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Housam Eidi[1]*, Janice Yoo[1≠], Suresh C. Bairwa[1≠], Michael Kuo[1], Eric C. Sayre[2], Lucija Tomljenovic [1], and Christopher A. Shaw[1,3,4]

[1] Neural Dynamics Research Group, Department of Ophthalmology and Visual Sciences, University of British Columbia, Vancouver, British Columbia, Canada
[2]Arthritis Research Canada, Richmond, BC, Canada.
[3] Program in Experimental Medicine, University of British Columbia, Vancouver, British Columbia, Canada
[4] Program in Neuroscience, University of British Columbia, Vancouver, British Columbia, Canada

[≠] These authors contributed equally to the present work

*Corresponding author: housam.eidi@gmail.com

Abstract

The present study was designed to evaluate the possible effects of the paediatric vaccination schedule in the United States on the central nervous system in a murine model. We compared the impact of treatment with the whole vaccines versus true placebo control. Seventy-six pups were divided into three groups: two vaccinated groups and unvaccinated control. The two vaccinated groups were treated between 7 and 21 post-natal days either with one or three times of the vaccine doses per body weight as used in children between newborn and eighteen months of age. The post-vaccination development, neuromotor behaviours and neurobehavioural abnormalities (NBAs) were evaluated in all mouse groups during the 67 post-natal weeks of mouse age. Mouse body weight was affected only in the vaccinated females compared to males and control. Some NBAs such as decreased sociability, increased anxiety-like behaviours, and alteration of visual-spatial learning and memory were observed in vaccinated male and female mice compared to controls. The present study also shows a slower acquisition of some neonatal reflexes in vaccinated female mice compared to vaccinated males and controls. The observed neurodevelopmental alterations did not show a linear relationship with vaccine dose, suggesting that the single dose gave a saturated response. The outcomes seemed to be sex-dependent and transient with age.

Keywords: *vaccine, neurodevelopment, behavioural tests, neurobehavioural abnormalities.*

1. Introduction

Most countries in the developed world tend to recommend the same general types of vaccines for paediatric use. However, absolute vaccination schedules vary from country to country. In the United States and Canada, for example, the influenza vaccine is recommended for everyone over 6 months of age, while in France this vaccine is targeted to those over 65 years of age and to special groups such as people with serious medical conditions. Some vaccines are not included in the UK routine program, but are in other countries. For example, the chickenpox (varicella) vaccine is recommended routinely in Australia and the United States, but not in the UK [1].

The number of vaccines given under the U. S. vaccine schedule has grown substantially since 1990 making it one of the highest mandated vaccine schedules in the world for children under 5 years old (36 vaccinations, double the Western world average of 18 in 2009) [2].

Some reports suggest that excessive immune stimulation can lead to autoimmune conditions and/or changes in the central nervous system (CNS) structure or function during early development [3-5]. One way this may occur is due to the use of various aluminum (Al) adjuvants which have been linked to diseases of the autoimmune-inflammatory syndrome induced by adjuvants (ASIA) [6]. A chronic condition, termed macrophagic myofasciitis (MMF), [7] occurs primarily in adult women following vaccination with aluminium oxyhydroxide adjuvant containing vaccines. Autoimmune disorders linked to some vaccines and adjuvant treatments have also been reported in animal studies [8-17], supporting some outcomes seen in human studies.

Many recent studies have underscored the tight connection between the development of the immune system and the CNS and thus the plausibility that disruption of critical events in immune development may play a role in neurobehavioural disorders [18,19].

In regard to particular vaccines, Li *et al* have shown that neonatal vaccination in rats with the hepatitis B vaccine alters hippocampal synaptic plasticity [20]. These data have been confirmed in mice by the same group showing that early hepatitis B vaccination induces neurobehavioural abnormalities and interferes with brain development, particularly hippocampal neurogenesis [21,22].

Ongoing reports from human studies suggest an increase in the prevalence of neurodevelopmental disorders [23,24] in developed countries, particularly the U.S., U.K., Canada and Australia which various authors attribute to vaccines, adjuvants, or both [25,26].

Various studies have shown that some past and present paediatric vaccine constituents such as mercury [27], Al [22,28-31] and formalin are neurotoxic and have been associated with adverse neurological and immune outcomes in animal models [9,32-35].

Masson *et al* have recently documented that the three available studies on Al adjuvant toxicokinetics [36-38] suffer from serious conceptual and methodological weaknesses [39]. In this context, Gherardi *et al* have recently emphasised the fact that animal studies are a key step toward a better understanding potential vaccine and Al adjuvant adverse effects [40].

Experimental evidence also shows that when individually administered in vaccine-relevant amounts for human exposure, Al is capable of causing serious adverse and persistent neuroimmune outcomes in animal models [8,9,41-45]. Moreover, substantial evidence suggests that Al vaccine adjuvants are strongly linked to CNS disorders and a variety of autoimmune/inflammatory conditions in human adults [6,22,33,34,46-51]. Since children receive much more Al (and others vaccine constituents) from the vaccination schedule per kg of body weight than adults, they may be at greater risk of auto immunity and vaccine-related adverse effects based solely on dose [22,33,52].

It is also important to recognize that the route of administration of most vaccines, namely via intramuscular (*i.m*) or subcutaneous (*s.c*) injection, considerably lowers the threshold doses of toxic constituents capable of causing harm compared to oral administrations of the same compound in diet or water [53-55]. Furthermore, the vaccine Al administration is considered as an acute exposure and an infant's physiology will react differently to such exposure to a high concentration of Al over a short period as compared to the Al diet intake [56].

In part as a consequence of the overall benefits and success of vaccination, any associated adverse effects may tend to be overlooked or trivialized by public health authorities. Additionally, it should be mentioned that potential oversights in vaccine safety trials may arise since the FDA considers vaccines to be biologics, not drugs. This categorization has typically led to a relatively short surveillance period (days to weeks) for possible adverse outcomes. Another issue that needs to be addressed is that in most clinical vaccine trials "controls" are not typically true placebo controls, but rather another vaccine or the Al adjuvant used for the vaccine in question [57].

With the above points considered, presumptions about vaccine safety should be supported by appropriate scientific evidence as urged in 2004 by the WHO Global Advisory Committee [58]. In this context, concerns about the overall safety of current childhood vaccination programs are worthy of further investigation.

Our current study aims to investigate, in a murine model using newly born pups, the impact of the combined U.S. paediatric vaccination schedule on reflex development, neuro- and motor behaviours (including social interactions), anxiety, memory, and cognitive functions.

2. Materials and Methods

2.1. Animals, breeding and experimental groups

All experimental procedures on mice were approved by the University of British Columbia's Animal Care Committee (protocol #A16-0125 and #A16-0052 for breeding and experimental procedures, respectively) and were in compliance with the Canadian Council on Animal Care regulations and guidelines.

Sixteen female and eight male C57BL/6 breeders purchased from Jackson Laboratory were used with one male mating with two females. Female and male mice were six and five weeks old, respectively. The weight averages of the received breeder males and females were 20 and 18 g, respectively. The females and males were housed separately for one week of acclimatization in a room with an ambient temperature of 22 °C and with a 14/10 h light/dark cycle. Purina mouse chow and water were available to mice *ad libitum*. After impregnation, the females were separated from males and monitored closely for the parturition date which was considered in the following to be postnatal day (PND) 0. Pregnant female mice were housed individually after the second week of gestation. A total of 76 pups (twelve litters) were obtained from our mouse breeding protocol. Pups were divided into two experimental groups and one control group of 4 litters each (see Table S1 for vaccine types and details): *i*) vaccine group (V1): 25 mice (11 males and 14 females), *ii*) vaccine×3 group (V3): 25 mice (13 males and 12 females), and *iii*) control group: 26 mice (19 males and 7 females). *i*) V1 group: mice vaccinated with mouse weight equivalent of the current U.S. Centers for Disease Control and Prevention (CDC) paediatric vaccination schedule for newborn to 18 months old infants *ii*) V3 group: mice vaccinated with, the mouse weight equivalent of a triple dose of the U.S. paediatric schedule, i.e., at PND7, 8, 9, 10, 14, 18 & 21, the mice pups received triple of each vaccine dose (combined in one shot), and *iii*) saline control group: mice injected with the same total volume of phosphate buffered saline (PBS).

In terms of comparison to humans, in mouse or rat pups a postnatal period of 4 to 21 days corresponds roughly to the period of human neurodevelopment from late gestation to the first one to two years after birth [59]. Based on several papers assessing the impact of administration of various well known neuro- and immune-toxicants in early postnatal periods of heightened developmental vulnerability in comparative rodent models [60,61], we have calculated what we consider to be reasonable mouse-age equivalence (see the Table S1) given that mice live only about 2 years [40].

At PND 22, pups were weaned and males were separated from females. Mouse weights were measured weekly throughout the study.

All behavioural tests and weighing started at PND7 to minimize any intervention that can adversely affect maternal behaviour.

2.2. Vaccine administration

Animals were injected according to the U.S. CDC 2017 recommended vaccination schedule for preschool children shown in Table S1. A total of 21 injections of eight paediatric vaccines were administered by *i.m* or *s.c* injections according to the vaccine manufacturer's instructions. The Rotateq vaccine was administered orally. The pups were

weighed in the morning of the scheduled injection days and, based on the average of measured weights, the corresponding vaccine doses per body weight were calculated regardless of the human/mouse conversion (as the latter takes into consideration the differences between mouse and the human metabolism). The total *i.m* and *s.c* injected volume of vaccine dilutions in PBS was adjusted to 10µl total volume per administration. Vaccines were *s.c* injected in mice into the "scruff" to achieve an *s.c*-like injection in mice, or via *i.m* injections into the left or right tibialis anterior muscles and/or the right and left caudal thigh muscle. Thus, we sought to mimic as closely as possible human infant vaccination as regards the mode of vaccine administration.

