Journal of Inorganic Biochemistry 128 (2013) 237–244

Contents lists available at ScienceDirect

Journal of Inorganic Biochemistry

journal homepage: www.elsevier.com/locate/jinorgbio

# Administration of aluminium to neonatal mice in vaccine-relevant amounts is associated with adverse long term neurological outcomes

C.A. Shaw [a,b,c,*], Y. Li [a], L. Tomljenovic [a]

[a] Dept. of Ophthalmology and Visual Sciences, University of British Columbia, Vancouver, British Columbia, Canada
[b] Program in Experimental Medicine, University of British Columbia, Vancouver, British Columbia, Canada
[c] Program in Neuroscience, University of British Columbia, Vancouver, British Columbia, Canada

ARTICLE INFO

*Article history:*
Received 6 May 2013
Received in revised form 10 July 2013
Accepted 13 July 2013
Available online 19 July 2013

*Keywords:*
Autism
Aluminium
Adjuvants
Vaccines
Neurotoxicity
Neurodevelopmental disorders

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.



## 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 ($Al(OH)_3$) gel suspension, was used as a source of aluminium hydroxide. Alhydrogel is manufactured by

* Corresponding author at: Neural Dynamics Research Group, 828 W. 10th Ave., Vancouver, British Columbia V5Z1L8, Canada. Tel.: +1 604 875 4111x68373; fax: +1 604 875 4376.
*E-mail address:* cashawlab@gmail.com (C.A. Shaw).

0162-0134/$ – see front matter © 2013 Elsevier Inc. All rights reserved.
http://dx.doi.org/10.1016/j.jinorgbio.2013.07.022

**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‡ | | 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).
‡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 microneedle 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 Al injected (μg/kg bw) |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | 2 | 3 | 4 | 5 | 6 | 7 | 8 | 9 | 10 | 11 | 12 | 13 | 14 | 15 | 16 | 17 | |
| High Al (U.S.) | | x | | x | x | | | | x | | x | | | | | x | 550 |
| Low Al (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 *Klebisella*). 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

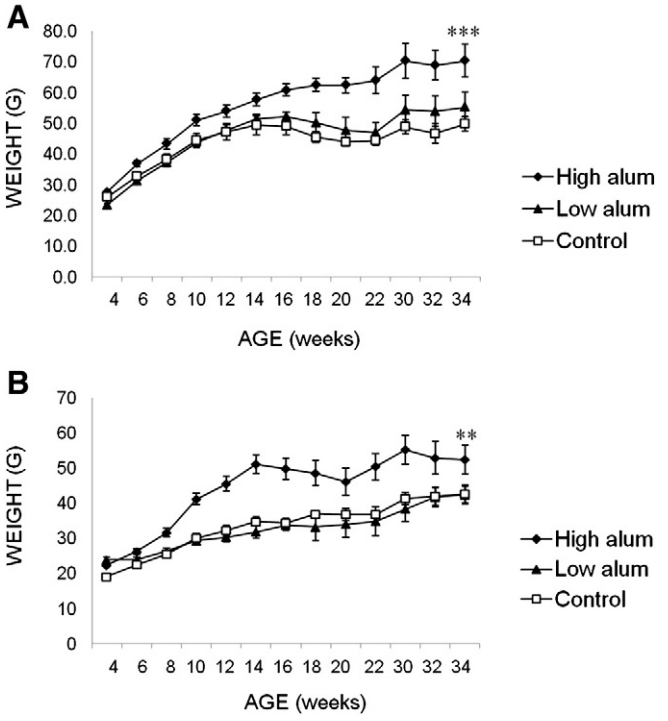


**Fig. 1.** The effects of Al adjuvant injections on body weight in young male (A) and female (B) CD-1 mice. Data are mean ± S.E.M. (animals per group, n = 7–10 males; n = 4–7 females). Mice were weighed once a week post-weaning. Both male and female mice injected with high Al showed a highly significant 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 ($p < 0.034$).

