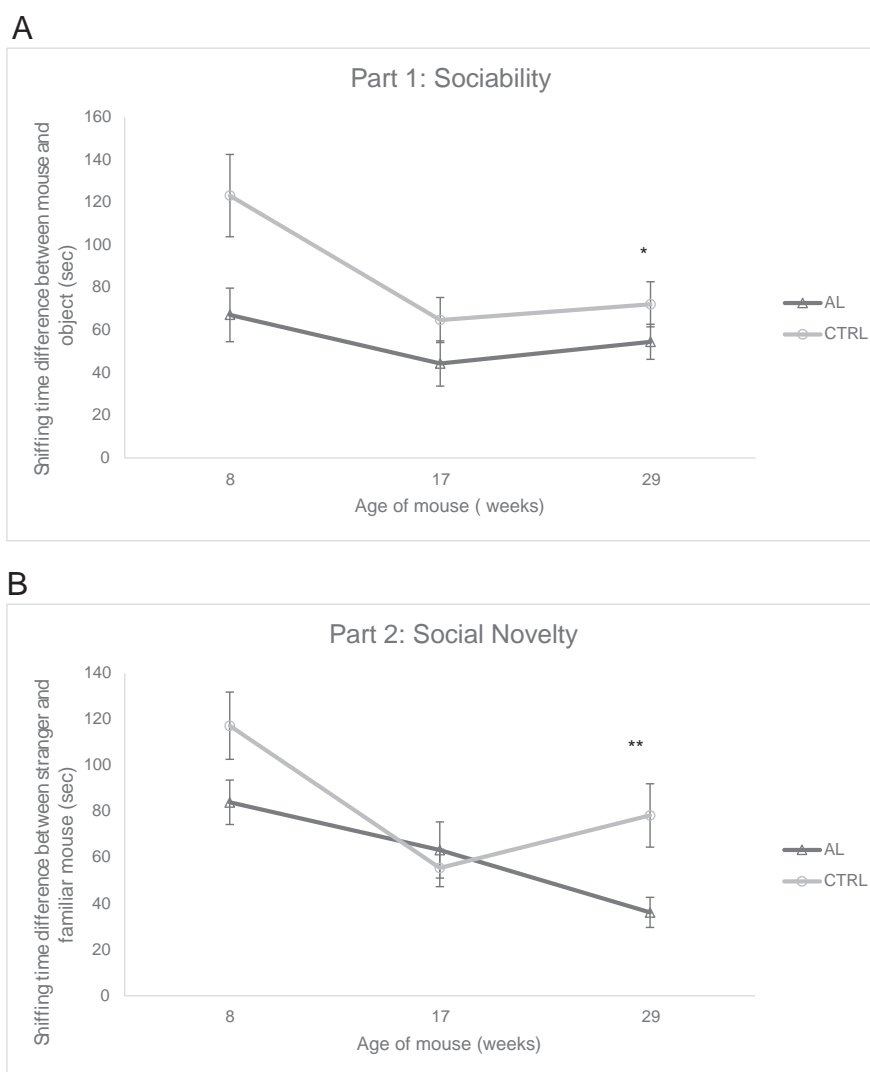
Table 3

Differences in the preferences of the control vs. aluminum-treated animal groups in two sociability tests performed at each of the three time points. Note that a certain time-point was considered to bear significance when the difference in sniffing time between controls and treated animals was significant ( $p < 0.05$ ) on either of the two stimuli they were exposed to in test 1 and 2, respectively.

Sociability test	Time points post Al-treatment		
	1 (week 8)	2 (week 17)	3 (week 29)
n $\rightarrow$	23 (Ctrl); 26 (AI)	23 (Ctrl); 25 (AI)	23 (Ctrl); 25 (AI)
Part 1 <sup>a</sup> - Preference: mouse <sup>c</sup> vs object	Significant difference: Al-treated prefers mouse less than controls ( $p = 0.016$ )	Significant difference: Al-treated prefers mouse less than controls ( $p = 0.012$ )	No difference between groups
Part 2 <sup>b</sup> - Preference: familiar mouse <sup>c</sup> vs stranger mouse <sup>d</sup>	Significant difference: Al-treated prefers stranger mouse less than controls ( $p = 0.002$ )	No difference between groups	Significant difference: Al-treated prefers familiar mouse more than controls ( $p = 0.042$ )

Significance value:  $p = 0.05$  using Mann-Whitney test.