The same volumes of PBS were injected in mice of saline control group in the same manner as any of the scheduled vaccines. The oral administrations were conducted gently putting 10 µl of the vaccine suspension inside the pup mouth using a micropipette and sterile plastic tips ensuring that all the droplets were consumed.

Mouse pups were observed for general health and behaviour immediately after injections to ensure that there were no overt signs of adverse effects, such as tremors, seizures, or respiratory problems. Following treatments, pups were returned to their home cages.

2.3. Mouse weights

Mouse body weights were monitored weekly during the post-weaning period from four to 67 weeks of mouse age.

2.4. Behavioural tests

In each test, the experimenter conducting the tests was blinded to the identity of the animals from all treatment groups. All behavioural tests were done between 9 am and 5 pm. No specific order was followed in animals testing since the experiments were randomized and blinded.

Table 1 summarizes all the behavioural tests conducted in this study.

Table 1. Summary of pre- and post-weaning behavioural tests. The same number of mice was used for all tests, namely a total of 76 pups divided into two experimental groups and one control group of 4 litters each.

Behaviour test	**Age of mice**	**Elements to be checked**	**Main parameters analyzed**
Pre-weaning tests			
Reflex righting (RR)	7, 8, 9, 10, 11, 14, and 15 days	Reflex development and neuromotor	The time to right onto all 4 paws from the supine position

Cliff avoidance (CA)	7, 8, 9, 10, 11, 14, 15, 16, 17, 18, 21, and 22 days	abilities	The time to turn 180° away from the cliff face
Negative geotaxis (NG)	7, 8, 9, 10, 11, 14, 15, 16, 17, and 18 days		Time to turn 180° to face upward from the downward position
Post-weaning tests			
Open field (OF)	OF 1: Week 6-7 OF 2: Week 28-31 OF 3: Week 48-52	General locomotion	Distance moved; velocity; duration of moving
Social interaction test (SIT)	SIT 1: Week 8-17 SIT 2: Week 43-47	Social recognition skills	Frequency of entering each chamber; duration in each chamber
Light dark box (LDB)	LDB 1: Week 20-22 LDB 2: Week 36-39	Anxiety-like behaviour	Duration in dark box
Novel object recognition (NOR)	NOR 1: Week 28-31 NOR 2: Week 48-52	Learning and recognition memory	Discrimination ratio
Barnes maze (BM)	Week 56-67	Visual-spatial learning and memory	Primary latency; primary error; total latency; total error; search strategy

2.4.1. Pre-weaning tests for developmental milestones

The pre-weaning tests were carried out as described by Cole *et al* [60]. Briefly, pups were tested for reflex development and neuromotor abilities using tests of reflex righting (RR), cliff avoidance (CA) and negative geotaxis (NG). Reflex tests were commenced in the morning and concluded at least one hour before injections. All pups were tested in a room separated from their mothers.

All the pre-weaning tests were started at PND7 and pups were tested daily. In the RR test, the pups were placed in a supine position and the time it took them to right themselves onto all four paws was measured. This test was continued with one trial per day until the mice met the criterion of a 3-second latency or less for two consecutive days.

The CA test was measured using a flat plexiglass surface raised to a height of 23 cm above a lab laboratory bench. Each pup was placed with their front paws and snout over the edge and the time to turn 180° away from the cliff face was recorded. The CA test was measured daily until the mice achieved a 6-second latency or less for two consecutive days.

For the NG test, the pups were placed head-downward on a 30° mesh incline and the time it took them to turn 180° to face upward was measured. Mice were tested daily until they achieved a 6-second latency or less for two consecutive days.

For all reflex tests, if the mice did not complete the task within 30 seconds, the test was terminated and the mice were scored as "criterion not achieved" and returned to their home cages. The orders of reflex testing for each day consisted of CA followed by NG and then RR with a rest period of at least 15 minutes between tests.

2.4.2. Post-weaning tests

By using a battery of post-weaning behaviour tests, we tested for behavioural abnormalities including impaired social interaction, anxiety-like behaviours, and memory deficits. At least a break of two weeks was given to each animal before each new test.

The open field test was conducted at one month of age (OF1) and repeated two times in order to analyze mouse locomotor activity as a control of other behavioural tests. During the OF test, distance moved, velocity, and duration of movement were evaluated.

2.4.2.1 Social interaction test

The Social interaction test (SIT) was designed to assess components of social affiliation, social recognition [62]. The SIT apparatus is illustrated in Figure 3. The SIT was conducted twice (SIT1 and SIT2, see Table 1). In both SIT1 and SIT2, the number of mice to be tested was greater than the number that could reasonably be tested in any one day. For this reason, mice from different treatment groups were assigned randomly to different days of testing.

The SIT took around two months to complete (2-4 months of age) given the large number tested animals and the time involved (~40 min/animal/day for 3 days). This delay period is also due to personnel and other logistic considerations.

Habituation: A test mouse was placed in the middle chamber and allowed to explore for 10 minutes with the doorways open into the side chambers open. Each of the two side chambers contained an empty wire cage. The time spent in each side chamber was measured to confirm the absence of a side preference bias for either of the side chambers.

Sociability: After the habituation session, the test mouse was transferred to a temporary cage and an unfamiliar mouse (stranger 1) was enclosed in one of the wire cages and placed in one of the side chambers. Stranger animals were sex and age-matched C57BL/6J mice with no previous contact with the subjects after birth. The animals serving as strangers were habituated to the wire cages in the test box for 10 minutes per day for 5 days before the start of SIT. During the test, the location for the stranger alternated between the left and right sides of the test box with different animals tested. Following placement of stranger, the test animal was put back into the middle chamber and allowed to explore the entire box for 10 minutes. A Noldus Ethovision system (Noldus Information Technology, Seattle, WA) was used to automatically score the frequency of entering the center, left and right chambers and time spent in each chamber.

2.4.2.2 Light dark box test

The Light dark box (LDB) test provides a means of examining anxiety-like behaviours. The LDB paradigm is based on the innate aversion of mice to brightly illuminated areas and on their spontaneous exploratory behaviour applying mild stressors, namely a novel environment and light [63]. This test was performed in a standard two-compartment chamber as described previously [64]. The LDB test was conducted twice with an interval of four months, at 4-5 months and repeated at 8-9 months of mouse age (LDB1 and LDB2, respectively) with the same randomization of tested mice as cited above. The dark box insert was made of black Perspex designed to cover one third of the area of the activity chamber (45 cm X 30 cm X 21 cm) with a 7 cm × 7 cm hole placed in the middle of the wall at floor level. At the beginning of the test, the test subject was placed in the light box facing the wall farthest apart from the dark box. Mice were allowed to move freely between two chambers for 10 minutes. Time spent in the dark box was automatically scored by the EthoVision system.

2.4.2.3 Novel object recognition test

The novel object recognition (NOR) test is based on the spontaneous tendency of rodents to spend more time exploring a novel object than a familiar one [65] (see Figure S.3a). The NOR test was used to evaluate cognition, particularly recognition memory, in rodent models of CNS disorders [66].

Mice were tested at 5-6 months of age with randomization as above. Three test sessions (habituation, familiarization, and recognition) were conducted on three consecutive days. Twenty-four hours before the familiarization phase, the mice were habituated to the open field for 5 minutes (day 1). In the habituation phase, the open field tests 2 and 3 (OF2 and OF3) were conducted to analyze the locomotor activity of mice as a control of NOR and other tests. During the familiarization session (day 2), two identical objects, either a tower of Lego® bricks or a Falcon tissue culture flask, were placed approximately 5 cm from the wall and 20 cm away from each other (symmetrically) and then the individual animal was allowed to explore them for 5 minutes [65]. The pair of objects was randomized between each mouse tested. Exploration of an object was defined as directing the nose to the object at a distance of less than 2 cm and/or touching it with the nose and rearing at the object. Turning around or sitting near the object was not considered as an exploratory behaviour. The minimal exploration time for both objects during the familiarization phase (~20 s) was used to ensure a similar exploration time of the two identical objects and between animals. During the recognition phase (day 3), one of the familiar objects used in the previous phase was replaced by a novel object. After this, the animals were placed back into the box and allowed to explore the objects for 5 minutes. During the familiarization and recognition sessions, video recordings were made by a camera placed above the arena and video analysis was done manually by an experimenter who was blinded to the treatment groups. We measured the absolute time

spent exploring each object during each session. A discrimination ratio (DR), an index of the amount of time spent exploring the unfamiliar object over the total time spent exploring both objects, was used to measure recognition memory (DR = time spent with the novel object / total exploring time).

2.4.2.4 Barnes maze

The Barnes maze (BM) test was designed for testing visual-spatial learning and memory in rats and mice [67]. This dry-land maze was made of two circular platform elevated at the height of 87 cm (Figure S.1). The maze was placed near the corner in the test room and extra-maze visual cues were placed around the maze so mice could use them as references and learn the position of the escape hole. Two 100-watts fluorescent bulbs facing towards the maze were used as aversive stimuli.