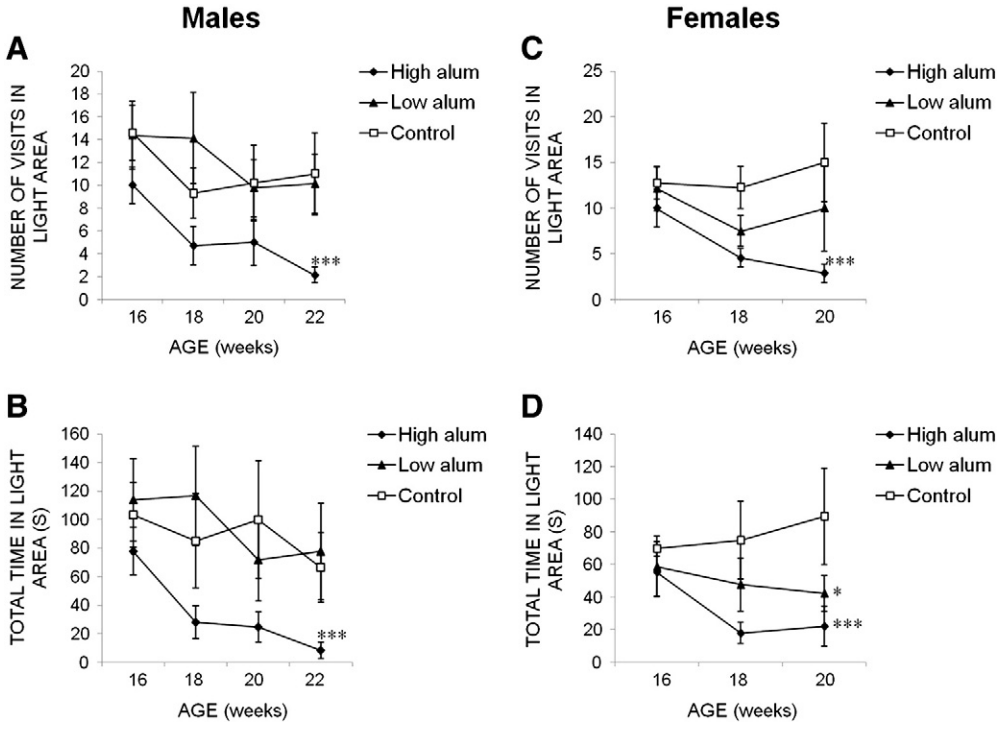


**Fig. 2.** The effects of Al adjuvant injections on indices of anxiety and exploratory behaviour in the light/dark box test in young CD-1 mice. Data are mean ± S.E.M. (n = 7–10 males; n = 4–7 females). Mice were tested at 14 weeks of age for a total of four tests, once every two weeks. Male (A) and 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 μg/kg of body weight administered in four injections (for a total of 48 μg/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 μg/kg (spread out over four injections; Table 2).