- <sup>a</sup> Preference for mouse infers intact social interest in subject mouse.  
<sup>b</sup> Preference for stranger mouse infers intact social memory and novelty in subject mouse.  
<sup>c</sup> The same mouse used, thus considered familiar mouse in part 2.  
<sup>d</sup> A new stimulus mouse is used in each time point in both parts.



**Fig. 2.** Sniffing time differences ( $\pm$  S.E.M.) in controls vs. aluminum-treated mice in A) Sociability test ( $p < 0.05$ ) and B) social novelty test ( $p < 0.01$ ) performed across three time points. p-Values obtained using the Kruskal-Wallis Test. Note that we were unable to perform the Friedman's test due to unexpected mortality in some of our mice.

observed between controls and aluminium-injected mice. However, there were notable differences between the weights of aluminium-injected mice and control mice. In both males and females, the aluminium-injected mice weighed significantly lesser over time than controls ( $p < 0.001$ ). While both groups weighed about the same at week 3, the difference in weights between the two groups were initially noted at week 13 (in males) and week 7 (in females). This difference was consistently greater over time, and most significant at week 31 ( $p < 0.001$ ) (Fig. 1).

### 3.2. Social interaction test

In part 1, aluminium-treated mice spent significantly less time sniffing the 'mouse' stimulus compared to controls at both, week 8 ( $p = 0.016$ ) and week 17 ( $p = 0.012$ ). However, there was no significant difference in the time spent sniffing the mouse stimulus between the two groups by the final time-point at week 29. Also, there were no significance differences between groups in the time spent

sniffing the 'object' stimulus. In Test 2, aluminium-treated mice spent significantly less time than controls sniffing the 'stranger mouse' in week 8 ( $p = 0.002$ ) and significantly more time sniffing the 'familiar mouse' in week 29 ( $p = 0.042$ ) as compared to controls. There was no significant difference in sniffing time between aluminium-injected mice and controls in week 17. There were no significant differences between males and females, and were hence, combined for the purposes of data analysis. The results are summarized in Table 3 and Fig. 3.

### 3.3. Olfactory test

The analysis for males and females was done separately as the data was collected at different ages. No significant difference in sniffing time was noted between injected and control mice across time as shown in Fig. 3.