The Barnes test consisted of four phases: adaptation, acquisition, 1st probe trial and 2nd probe trial. Adaption was a pre-acquisition phase in which a mouse was allowed to explore the maze for 3 min, after which the mouse was guided towards the escape hole and allowed to enter and sit in the box for 2 min. Thus, the mice became familiar with the maze and they know that there is an escape hole in the apparatus environment. In the acquisition phase, mice were trained four times a day with 15 min interval for four days. Mice were kept in their home cage until the end of their test and were allowed to habituate in the testing room for 30 min before test onset. Before the start of each test, the tested mouse was placed in the center of the maze in a black round start box for 10 sec. When the training started, the box was lifted and mouse was allowed to explore the platform for 3 min. Mice were allowed to stay in the box for 1 min before being placed in their home cages. The test ended when mice entered the escape box or after 3 min had elapsed. The top platform was rotated and wiped with 70% ethanol after each test in order to minimize intra-maze cues and residual olfactory cues. The escape box was thoroughly cleaned with 70% ethanol for removing any residual mouse scent.

Five parameters were measured in the Barnes test (Figure S.2): primary latency, primary error, total latency, total error, and the search strategy used. Primary latency was the time taken when a mouse first encountered the escape hole. The primary error was the number of incorrect holes the mouse sniffed or turned its head towards before finding the escape hole. Sometimes mice did not enter the escape hole after finding it and continued to explore the maze. The total latency was the time taken to fully enter in the hole. The total error was the number of holes searched before entering the escape hole. The search strategies were the patterns that mice utilized during the search of the escape hole. We distinguished among three types of search strategy: *i*) Serial, at least two holes were searched before finding the target hole and searching was done in a serial fashion hole by hole, clockwise or counter clockwise. *ii*) Direct (spatial), directly heading to the escape hole or visiting one adjacent hole was done after visiting to one adjacent hole. *iii*)

Mixed, unorganized search of the escape hole or searching holes by crossing through the center of the maze.

The 1st probe trial was done on day 5 after the last acquisition training to test short-term retention memory. The 2nd probe trial was done on day 12 to test long-term retention memory. Each test was recorded by a camera placed on top of the maze and recordings were made with Noldus Ethovision tracking software.

The mice were observed for any aggression-like behaviours, such as the barbering, through the study.

2.5. Statistical analyses

The numbers of mice used were based on power analysis with additional mice added in order to account for unexpected morbidity and mortality due to both study and non-study conditions. In the present study, 24-26 animals were used per treatment group. In developmental studies assessing the impact of developmental xenobiotics in rodent models, typically 2 to 11 litters were used per experimental group [60,68-70]. Therefore, we opted for four litters per group to satisfy both a sufficient number of individual animals and litters in consideration. Furthermore, the power analysis (nQuery Advisor 5.0 power analysis software, Los Angeles, CA) showed that when the sample size in each of the experimental groups is 10, a one-way analysis of variance will have 95% power to detect significance at the 0.05 level. In the present study, the total number in all experiment groups is more than 20 female and male mice (see the Section 3.1).

In all models, generalized estimating equations (GEE) were used to account for potential correlations among animals of the same litter. To assess model fit (appropriateness of GEE specification), we compared working correlation specifications exchangeable versus independence, and independence had the lowest quasi information criterion (QIC), absolute lowest in the majority of models, <1 above in a minority of models and hence considered not substantially different. Therefore GEE models were fit with the independence correlation structure. This also served as confirmation that treating individual animals as the experimental unit (and not litters) in the final models was appropriate (i.e., no litter effect).

Pre-weaning tests: We modeled latency vs. treatment (reference=control). Kaplan Meier curves were used to characterize time to complete tests, stratified by treatment. Log-rank tests were performed for pair-wise comparisons between the curves for treatment pairs vs. control group. A *p-value* of < 0.05 was considered significant.

Mouse weights: We fit models predicting body weight at week 67 postnatal age. Additionally, the curve models of mouse body weights were fit to help assess possible differences in growth rates, even with the assumption that mice may end up the same weight.

In pre-weaning tests and mouse weights measurements, all of the GEE models were repeated in the whole sample, as well as males and females separately. A litter effect was

deemed unimportant in the GEE models as assessed by QIC statistics. Therefore, GEE models were fit via the independence structure. A *p-value* of <0.05 was considered significant.

Post-weaning tests: In the three model sets described below (SIT, LDB, and NOR), we compared GEE working correlation specifications exchangeable versus independence, and independence had the lowest QIC. Therefore, GEE models were fit with the independence correlation structure. A *p-value* of <0.05 was considered significant.

- SIT: The SIT data analyses were based on the fact that, in animal models, sociability is a binary yes/no phenotype ("social"/"non social") that can be evaluated by comparing the duration in the two chambers of the SIT apparatus within each group. Furthermore, SIT is not a graded parameter for quantitatively comparing chamber times across groups [71]. Thus, no comparison among treatment groups was made during analysis of the social interaction test data as suggested by the original developer of this test [71]. We used logistic regression to compare sex and/or treatment groups on the odds of asocial behaviours (e.g., longer duration spent in empty cage vs. with a stranger).

- LDB: Poisson regression models were used to compare sex and/or treatment groups on the number of full body transitions, and linear regression models were used to compare groups on their time spent in the dark box and the latency to enter the dark box. In the Poisson models, exponentiated regression coefficients were count ratios, or factors, by which the expected counts differed from the reference expected counts. We reported p-values and confidence intervals around the count ratios. Residuals from the linear models were plotted in normal quantile-quantile plots (QQ-plots) and demonstrated adequate normality.

- NOR: We modeled discrimination ratio (DR) with linear regression. In the linear models, regression coefficients estimated the additive differences in expected duration in each level compared to the reference categories, along with p-values and confidence intervals around the differences. Residuals from the linear models were plotted in normal QQ-plots, and demonstrated adequate normality.

- BM: we performed day to day comparisons, by treatment; treatment comparisons, by day; interaction models for treatment by day. We repeated these on all data, females, and males, and all the above on all four outcomes (primary errors, primary latency, total errors, total latency). We repeated all the above again but using days 5 and 12 data only, with reference day 5 (primary errors and primary latency only). Finally, we performed chi-square tests and cross-tabs between strategy and treatment, stratified by day, and chi-square tests and cross-tabs between strategy and day, stratified by treatment.

Barbering: We performed Fisher's Exact Test to test whether barbering was related to either sex or each treatment group (*vs.* control). In the latter, we analyzed both overall, and stratified the results by sex.

All statistical analyses were performed using SAS v9.4 (SAS Institute, Cary, North Carolina).

3. Results

3.1. Overall mouse development

No abnormal maternal behaviours were observed. No leg limp or motor disabilities caused by the *i.m* injections were observed in any animals.

3.2 Mouse weight

Both experimental groups started off at the similar body weight but increased at a significantly lower rate compared to controls (Figure 1a). When data were stratified by sex, significant differences in body weight were observed in female mice from the V1 and males from the V1 and V3 group vs controls (Figure 1b and 1c). No significant differences in body weight were observed between the two vaccinated groups (V1 vs V3).

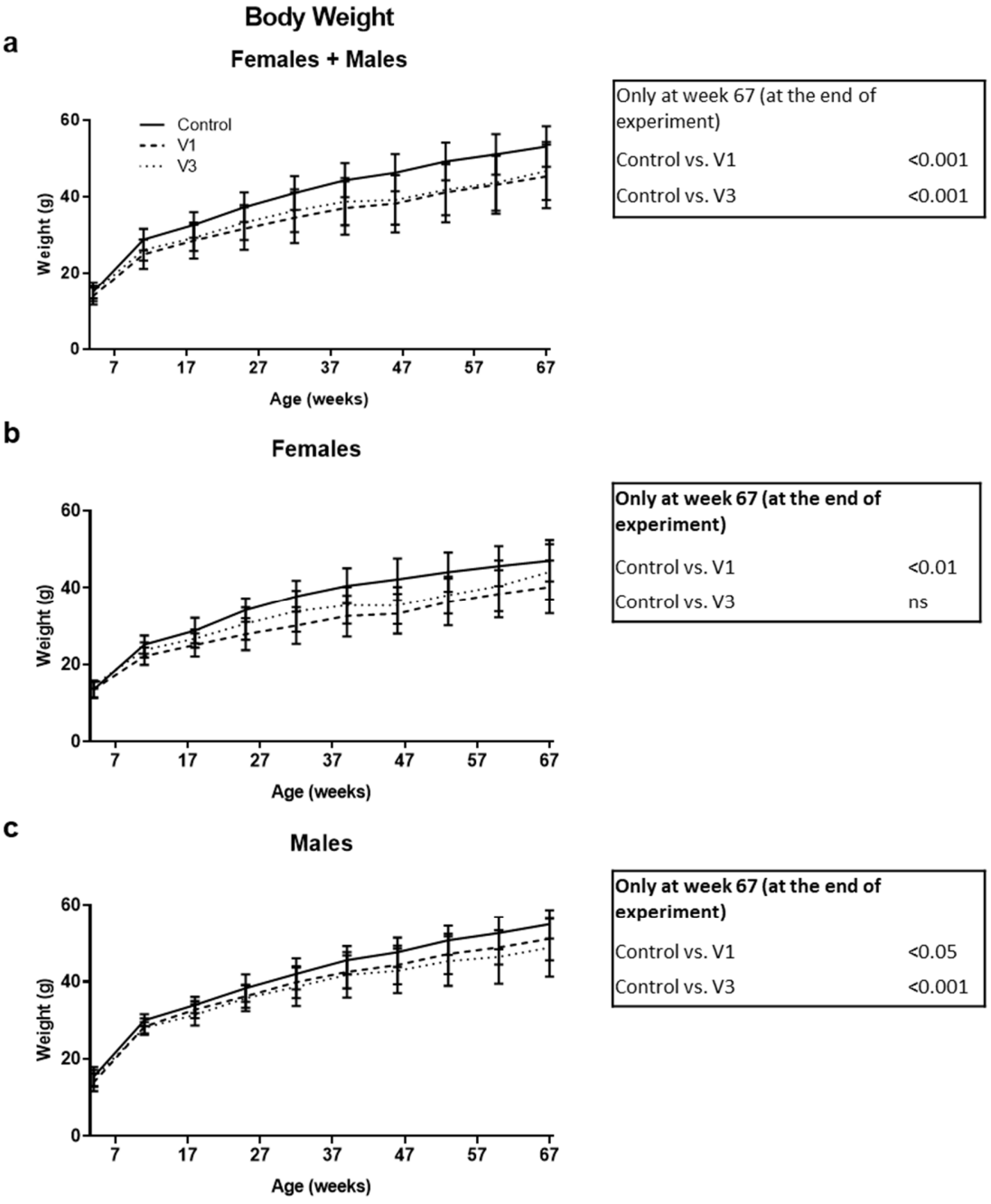


Figure 1. The effect of vaccine administration on body weight in mice. Data are expressed as mean ± SD weight of mice between 4 and 67 weeks of age for females and males. ns: no significant difference.