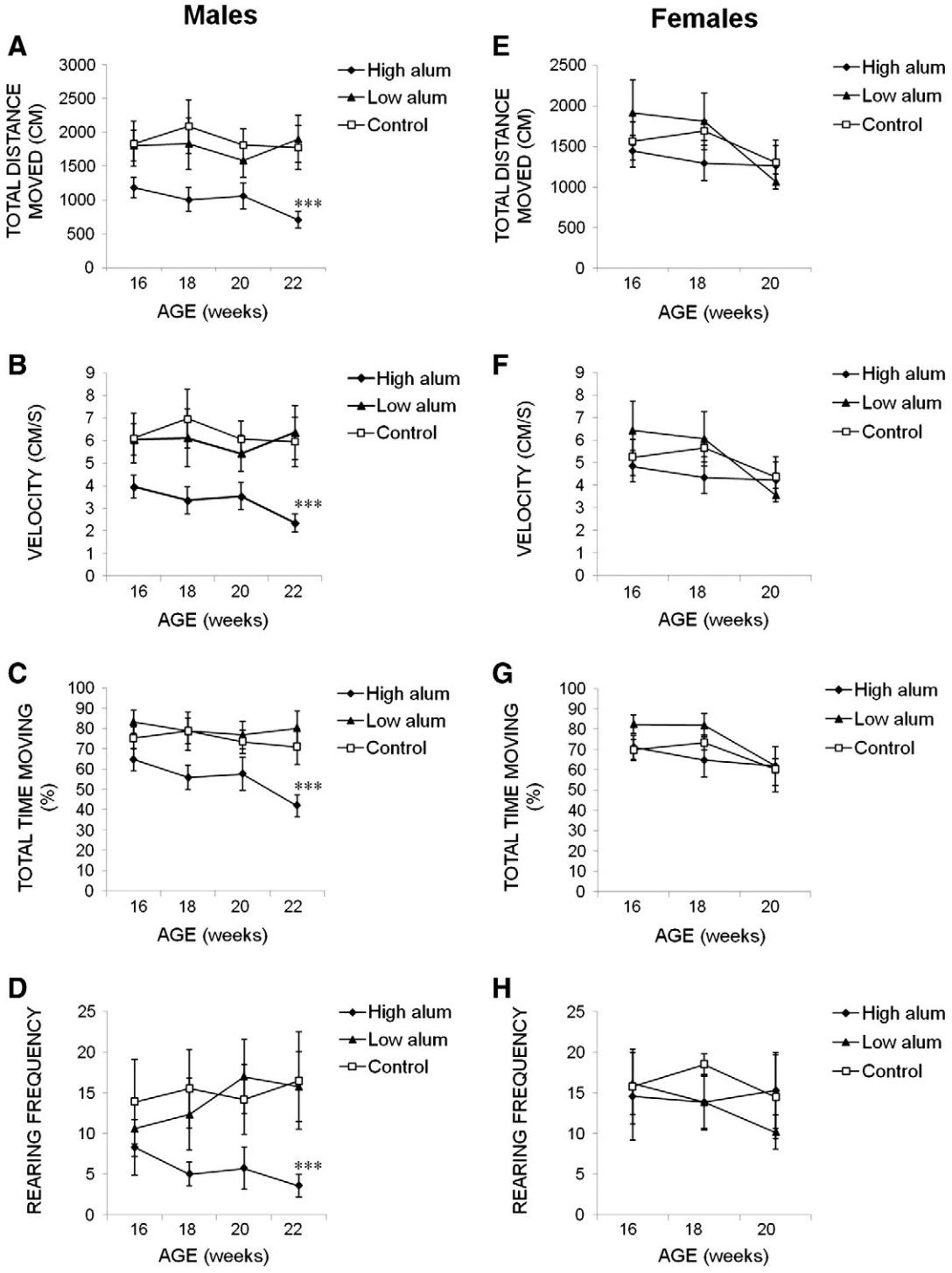


**Fig. 3.** The effects of Al adjuvant injections on locomotor activity in the open field test in young CD-1 mice. Data are mean ± S.E.M. (n = 7–10 males; n = 4–7 females). Mice were tested at 14 weeks of age for a total of four tests, once every two weeks. Male but not female mice injected with high 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 NLPR3 inflammasome pathway (and its downstream mediators caspase-1 and IL-1β), which is the principal

immunostimulatory pathway through which Al adjuvants operate [36,37]. Unfortunately, activation of the NLPR3 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 NLPR3 activation triggers type 2 diabetes is through interference with insulin signalling and promotion of insulin resistance. For example, using NLPR3 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-1β 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 NLPR3 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 biopersistence 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.

## Acknowledgements

The authors thank the Dwoskin Family Foundation and the Katlyn Fox Foundation for their financial support. We are also grateful to Agripina Suarez and other laboratory members for their assistance.