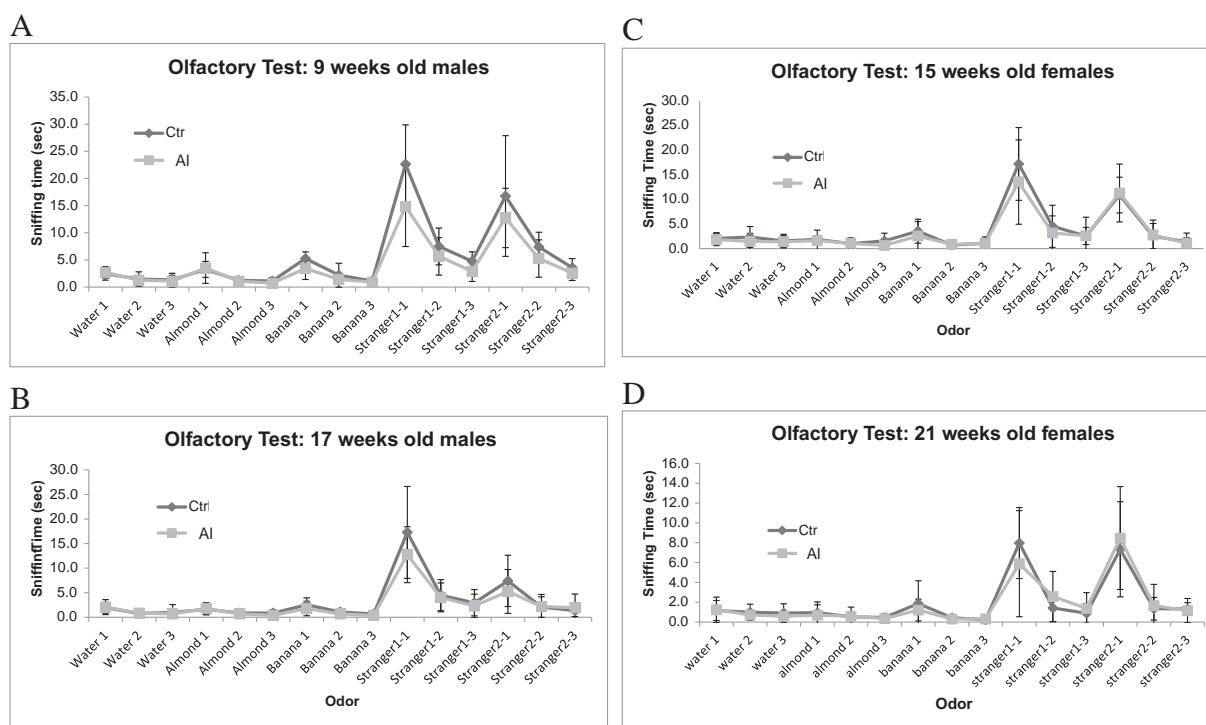


Fig. 3. Time spent sniffing ( $\pm$  S.D.) social (stranger 1, stranger 2) vs. non-social (almond, banana) odors in seconds. A) 9 week males B) 17 weeks males C) 15 weeks females D) 21 weeks females.

#### 4. Discussion

Several studies have demonstrated the neurotoxic effects of aluminium compounds [6,7,9,10,12]– [17,23]. Some studies have associated the neurotoxic effects of aluminium with ASD in a human ecological study [31] and in animal models [16,32]– [37]. Social interaction deficits are a key symptom of ASD. This is the first study, to our best knowledge, to investigate social behaviours in mice upon exposure to aluminium adjuvants. The results from our pilot study have shown that aluminium moderately impairs social interaction in CD-1 mice when injected subcutaneously to neonatal pups. We also noted that mice treated with aluminium weighed lesser on average than controls. This result differs from our previous study [32], however, is consistent with several other studies which have shown a reduction in body weight post exposure to aluminium [48–51]. This inconsistency, which is also present in the literature, could be attributed to several factors including the low sample sizes in both studies and the large variation in genetic profiles amongst CD-1 mice [52,50,53].

In both parts of the social interaction test, the difference in the time spent sniffing the two stimuli ('object' vs. 'mouse'; 'familiar' vs. 'stranger') was significant between controls and treated groups over time (see Fig. 2). In part 1, aluminium-injected mice consistently showed a significantly decreased interest in interacting with the 'mouse' stimulus as compared to controls at both time-point1 and time point2. However, this effect diminished at Time-point3 (see Table 3). These data suggest that the early impact of aluminium injection diminishes with time and age, a result that may mirror a feature of human ASD [54]. Our results from the first two timepoints in part 1 are consistent with Dawson et al. who showed that ASD children orient significantly less to social stimuli than to non-social stimuli compared to typically developing children [55]. Both groups showed similar 'interest' in the 'object' stimulus, suggesting that the anomaly in social interaction amongst the aluminium-treated animals resulted from a diminished interest in the social stimulus. This observation was most pronounced at week 8 (see Fig. 2). In part 2 of the social interaction test, we saw significant differences in social novelty between groups at time-point 1

and time-point 3, but not at time-point 2 (see Fig. 2 and Table 3). Also, there is a significant difference overall between groups over time (see Fig. 2); however, it is difficult to interpret if the anomaly in social novelty results from diminished interest in the socially-novel or familiar pathways due to the inconsistency in the trend across time-points (see Table 3).

While the underlying pathways through which aluminium impairs social behaviour are unknown, it seems plausible that aluminium impacts different behaviours at varying severities and neurodevelopmental stages because of its interactions with multiple neural pathways and immune molecules. In this study, while it seems to affect social interaction, both in early development and adolescence, social novelty appears to be spared at adolescence. Overall, it appears to be that aluminium treatment in this model system impairs both sociability and social novelty early-on in development, however, the effects of aluminium on social behaviour over time are less consistent and clear. Many studies have shown that exposure to aluminium is linked with memory deficits in humans and rodents [8,17,20,48,56,57]. Chronic exposure to aluminium has also been associated with decrease in acetylcholine levels which is an important neurotransmitter for memory and learning processes [58]. We found that aluminium injected mice were not very "interested" in sniffing the stranger mouse. While lack of such interest in the 'stranger' is one explanation for this finding, another one could be that the subject mouse has forgotten the 'familiar' mouse; hence, making them both equally novel for the subject mouse. While this study does not directly assess visual working memory, it seems to be a plausible explanation for the trend in test 2 based on previous studies. Future work can include the novel object recognition test along with social interaction tests to clarify whether the results from test 2 are tapping into anomalies in social memory or social novelty.