3.3. Behavioural tests

Table 2 summarizes the behaviour outcomes for all of the experiments.

3.3.1. Pre-weaning tests

There were no significant differences among the groups in the development of neonatal reflexes and neuromotor abilities except for the CA reflex (Figure 2). Our data obtained from the cliff avoidance reflex demonstrated that female mice from both treated groups achieved the criteria of this test later than the control group (Figure 2e). However, these differences between treated and control females were significant in V1 (p<0.01) group whereas the differences were insignificant in the V3 group (p=0.054) (Figure 2e).

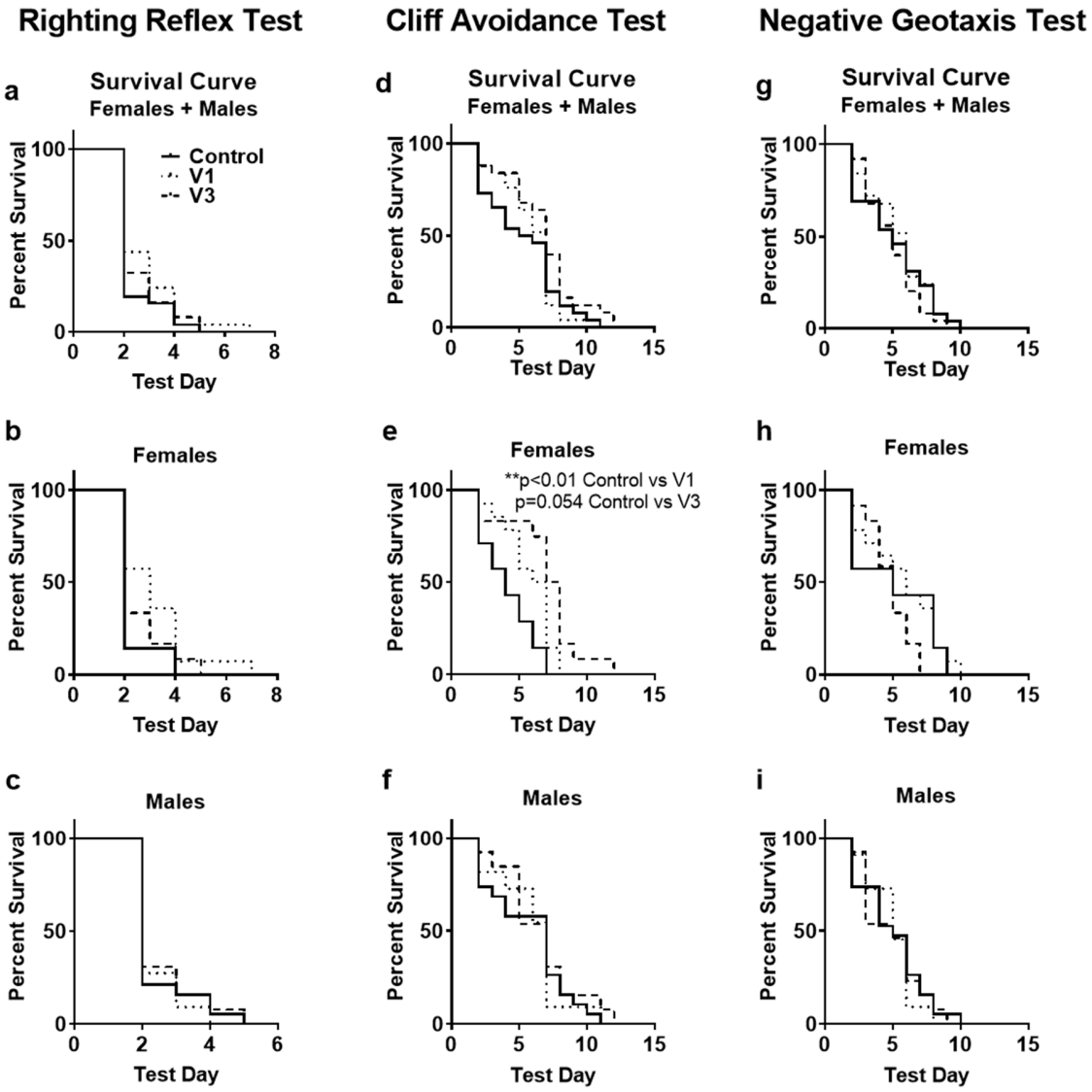


Figure 2: Evaluation of neonatal reflexes in pups during the first three weeks of postnatal age. Three pre-weaning tests were conducted: reflex righting (RR), cliff

avoidance (CA) and negative geotaxis (NG). **a**, **d** and **g**: Data from both sexes are analysed together and stratified by sex (females: **b**, **e** and **h**; males: **c**, **f** and **i**). In the RR and NG test, no significant difference was observed between mouse groups. **e**: In the CA test, female pups from all treated groups achieved the test criteria later than controls.

3.3.2. Post-weaning tests

Our data of the open field test (OF) showed that there was no difference in locomotor activity between the animals from all groups (data not shown).

3.3.2.1. Social interaction test

The total exploration time and the percentage of the time spent with the stranger mouse are shown in the supplementary data section (Table S.2 and Table S.3).

The administration of a single vaccine in V1 vs the V3 group, in the combined male and female groups, did not affect the mouse sociability (Figure 3a). When data were stratified by sex, the only group that did not achieve significant differences was the male V1 group in the time spent with the stranger 1 vs the empty cage (Figure 3b and c).

The data of SIT2 demonstrated that no significant effect on the mouse sociability was observed in any of the groups at 9-10 months of age (Figure 3d-f).