## References

[1] B.L. Carson, Integrated Laboratory Systems, Research Triangle Park, North Carolina, 2000, pp. 84. http://ntp.niehs.nih.gov/ntp/htdocs/Chem_Background/ExSumpdf/Aluminum.pdf.
[2] L. Tomljenovic, J. Alzheimers Dis. 23 (2011) 567–598.
[3] J.R. Walton, Curr. Inorg. Chem. 2 (2012) 19–39.
[4] J.R. Walton, Neurotoxicology 27 (2006) 385–394.
[5] C. Exley, E. House, Monatsh. Chem. 142 (2011) 357–363.
[6] C. Exley, Aluminium and medicine, in: A.L.R. Merce, J. Felcman, M.A.L. Recio (Eds.), Molecular and Supramolecular Bioinorganic Chemistry: Applications in Medical Sciences, Nova Science Publishers, Inc., New York, 2008, pp. 45–68.
[7] W.J. Gies, JAMA 57 (1911) 816–821.
[8] W.A. Banks, A.J. Kastin, Neurosci. Biobehav. Rev. 13 (1989) 47–53.
[9] C. Exley, J. Alzheimers Dis. 3 (2001) 551–552.
[10] C. Exley, J. Inorg. Biochem. 76 (1999) 133–140.
[11] J.R. Walton, M.X. Wang, J. Inorg. Biochem. 103 (2009) 1548–1554.
[12] J.R. Walton, Neurotoxicology 30 (2009) 182–193.
[13] J.R. Walton, J. Inorg. Biochem. 101 (2007) 1275–1284.
[14] A.T. Glenney, C.G. Pope, H. Waddington, U. Wallace, J. Pathol. Bacteriol. 29 (1926) 38–39.
[15] M.S. Petrik, M.C. Wong, R.C. Tabata, R.F. Garry, C.A. Shaw, Neuromolecular Med. 9 (2007) 83–100.
[16] C.A. Shaw, M.S. Petrik, J. Inorg. Biochem. 103 (2009) 1555–1562.
[17] L. Tomljenovic, C.A. Shaw, Lupus 21 (2012) 223–230.
[18] C.A. Shaw, L. Tomljenovic, Immunol. Res. (2013), http://dx.doi.org/10.1007/s12026-013-8403-1.
[19] L. Tomljenovic, C.A. Shaw, Curr. Med. Chem. 18 (2011) 2630–2637.
[20] E. Passeri, C. Villa, M. Couette, E. Itti, P. Brugieres, P. Cesaro, R.K. Gherardi, A.C. Bachoud-Levi, F.J. Authier, J. Inorg. Biochem. 105 (2011) 1457–1463.
[21] M. Couette, M.F. Boisse, P. Maison, P. Brugieres, P. Cesaro, X. Chevalier, R.K. Gherardi, A.C. Bachoud-Levi, F.J. Authier, J. Inorg. Biochem. 103 (2009) 1571–1578.
[22] F.J. Authier, P. Cherin, A. Creange, B. Bonnotte, X. Ferrer, A. Abdelmoumni, D. Ranoux, J. Pelletier, D. Figarella-Branger, B. Granel, T. Maisonobe, M. Coquet, J.D. Degos, R.K. Gherardi, Brain 124 (2001) 974–983.
[23] A. Batista-Duharte, E.B. Lindblad, E. Oviedo-Orta, Toxicol. Lett. 203 (2011) 97–105.
[24] Y. Shoenfeld, N. Agmon-Levin, J. Autoimmun. 36 (2011) 4–8.