Behavioural studies alone, especially in rodents, are often insufficient to make inferences about human disorders. One of the limitations of behavioural studies is that they often have several variables that cannot always be fully controlled for. A few examples, of these which may have also impacted the current study, are observer bias, learning bias, time of the day, transportation from housing to behaviour



room, room temperature changes, ambient noise, inter-individual differences, residual scents and other factors. Care had been taken to minimize many of these in the current experiments, but they may not have been fully eliminated. Apart from these variables, which may commonly impact in vivo behavioural studies, the size and characteristics of the stimulus mouse may have impacted the study. The stimulus mouse was sex matched and weight matched to the average weight of the cohort. However, it is possible that it may have appeared too aggressive or too shy for a certain subject mouse, thus influencing the results. Another caveat, which is present in most rodent studies, is the inherent species-specific differences in timing of crucial embryonic and neonatal developmental processes which makes it difficult to find an exact age correspondence between humans and rodents. Our methods of analysis varied from those of Yang et al. who suggested that sociability is a yes-or-no phenotype determined by comparing the time spent in the different chambers of the social interaction apparatus *within* groups, rather than between groups. The rationale for their suggestion seemed unclear. So, we did a quantitative analysis between groups of comparing sniffing times, which may arguably be a more accurate measure of social interaction than chamber time. This is because it was often observed that the subject mouse was grooming itself or exploring a certain chamber, rather than sniffing the stimulus. Importantly, while this study has shown impaired social behaviour after exposure to aluminium, this study alone cannot make any substantive claims regarding the link between aluminium and ASD in humans. Future studies that look for changes in biomarkers (such as thyroid-stimulating hormone, interleukins, etc.) and gene expression changes alongside behavioural outcomes will be more informative in establishing this link.

While the etiology of ASD remains unknown, it is often considered to be a multi-factorial spectrum of conditions resulting from a complex interaction between genes and environmental triggers [30,59]. For example, exposure to prenatal and perinatal xenobiotics is now strongly implicated in the pathogenesis of disorders of the ASD [60,61]. Immune system dysfunction has been shown to be strongly correlated with cognitive and behavioural changes, including increased fear and anxiety, impaired social interactions, deficits in object recognition memory and sensorimotor gating deficits, many of which are present in ASD [62,63,64]. As mentioned above, a previous study from our laboratory has demonstrated a correlation between ASD prevalence and exposure to Al adjuvants [31]. Consistent with these findings, at least two other studies have shown positive relationships between rates of developmental disabilities, such as autism, and dosages of early-childhood vaccinations [65,66]. Moreover, an analysis of the VAERS database suggested significant toxic effects of Al on vulnerable children, positing its potential influence in the pathogenesis of autism [67]. However, we acknowledge that the VAERS data base may not always provide accurate rates of adverse reactions. Also, another recent study reported particularly high blood levels of Al in children with autism compared to those of typically developing children, citing that 90% of them had taken all pediatric vaccines [68]. Interestingly, 70% of their mothers had received vaccinations while pregnant, and 80% had been eating canned food and fish during that time; these results suggest that not only pharmaceutical but also dietary Al exposure may be a risk factor in developmentally vulnerable perinatal periods [68]. These findings support our hypotheses that Al is a suitable addition to the list of xenobiotics associated with developmental immunotoxicity and potential risk factor in the development of ASD [62].

## 5. Conclusion

Previous work has linked aluminium exposure to ASD. Social interaction deficits are one of the three core symptoms of ASD. To our knowledge, our current results represent the first study on social behaviour in mice after early exposure to aluminium adjuvants. We found that aluminium impairs social interaction in mice in some instances.

## Conflicts of interest

This study has received funding from Children's Medical Safety Research Institute (CMSRI) and the Dwoskin Family Foundation.

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