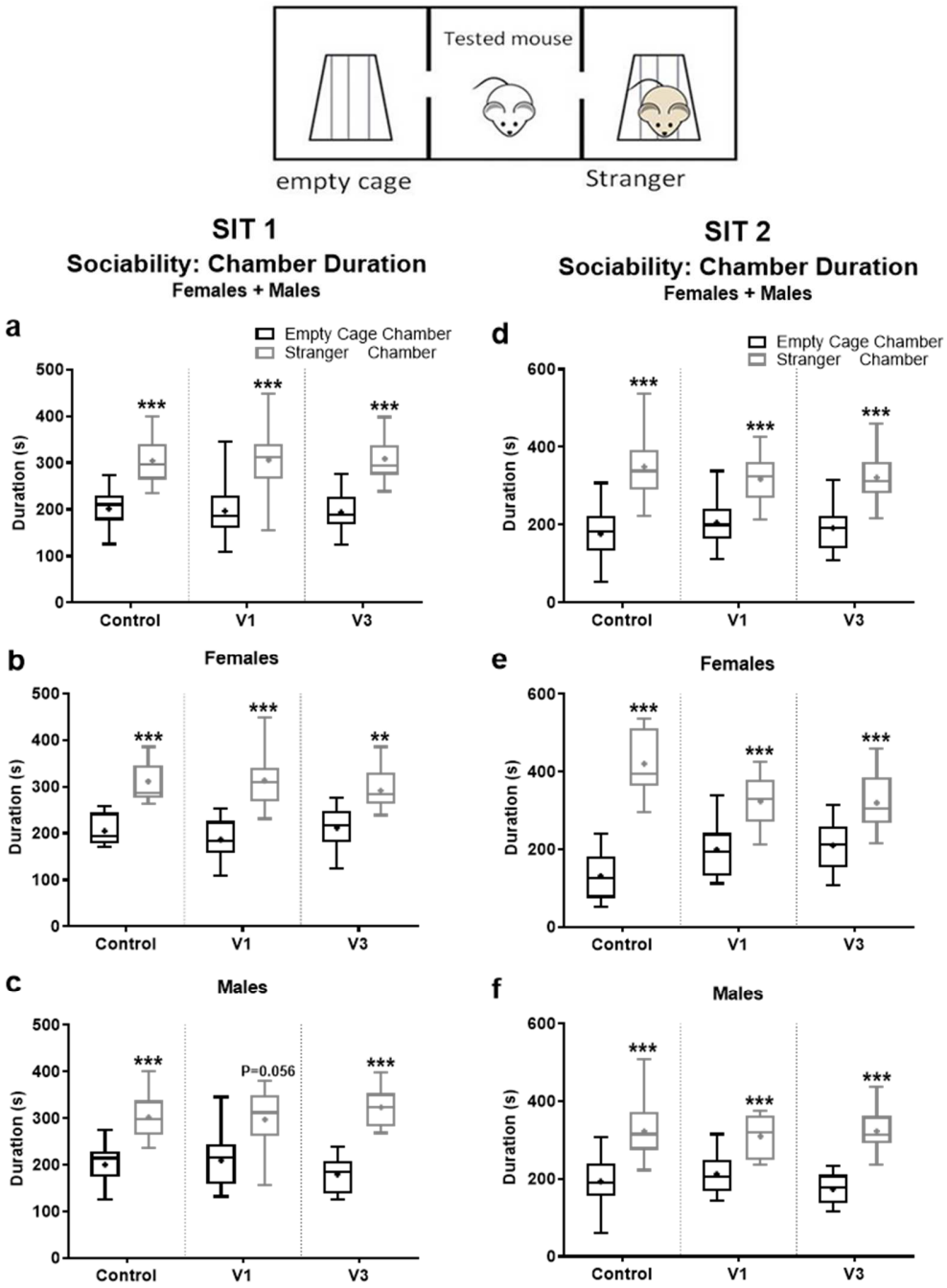


Figure 3. Social skills assessment was conducted twice in mice by the Social Interaction Test (SIT): SIT1 (2 - 4 months of age) and SIT2 (9 - 10 months of age). Sociability differences were expressed by the chamber duration within mouse groups and no comparison was done between treated groups and control. (**a, b and d - f**) No sociability anomaly was observed in any mouse groups and no differences were seen between males and females as all mice spent significantly more time with the stranger *vs*. the

472 empty cage. (**c**) Males from the V1 group showed no significant difference between the
473 time spent with the stranger 1 vs the empty cage. **p<0.01 and ***p<0.001.
474
475 3.3.2.2. Light dark box test
476 At 4-5 months of age, (LDB1), the results showed a significant anxiety increase and
477 a decrease in exploratory behaviour in both treatment groups as they spent more time
478 in the dark chamber (Figure 4a). When the data were stratified by sex, the same results
479 were obtained for the female mice in both treatment groups (Figure 4b). There was
480 moreover a sex-dependent effect in the V1 group as only the vaccinated females
481 showed increased anxiety when compared to the unvaccinated controls, while there
482 was no difference between the in the unvaccinated and vaccinated males (V1). In the V3
483 group however, males were also affected and showed significantly increased anxiety
484 (p<0.05), although to a lesser extent than females (p<0.01), compared to unvaccinated
485 animals of the same sex group since male mice need a higher dose of vaccines to
486 experience the effect as compared to females (Figure 4b and c). When this test was
487 repeated at 8-9 months of mice age (LDB2), no significant difference was observed
488 between two treated groups and the control (Figure 4d-f).
489 In both LDB tests, no significant differences were observed between V1 and V3
490 groups (Figure 4a-f). The numbers of entries in the dark and light box and the
491 percentage of time spent in each box of LDB1 and LDB2 are shown in Table S.4 and S.5.

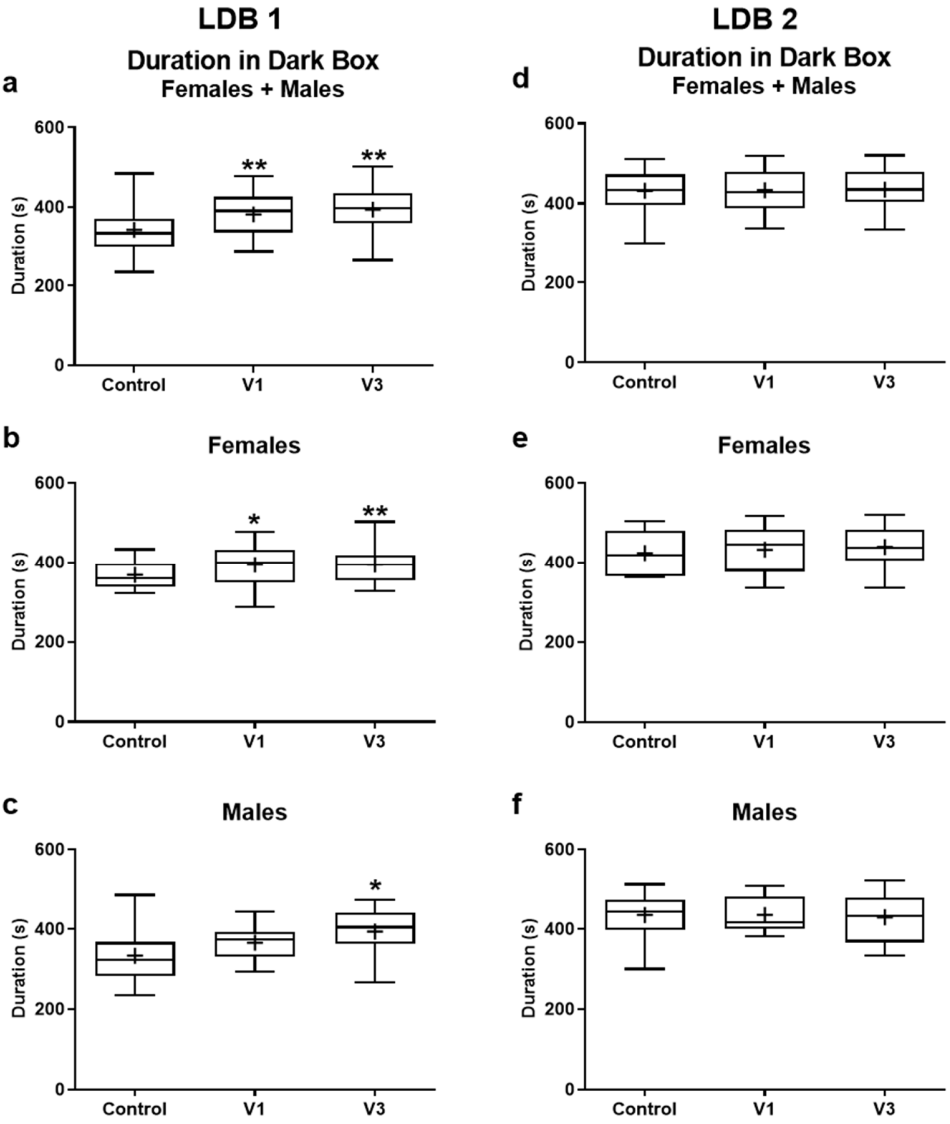


Figure 4. Anxiety and exploratory behaviour evaluation by the light dark box (LDB) test. The LDB test was conducted twice: at 4-5 and 8-9 months of age (LDB1 and LDB2, respectively). (**a**) LDB1: All treated groups showed a significant increase in the spent time in the dark chamber. (**b** and **c**) Females from all treated groups and only males from the V3 groups spent more time in the dark chamber compared to controls. (**d,e,f**) LDB2: No significant difference was observed between treated groups and control when the data were stratified by sex. *p<0.05 and **p<0.01 *vs.* control.

3.3.2.3. Novel object recognition test

NOR1 and NOR2 data showed that no negative effect on the recognition memory was observed in any group compared to controls (see the supplementary data section:

Figure S.3). The increased DR values, as compared to control, observed in NOR1 in V1 group females (Figure S.3c) and in NOR2 in V1 group males (Figure S.3g) are considered as "normal" recognition memory.

3.3.2.4. Barnes maze test

See the supplementary data section (Figure S.4, S.5, S.6 and S.7)

3.3.2.4.1. Acquisition phase

Primary latency and primary errors

During the 4 days of acquisition training, a significant reduction in primary latency in both sexes combined (Figure S.4a) on day 4 compared with day 1 was observed in the control and the both treatment groups ($p<0.001$). Similarly, when stratified by sex, both treatment groups in female and male mice showed a significant reduction in primary latency on day 4 compared with day 1 ($p\leq0.001$) (Figure S.4b and S.4c). When compared with the control on day 4, V1 group in both sexes combined (Figure S.4a) and in male mice (Figure S.4c) showed significant decrease in primary latency ($p<0.05$).

Primary errors in control and both the treatment groups showed a significant decrease ($p\leq0.001$) when both sexes combined (Figure S.4d) on day 4 compared with day 1 which was similarly observed in all the groups of male mice ($p \leq0.01$; Figure S.4f). During the 4-day acquisition trial in female mice, only control and V1 group showed a significant reduction in primary errors on day 4 compared with day 1 ($p<0.001$), while V3 females showed no significant reduction (Figure S.4e). No significant difference was observed in both treatment groups compared to control on day 4 (Figure S.4d, S.4e and S.4f).

Total latency and total errors

The total latency was significantly decreased ($p<0.001$) on day 4 compared with day 1 in control and in the two treatment groups in both sexes combined over the 4 days of the acquisition training period (Figure S.5a). Similarly, when separated by sex, all the groups of female (Figure S.5b) and male mice (Figure S.5c) showed a significant reduction in total latency ($p\leq0.001$). Total errors were likewise reduced significantly in control and all the treatment groups on day 4 compared with day 1 during 4 days of the acquisition training in both sexes combined ($p \leq0.01$) (Figure S.5d). When stratified by sex, control and V1 groups of female mice made significant improvements in total errors ($p<0.001$; (Figure S.5e) on day 4. During the 4 days of acquisition training, control and V3 groups in male mice showed a significant reduction in total errors on day 4 compared with day 1 ($p \leq0.01$) (Figure S.5f). Only female mice from V1 group made significantly more total errors on day 4 compared to control ($p<0.001$; Figure S.5e). No significant difference in total errors between the treatment groups and the control group was observed on day 4 when both sexes combined and in male mice.