[25] N. Agmon-Levin, G. Hughes, Y. Shoenfeld, Lupus 21 (2012) 118–120.
[26] I. Zivkovic, V. Petrusic, M. Stojanovic, A. Inic-Kanada, I. Stojicevic, L. Dimitrijevic, Innate Immun. 18 (2012) 333–342.
[27] L. Lujan, M. Perez, E. Salazar, N. Alvarez, M. Gimeno, P. Pinczowski, S. Irusta, J. Santamaria, N. Insausti, Y. Cortes, L. Figueras, I. Cuartielles, M. Vila, E. Fantova, J.L. Chapulle, Immunol. Res. (2013), http://dx.doi.org/10.1007/s12026-013-8404-0.
[28] L. Tomljenovic, C.A. Shaw, J. Inorg. Biochem. 105 (2011) 1489–1499.
[29] A.B. Hill, Proc. R. Soc. Med. 58 (1965) 295–300.
[30] S. Seneff, R.M. Davidson, J. Liu, Entropy 14 (2012) 2227–2253.
[31] L. Melendez, D. Santos, L. Luna Polido, M.L. Mendes, S. Sella, L.Q. Caldas, E. Silva-Filho, Clin. Exp. Pharmacol. 3 (2013), http://dx.doi.org/10.4172/2161-1459.1000120.
[32] K.R. Bailey, J.N. Crawley, Anxiety-related behaviors in mice, in: J.J. Buccafusco (Ed.), Methods of Behavior Analysis in Neuroscience, CRC Press, Boca Raton (FL), 2009.
[33] K.R. Bertges, L.C. Bertges, J.O. Tavares de Souza, J.C. Machado, C.A. Alberto Mourao Jr., Rev. Neurosci. 19 (2011) 34–38.
[34] M. Olczak, M. Duszczyk, P. Mierzejewski, K. Meyza, M.D. Majewska, Behav. Brain Res. 23 (2011) 107–118.
[35] T. Toimela, H. Tahti, Arch. Toxicol. 78 (2004) 565–574.
[36] S.C. Eisenbarth, O.R. Colegio, W. O'Connor, F.S. Sutterwala, R.A. Flavell, Nature 453 (2008) 1122–1126.
[37] C. Exley, P. Siesjo, H. Eriksson, Trends Immunol. 31 (2010) 103–109.
[38] S. Jha, S.Y. Srivastava, W.J. Brickey, H. Iocca, A. Toews, J.P. Morrison, V.S. Chen, D. Gris, G.K. Matsushima, J.P. Ting, J. Neurosci. 30 (2010) 15811–15820.
[39] S. Chakraborty, D.K. Kaushik, M. Gupta, A. Basu, J. Neurosci. Res. 88 (2010) 1615–1631.
[40] K. Rajamaki, J. Lappalainen, K. Oorni, E. Valimaki, S. Matikainen, P.T. Kovanen, K.K. Eklund, PLoS One 5 (2010) e11765.
[41] H. Wen, D. Gris, Y. Lei, S. Jha, L. Zhang, M.T. Huang, W.J. Brickey, J.P. Ting, Nat. Immunol. 12 (2011) 408–415.
[42] C. Bauer, P. Duewell, C. Mayer, H.A. Lehr, K.A. Fitzgerald, M. Dauer, J. Tschopp, S. Endres, E. Latz, M. Schnurr, Gut 59 (2010) 1192–1199.
[43] C.J. Tack, R. Stienstra, L.A. Joosten, M.G. Netea, Immunol. Rev. 249 (2012) 239–252.
[44] A.R. Moschen, C. Molnar, B. Enrich, S. Geiger, C.F. Ebenbichler, H. Tilg, Mol. Med. 17 (2011) 840–845.
[45] E. Israeli, N. Agmon-Levin, M. Blank, Y. Shoenfeld, Lupus 18 (2009) 1217–1225.
[46] R.K. Gherardi, Rev. Neurol. (Paris) 159 (2003) 162–164.
[47] R. Gherardi, F. Authier, Lupus 21 (2012) 184–189.
[48] C.J. Newschaffer, L.A. Croen, J. Daniels, E. Giarelli, J.K. Grether, S.E. Levy, D.S. Mandell, L.A. Miller, J. Pinto-Martin, J. Reaven, A.M. Reynolds, C.E. Rice, D. Schendel, G.C. Windham, Annu. Rev. Public Health 28 (2007) 235–258.
[49] D.L. Vargas, C. Nascimbene, C. Krishnan, A.W. Zimmerman, C.A. Pardo, Ann. Neurol. 57 (2005) 67–81.
[50] M. King, P. Bearman, Int. J. Epidemiol. 38 (2009) 1224–1234.
[51] H. Yazbak, J. Am. Phys. Surg. 8 (2003) 103–107.
[52] C.J. Newschaffer, M.D. Falb, J.G. Gurney, Pediatrics 115 (2005) e277–e282.
[53] C.G. Miller, Isr. Med. Assoc. J. 12 (2010) 711–712.
[54] R.R. Dietert, J.M. Dietert, J. Toxicol. Environ. Health B Crit. Rev. 11 (2008) 660–680.
[55] I. Hertz-Picciotto, H.Y. Park, M. Dostal, A. Kocan, T. Trnovec, R. Sram, Basic Clin. Pharmacol. Toxicol. 102 (2008) 146–154.
[56] M.K. Belmonte, G. Allen, A. Beckel-Mitchener, L.M. Boulanger, R.A. Carper, S.J. Webb, J. Neurosci. 24 (2004) 9228–9231.