Search strategies

When stratifying by treatments, the association between strategy and days (1 to 4) was significant in control and the both treatment groups when both sexes combined (p<0.001) (Figure S.6a) and separated (female: p<0.05; Figure S.6b, male: p<0.001: Figure S.6c). As the experiment progressed (from day 1 to day 4), mice used the direct strategy more often and the mixed strategy less often in all the groups when both sexes combined and separated. When stratifying by days, the association between strategy and treatments was not significant on any day (p>0.05). The same results were also seen in the female (Figure S.6b) and male mice (Figure S.6c).

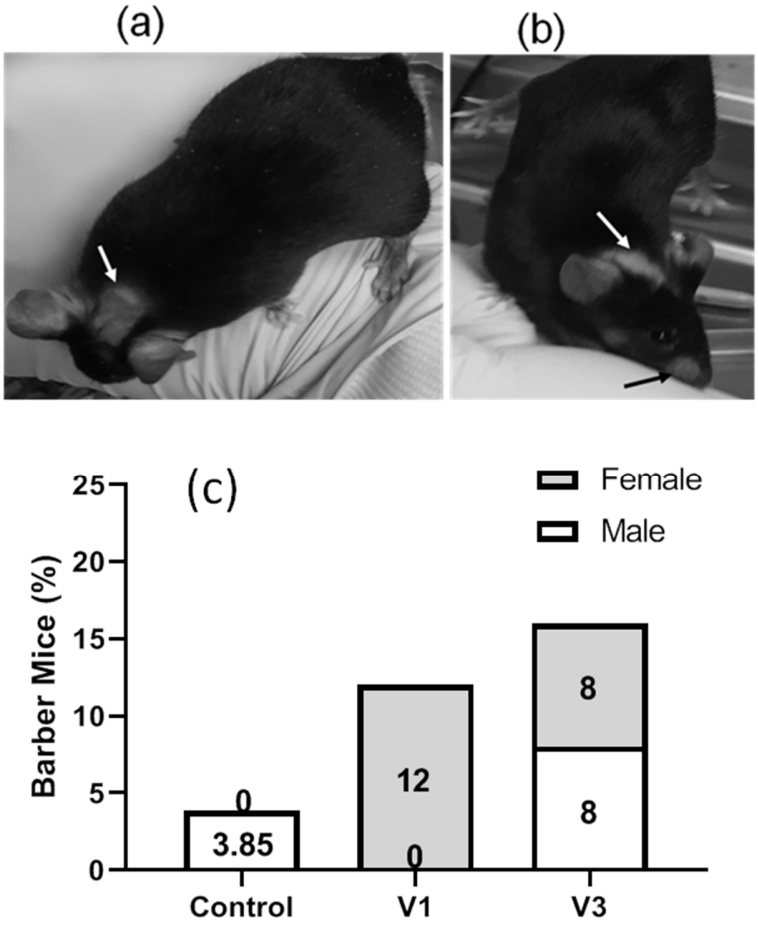
3.3.2.4.2. Short- and long-term memory retention

During the 1st (short-term) and 2nd (long-term) probe trial (day 5 and 12 after the last acquisition training, respectively), mice in both vaccine groups when both sexes combined (Figure S.7a) as well as females separately from males (Figure S.7b) did not show any significant improvement in primary latency compared to control on either day 5 or day 12, or compared between day 5 and day 12. However, on day 12, the V3 group showed significantly increased primary latency compared to their performance on day 5 (Figure S.7c).

Primary errors on day 5 were not significantly different in all the treatment groups compared to controls (Figure S.7d). On day 12, V1 and V3 group made significantly more primary errors compared with controls (p<0.05) (Figure S.7d). When comparing between the two probe trials, the V3 group had significantly increased primary errors on day 12 in comparison to day 5 (Figure S.7d). When stratified by sex, the data showed that there were no significant difference in primary errors in female (Figure A.7e) and male (Figure S.7f) mice in any of the treatment groups compared to control on day 5 or on day 12. Nor did any group show a difference in primary errors between days 5 and 12. These results could be due to a sample size issue which could have made the test under-powered to detect this particular difference according to sex.

3.4. Repetitive/aggressive-like behaviours

Barbering, a fur and whiskers tearing behaviour, was clearly observed by the end of the 3rd month of age of vaccinated mice compared to control (Figure 5a-b). Mice from V3 showed the highest barbering percentage (16%) followed by V1 group (12%) at 10 months of age (Figure 5c). However, the statistical analysis of these data showed that there was no significant difference between the two treated groups compared to control. Unlike the treated mice, all mice showing barbering behaviour in the control group were males, representing only 3.85% of this group. Females seemed to be more affected by barbering as the percentage of female barber mice from V1 groups was higher than males with no male barbers in the V1 group (Figure 5c).



584

585 **Figure 5**. Barbering was observed in all mouse groups between 3 and 10 months of age.
586 (**a)** and (**b)**: Two victim mice of barbering, a female from the V1 group and a male from
587 the V3 group with aggressive fur barbering induced by one (or more) of its cage mates
588 (a barber mouse) leading to a fur loss in the neck and head area (see the arrows) (**c**) The
589 percentages of barber mice at 10 months of age: 16% in the V3 group followed by V1
590 group (12%) and control group (3.85%).

Table 2.

Table 2a. Qualitative summary of the overall vaccine impacts on body weight, reflex development, neuromotor behaviours, and neurobehavioural abnormalities in mice.

Group	**Sex**	**Body weight**	**Cliff avoidance**	**Negative geotaxis**	**Reflex righting**	**SIT1**	**SIT2**	**LDB 1**	**LDB 2**	**NOR 1**	**NOR 2**	**Barber mice (%)**
			Reflex development and neuromotor abilities			Sociability		Anxiety-like behaviour		Learning and recognition memory		Aggressive behaviours
V1	F	**S**[1]	**S**	NS		Normal		**S**	NS			
	M	NS[2]	NS			**Abnormal**	Normal	NS				
	F+M	**S**	NS			Normal						
V3	F	NS	**S**					**S**				
	M	**S**	NS									
	F+M	**S**	NS									

LDB: light dark box test; *NOR*: novel object recognition; *F*: females; *M*: males.

1: a statistically significant effect compared to control ($p \leq 0.05$).

2: no statistically significant effect compared with control ($p > 0.05$).

Table 2b. Qualitative summary of the overall vaccine impacts on visual-and spatial learning and memory parameters in the Barnes maze (BM) trial. We qualitatively assessed a "global impact" of the various treatments by giving a point for every tested measure that was significantly altered [72]. Note here that it is normal that latency and errors should decrease significantly over the course of 4 days in the acquisition trial so that the designation "Normal" refers to cases where such decrease was observed. The designation "Increased" represents cases where an increase was observed in either latency or errors in the vaccinated compared to the control group. The designation "Decreased" on the other hand represents cases where significant decrease in either latency or errors was observed in vaccinated compared to control animals. If however the within-treatment group increases or decreases in latency or error values did not statistically differ from those observed in the control animals on day 12, they were not counted in the global score (for details see Discussion).

BM (visual-spatial learning and memory)									
Group	**Sex**	**Acquisition phase trial**[1]					**1st and 2nd probe trial** (day 5 and day 12 after the last acquisition training respectively)		**Global score (Tables 2a and 2b)**
		Primary latency	Primary errors	Total latency	Total errors	Search strategies	Primary latency	Primary errors	
V1	F	Normal	Normal		Increased**	Normal	Normal	Normal	**4**
	M	Decreased*			Normal				**1**
	F+M	Decreased*						Increased*[1]	**2**
V3	F	Normal						Normal	**2**
	M						Normal†		**2**
	F+M						Normal	Increased*[2]†	**3**

610 * a statistical significant effect compared with control ($p \leq 0.05$).

611 ** a statistical significant effect compared with control ($p \leq 0.001$).

612 †: a statistical significant effect within the same group between day 5 and 12.

613 [1] V1 males and females on day 12 compared to control male and females on day 12.

614 [2] V3 males and females on day 12 compared to control males and females on day 12.

4. Discussion

In the present study, we detected abnormalities in the vaccinated mice in some of the parameters tested: body weight, reflex development and neuromotor abilities (as measured by the CA test), anxiety, visual-spatial learning and memory and aggressiveness. Females from the V1 group and males from the V3 group were the most affected. While most of the abnormalities detected did not persist until the final evaluations at 67 weeks of age, some persisted into adulthood, including decreased body weight (in both vaccine treatment groups). Indeed, according to our raw data of SIT, NOR and LDB tests, 12 – 54% of affected mice at the 1st trial were still showing abnormalities during the 2nd trial of these tests (data not shown). However, most of these observed percentages were not statistically significant compared to control.

The differences between the data obtained in the two treated groups (V1 vs V3) could indicate that not all targets were saturated with a single vaccine dose.

Concerning the sample sizes of the control and treatment groups, our power analysis suggests that the numbers in each group were adequate to detect significant changes in weight, but it must be acknowledged that even so the capacity for relatively small sample sizes to unduly impact the outcomes cannot be totally dismissed.

With respect to how our study results relate to other similar investigations we observe the following:

i) in regard to body weight, our results showed that the mice from both experimental groups deviated from the expected body weight which was significantly lower than controls. The body weight has been suggested as one of the best indicator of the physical development of pups and highly correlated with the pre-weaning landmarks of development [73]. Our result of body weight is in line with those from our previous study [74] and others [75-77] which have shown a reduction in body weight post exposure to vaccine Al adjuvants that can be found in some of the vaccine formulations used in the present study. However, another study from our laboratory demonstrated a weight increase in male and female mouse pups injected with vaccine Al adjuvant alone between 2 and 17 PND [78]. These inconsistencies may be linked to several factors including mouse strain, the sample size in these studies, mouse age, the administrated Al adjuvant type, the dose and the route of administration and the Al adjuvant effect vs the whole vaccine one.

Although the mechanism behind the post-vaccination alterations of body weight is still unclear, various studies have suggested that body weight decrease can be due to nutritional changes caused by changes in animal water consumption after vaccination [79]. Other studies suggested that body weight reduction occurred as a result of changes in the expression of several genes in vaccinated animals [79,80].

ii) Females in both treatment groups showed a significantly late development of the cliff avoidance reflex compared to the control group. However, the two other

development reflexes tested in our study, namely the negative geotaxis and reflex righting, were not significantly affected by the vaccine treatments. These data indicate early effects on some development reflexes in the vaccinated pups compared to unvaccinated ones. These results are in line with Hewitson *et al* findings as they reported a significant delayed acquisition of neonatal reflexes in male newborn primates receiving a hepatitis B vaccine [45].

iii) Our data of SIT performed at 2-4 months of mouse age (SIT1) showed that only male mice from V1 group showed a significant abnormal sociability as no significant difference was observed between the time spent with the stranger 1 vs the empty cage (p=0.056). At 9-10 months of mouse age, SIT2 outcomes showed no abnormal sociability in the two treatment groups compared to controls.

iv) The LDB data obtained in the present study showed increased anxiety and decreased exploratory behaviours in mice from V1 and V3 groups at 4-5 months of age (LDB1), an outcome which is consistent with the published data from our laboratory using CD1 mice injected with Al hydroxide adjuvant [78] and those recently obtained by Asin *et al* using the same vaccine Al adjuvant in sheep [81]. Furthermore, when the current data were stratified by sex, a significant difference was observed between the vaccinated and unvaccinated females, but no such difference was observed between vaccinated and unvaccinated males in the V1 group. However, in the V3 group, both sexes were significantly affected although males less significantly than females given that the later need a lower dose of vaccines to experience the effect as compared to males (Figure 4b and c). These findings indicate thus a sex-dependent as well as dose-dependent effect of vaccination on the anxiety and exploratory behaviours in mice, with females being more adversely affected than males even at lower doses. At 8-9 months of age (LDB2), no significant alterations in anxiety and exploratory behaviours were seen in V1 and V3 groups compared to controls.

v) Our BM data on long-term memory retention assessment demonstrate that V1 and V3 mice made significantly more errors in locating the target hole on the probe test on day 12. However, no significant differences were seen in time taken to find the target hole. V1 and V3 female mice similarly exhibited abnormal values in the acquisition of test parameters, the former showing a significant increase in total errors while the latter in total latency (Table 2b). Collectively these results suggest a negative impact of vaccine treatment on short-term visual-spatial learning, as well as a sex-dependent vulnerability, with females being more susceptible than males. In all of these cases, the net effect of this apparent improvement was scores of primary latency and errors. The observation that the same trend occurred in control mice can be interpreted as all of the animals of whatever group may reflect neuroplasticity such that the scores of all groups converged.

The improvement in the early neurobehavioural impairments observed by Curtis *et al* in primates [82] and those obtained from our SIT and LDB outcomes in mice mirror

those reported in human children with neurodevelopmental disorders [83,84]. Moreover, our BM data at 12 months of age seem to be in line with our hypothesis of possible age-dependant adverse outcomes of the paediatric vaccine schedule. Such changes in all three species (human, non-human primates and murine) might be due to compensation/neuroplasticity. However, the possible mechanism of such compensation is still poorly understood and has no unanimous definition [85]. Nevertheless, such outcomes in human children as well as in the other species, may suggest an age-dependent impact of such vaccination schedules.

vi) In the early stages of these tests, we observed a particularly notable behavioural abnormality, e.g., aggressive interactions, among treated mice between 3 and 10 months of age. This aggressiveness was observed as a hair and/or whisker barbering either by an aggressive cage mate or by themselves. Hauschka reported that the normal rate of overall female barbers in C57BL/6J mice is around 10.7 % at 7 -10 months of age [86]. In the present study, at this age window, barbering mouse percentage in treated groups was clearly greater than in controls and the normal ratio reported in the literature (0% vs 12% in control vs V1 females, respectively). Moreover, our data analysis of barbering demonstrated a sex-dependent effect since there was an increased percentage of female barbers in all treated groups in comparison to the control group where all the barbers were males. However, no statistical significance difference in barbering was observed in V1 and V3 group compared to controls. This statistical insignificance could be explained by the sample size which could have made the test under-powered to detect this particular difference. Thus, further experiments with more appropriate sample size should be conducted in order to shed light on these unexpected findings.

vii) The administration of triple the dose of the administered vaccines in a very short time period did not uniformly affect mouse behaviours more than that of a single dose of each. This findings are in accordance with what has been recently reported for vaccine Al-adjuvants, namely that vaccine adverse effects may not obey "the dose makes the poison" rule of the classical pharmacology [39,87,88]. This could be explained by immune system saturation in the V1 group. A manuscript currently in preparation on the cytokine profiles in the mice used in the present study supports this hypothesis as it shows some similar increases in cytokines levels, in particular IL-5, in the plasma of both sexes of V1 and V3 mice.

As mentioned above, our data analysis showed that most of the neuro-behavioural abnormalities observed in the present study were not identical across time. Our speculation is that there is a complex interplay of dose, age, and sex (Figure 6). This combination of variables might explain the different outcomes between animal groups and sexes. In addition, the route of administration, i.e., *i.m* or s.c can be added as a variable.

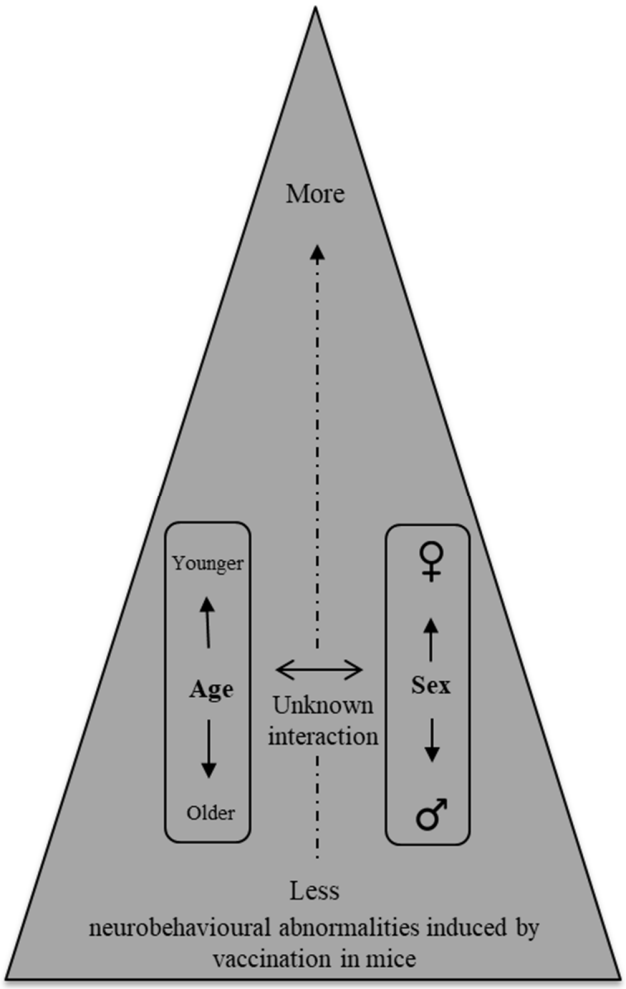


Figure 6. A schematic depicting the neurobehavioural effects of administering paediatric vaccines to mice. Not included in this schematic are the potential impacts of vaccine dose, mouse strain and Al adjuvant type.

To the best of our knowledge, apart from the study by Curtis *et al* [82], this is the only other study examining the impact on neurodevelopment, anxiety, learning and social behaviour of a combined routine paediatric vaccination schedule in an animal model.