[57] D. Ibi, T. Nagai, Y. Kitahara, H. Mizoguchi, H. Koike, A. Shiraki, K. Takuma, H. Kamei, Y. Noda, A. Nitta, T. Nabeshima, Y. Yoneda, K. Yamada, Neurosci. Res. 64 (2009) 297–305.
[58] G.W. Konat, B.E. Lally, A.A. Toth, A.K. Salm, Metab. Brain Dis. 26 (2011) 237–240.
[59] S.J. Spencer, J.G. Heida, Q.J. Pittman, Behav. Brain Res. 164 (2005) 231–238.
[60] M. Hornig, D. Chian, W.I. Lipkin, Mol. Psychiatry 9 (2004) 833–845.
[61] L. Shi, S.H. Fatemi, R.W. Sidwell, P.H. Patterson, J. Neurosci. 23 (2003) 97–302.
[62] T.C. Theoharides, D. Kempuraj, L. Redwood, Expert Opin. Pharmacother. 10 (2009) 2127–2143.
[63] M. Olczak, M. Duszczyk, P. Mierzejewski, T. Wierzba-Bobrowicz, M.D. Majewska, Folia Neuropathol. 48 (2011) 258–269.
[64] L. Tomljenovic, J.G. Dorea, A.C. Shaw, J. Dev. Disabilities 18 (2012) 29–37.
[65] G. Delong, J. Toxicol. Environ. Health A 74 (2011) 903–916.
[66] C.M. Gallagher, M.S. Goodman, J. Toxicol. Environ. Health A (2010) 1665–1677.
[67] N.W. Baylor, W. Egan, P. Richman, Vaccine 20 (Suppl. 3) (2002) S18–S23.
[68] P.A. Offit, R.K. Jew, Pediatrics 112 (2003) 1394–1397.
[69] R.J. Mitkus, D.B. King, M.A. Hess, R.A. Forshee, M.O. Walderhaug, Vaccine 29 (2011) 9538–9543.
[70] X. Li, H. Zheng, Z. Zhang, M. Li, Z. Huang, H.J. Schluesener, Y. Li, S. Xu, Nanomedicine 5 (2009) 473–479.
[71] D. Bonnefont-Rousselot, C. Chantalat-Auger, A. Teixeira, M.C. Jaudon, S. Pelletier, P. Cherin, Biomed. Pharmacother. 58 (2004) 516–519.
[72] R.K. Gherardi, A. Plonquet, C. André, L. Intrator, F. Poron, M. Coquet, Neurology 56 (Suppl. 3) (2001) A62.
[73] R.K. Gherardi, M. Coquet, P. Cherin, L. Belec, P. Moretto, P.A. Dreyfus, J.F. Pellissier, P. Chariot, F.J. Authier, Brain 124 (2001) 1821–1831.
[74] F.J. Authier, S. Sauvat, J. Champey, I. Drogou, M. Coquet, R.K. Gherardi, Arthritis Rheum. 48 (2003) 569–570.
[75] R.A. Yokel, C.L. Hicks, R.L. Florence, Food Chem. Toxicol. 46 (2008) 2261–2266.
[76] R.A. Yokel, P.J. McNamara, Pharmacol. Toxicol. 88 (2001) 159–167.
[77] P.M. Egan, M.T. Belfast, J.A. Gimenez, R.D. Sitrin, R.J. Mancinelli, Vaccine 27 (2009) 3175–3180.
[78] A.M. Ryan, N. Bermingham, H.J. Harrington, C. Keohane, Neuromuscul. Disord. 16 (2006) 867–869.
[79] A. Shivane, D.A. Hilton, R.M. Moate, P.R. Bond, A. Endean, Neuropathol. Appl. Neurobiol. 38 (2012) 734–736.
[80] C. Exley, L. Swarbrick, R.K. Gherardi, F.J. Authier, Med. Hypotheses 72 (2009) 135–139.
[81] R.E. Flarend, S.L. Hem, J.L. White, D. Elmore, M.A. Suckow, A.C. Rudy, E.A. Dandashli, Vaccine 15 (1997) 1314–1318.
[82] G.L. Morefield, A. Sokolovska, D. Jiang, H. HogenEsch, J.P. Robinson, S.L. Hem, Vaccine 23 (2005) 1588–1595.
[83] J.A. Hamilton, R. Byrne, G. Whitty, J. Leukoc. Biol. 67 (2000) 226–232.
[84] Z. Khan, C. Combadiere, F.J. Authier, V. Itier, F. Lux, C. Exley, M. Mahrouf-Yorgov, X. Decrouy, P. Moretto, O. Tillement, R.K. Gherardi, J. Cadusseau, BMC Med. 11 (2013) 99.
[85] A. Tourbah, O. Gout, R. Liblau, O. Lyon-Caen, C. Bougniot, M.T. Iba-Zizen, E.A. Cabanis, Neurology 53 (1999) 396–401.
[86] D. Konstantinou, C. Paschalis, T. Maraziotis, P. Dimopoulos, H. Bassaris, A. Skoutelis, Clin. Infect. Dis. 33 (2001) 1772–1773.
[87] H. Aydin, E. Ozgul, A.M. Agildere, Pediatr. Radiol. 40 (2010) 1281–1284.
[88] Y. Zafrir, N. Agmon-Levin, Z. Paz, T. Shilton, Y. Shoenfeld, Lupus 21 (2012) 146–152.
[89] C. Exley, Vaccine 29 (2011) 9289.