We stress that since we, like Curtis *et al*, used whole vaccines, any abnormal outcomes reported cannot easily be attributed to particular chemical agents in the vaccines themselves. For example, vaccines contain a specific antigen designed to mimic part of the natural pathogen. In addition, many vaccines contain Al adjuvants. Finally, there are various other trace molecules usually listed as excipients. Using a whole vaccine makes it impossible to define an independent role for any of these. In this regard, it is possible, but not certain, that some of the abnormal outcomes we have seen

749 in the treatment groups reflect primarily the impact of the Al adjuvant, an outcome
750 which would be consistent with our previous work and that of others [6,34,46-50,89].
751 The monkeys (*Macaca mulatta*) used in the Curtis *et al's* study, however, can be
752 considered as a wild type model in that individual variations can be extensive
753 compared to other laboratory species, such as colony bred mice, in which the mice are
754 designed to have a more homogeneous genotype and phenotype. One interpretation for
755 differences in these two data sets might be that in genetically similar populations any
756 impacts might be more prominent. However, the observation that across two
757 mammalian species abnormalities were seen may lend support to the notion that in
758 humans some level of neurobehavioural abnormality might be expected. While the
759 numbers of those so affected might be small, it would still be plausible to expect such
760 numbers to increase as the causative stimuli increased.
761 Nevertheless, the Curtis *et al* study had several limitations which may lead to
762 different profiles of toxicokinetics and toxiocodynamics of the vaccine constituents. The
763 first is the nature of colony animals that may be viewed as more outbred than colony
764 bred rodents. Further, only male animals were studied. In contrast, many of the
765 alterations we found in behaviour were in the treated female mice (see Table 2a and
766 Figures: 1b; 2b and 4b).
767 Some limitations inherent to our study should be noted as well. First, while we
768 avoided a variation in dose based on body weight, apart from the deliberate use of the
769 V3 treatment group, we acknowledge that the use of an appropriate dose is not a simple
770 matter to resolve. Second, as our data showed, the age of the animal is an issue given
771 that mouse neural and immune systems are more mature at birth compared to humans.
772 For this reason, age equivalents for treatments compared to humans are often
773 approximate. Our approach, as cited in the Materials and Methods section for the
774 treatments on certain postnatal days, is thus not precisely in register with humans, but
775 rather reflects a best approximation that arguably is more conservative.
776 Another caveat to the present study is that, similar to other fields of medical
777 sciences, animal models of neuro-developmental disorders should comply with the
778 three criteria of validity, namely: face, construct, and predictive validity [90,91]. Face
779 validity refers to the ability of a model to successfully capture aspects of the observed
780 phenotype in humans. While it is considered as a requirement for rodent model of ASD
781 to show social deficits in order to show demonstrate face validity, it still remains
782 questionable whether behavioral abnormalities in rodents truly mirror the social
783 deficient observed in ASD [91], and thus the results obtained from such tests should be
784 interpreted with caution. Construct validity refers to the use of a known cause for a
785 given biological disorder in the animal model such as the animal exposure to an
786 environmental factors in order to induce defined outcomes. Predictive validity is a
787 measure of the degree to which a treatment in a model system predicts outcomes in
788 humans.

In regard to models for ASD, these can be divided into three categories: i) genetic-based models ii) environmental-based models, which are produced by introducing an environmental factor that has been linked to ASD such as chemicals or infectious microorganisms and iii) behaviourally-based models [91]. In the present study, the mouse model injected with vaccines satisfies Buxbaum *et al's* recommendations [92] regarding the criteria of validity. This level of validity does not alone suggest that the US vaccination schedule is responsible for the apparent increase in ASD in recent decades. Neither, however, does it allow us to definitively eliminate this schedule as one of the etiological factors involved in the disorder. It is worthwhile to note that the current work was originally designed in order to only capture the first 18 months of the US schedule of paediatric vaccination and that the provision of the rest of the schedule at older ages may have given more pronounced outcomes.

Overall, these concerns reflect general caveats to any model system approach to human diseases [93]. Nevertheless, any such model approach is the best that can be done in place of any invasive studies on humans. Clearly, the latter is neither ethical nor feasible. In regard to primate studies such as those performed by Curtis *et al* [82], the likely broad genetic variations in a wild type population would render the number of animals used insufficient to see small population effects, at least unless the individual animals were 'deconvolved' to see individual variations across the overall test groups compared to humans. However, small numbers in such individual analyses would almost certainly not be sufficient to perform statistical analysis. In our work, we have tried to address this issue by looking at individual animals over time as shown in Table 2, recognizing that these represent qualitative data only.

While the underlying pathways through which vaccines provoke some neurobehavioural abnormalities in mice are still unclear, they seem to interfere with multiple neural and immune pathways during certain critical windows of nervous and immune system development, especially during the first two weeks of postnatal life when the brain-blood barrier is still immature and permeable [94].

The interaction assessment/confirmation of vaccine effects on the developing nervous and immune systems in mice is the next aim of our ongoing study using various histological and biochemical analyses. This study will be reported at a future date.

The current study has attempted to answer a question about vaccine adverse effects, a question that has pitted two apparently severely polarized camps with diametrically opposing views. The mainstream medical camp holds that vaccine adverse events are extremely uncommon, tending to discount reports to the contrary. Against this viewpoint are lay people and a number of scientists who maintain that adverse vaccine events are far more common and potentially devastating to CNS development than conventionally acknowledged.

The results presented in this paper will likely satisfy neither solitude, nor the "trolls" who constantly hover around this subject. At least in this *in vivo* model, accepting all the caveats to such models, the results reveal a far more nuanced outcome: While most of the behavioral assessments in vaccinated mice at early time post vaccination were within the normal range, some were significantly not. Many of these differences disappeared with age such that by one year of age, few significant differences in the tested populations remained. The results were also stratified by sex and our data showed that female mice seemed to be more vulnerable in certain parameters measured. Put more succinctly, vaccine adverse effects in this model system were neither trivial nor devastating at a population level. This outcome, again with the caveats to animal models, appears to mirror to some extent the range of outcomes seen in human populations.

5. Conclusions

In the present study, some neurobehavioural abnormalities (NBAs) were observed in mice treated with vaccines compared to true placebo controls. These NBAs were not identical across time, changing according to a complex 3×3 matrix where the key factors appear to be vaccine dose, animal age, and sex.

The majority of treated mice at the end of the experimental period did not significantly differ from the control population such that, most of the abnormalities detected did not persist until the final evaluations at 67 weeks of age, however some persisted into adulthood. Even if this last outcome is not statistically significant, it is worth noting that these outcomes reflect an average of population average statement, not an individual one with recovery not universal.

Vaccine impacts seem not to have a clear linear dose-relationship suggesting that classical toxicokinetics may be different. Thus, a single dose of vaccine antigens could simply saturate the system triggering often a greater impact than higher doses.

Such behavioural data reported here, particularly in a murine model, are not sufficient to make firm inferences about human neurodevelopmental disorders, in particular for subjects as contentious as the aetiology of ASD.

The current results suggest, however, that lay and professional concerns about vaccine safety may not be as unfounded as often claimed and that further investigations in this area are indeed still warranted.

Author Contributions: conceptualization: CAS, LT and HE; methodology: HE, JY and SCB; validation: HE, ECS and JY; formal analysis: HE, JY and ECS; investigation: HE and MK; data curation: HE, JY, and SCB; writing original draft preparation: HE; writing review and editing: CAS, LT, HE, MK, JY and SCB; supervision: CAS and HE; project administration: JY, MK and SCB; statistical analysis: ECS, JY, SCB and HE.

Funding: This research was funded by Children's Medical Safety Research Institute (grant number 20R73006) and the Katlyn Fox Foundation (grant number 20R47306).

Conflicts of Interest: This work received funding from Children's Medical Safety Research Institute (CMSRI) and the Katlyn Fox Foundation.

Acknowledgments:
We thank Sears Family Pediatrics for their kind help in acquiring the whole vaccines used in the present study.
We also thank Agripina Suarez and Jessica Morrice for their feedback on study design and data analysis, and Sara Wu for her help with the behavioural tests.

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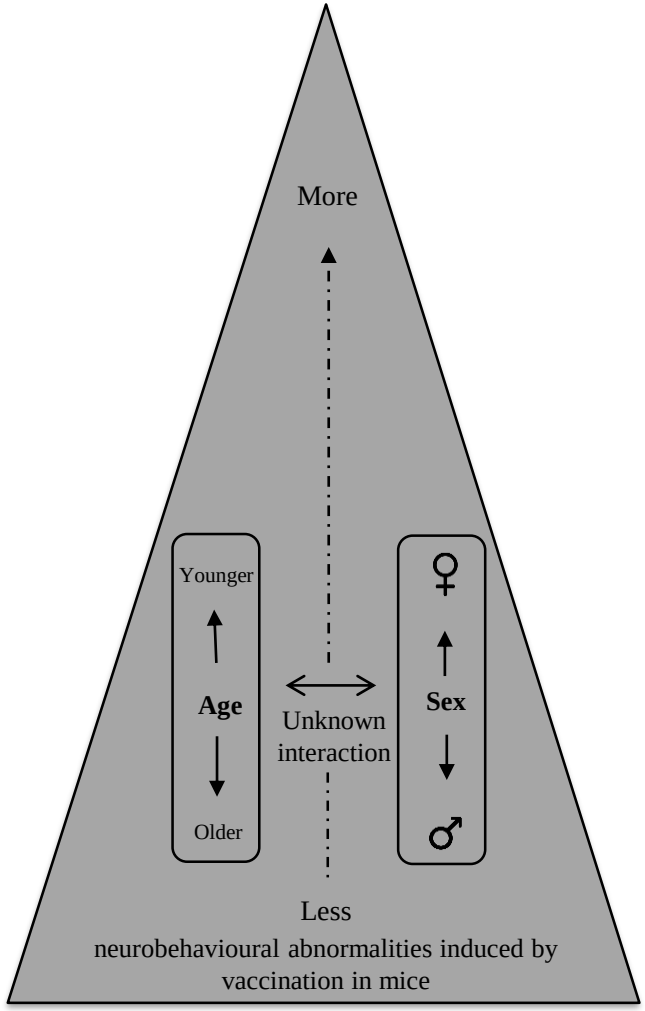
Synopsis for the Graphical Abstract

A schematic depicting the neurobehavioural effects of administering paediatric vaccines to mice. Not included in this schematic are the potential impacts of vaccine dose, mouse strain and Al adjuvant type.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neurobehavioural abnormalities induced by
vaccination in mice