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1 **Early postnatal injections of whole vaccines compared to placebo controls:**  
2 **differential behavioral outcomes in mice**

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17  
18 **Abstract**

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

37  
38 **Keywords:** *vaccine, neurodevelopment, behavioural tests, neurobehavioural abnormalities.*

39  
40

41 **1. Introduction**

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

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

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

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

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

72 Ongoing reports from human studies suggest an increase in the prevalence of  
73 neurodevelopmental disorders [23,24] in developed countries, particularly the U.S., U.K.,  
74 Canada and Australia which various authors attribute to vaccines, adjuvants, or both  
75 [25,26].

76 Various studies have shown that some past and present paediatric vaccine  
77 constituents such as mercury [27], Al [22,28-31] and formalin are neurotoxic and have  
78 been associated with adverse neurological and immune outcomes in animal models  
79 [9,32-35].

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

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

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

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

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

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

116

117 **2. Materials and Methods**

118 2.1. Animals, breeding and experimental groups

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

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

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

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

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

## 154 2.2. Vaccine administration

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

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

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

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

176  
 177 2.3. Mouse weights

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

180  
 181 2.4. Behavioural tests

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

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

187  
 188 Table 1. Summary of pre- and post-weaning behavioural tests. The same number of  
 189 mice was used for all tests, namely a total of 76 pups divided into two experimental  
 190 groups and one control group of 4 litters each.

191

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

192

#### 193 2.4.1. Pre-weaning tests for developmental milestones

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

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

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

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

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

215

#### 216 2.4.2. Post-weaning tests

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

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

223

##### 224 2.4.2.1 Social interaction test

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

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

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

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

250



#### 251 2.4.2.2 Light dark box test

252 The Light dark box (LDB) test provides a means of examining anxiety-like  
253 behaviours. The LDB paradigm is based on the innate aversion of mice to brightly  
254 illuminated areas and on their spontaneous exploratory behaviour applying mild  
255 stressors, namely a novel environment and light [63]. This test was performed in a  
256 standard two-compartment chamber as described previously [64]. The LDB test was  
257 conducted twice with an interval of four months, at 4-5 months and repeated at 8-9  
258 months of mouse age (LDB1 and LDB2, respectively) with the same randomization of  
259 tested mice as cited above. The dark box insert was made of black Perspex designed to  
260 cover one third of the area of the activity chamber (45 cm X 30 cm X 21 cm) with a 7 cm ×  
261 7 cm hole placed in the middle of the wall at floor level. At the beginning of the test, the  
262 test subject was placed in the light box facing the wall farthest apart from the dark box.  
263 Mice were allowed to move freely between two chambers for 10 minutes. Time spent in  
264 the dark box was automatically scored by the EthoVision system.

#### 265 2.4.2.3 Novel object recognition test

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

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

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

295

#### 296 2.4.2.4 Barnes maze

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

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

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

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

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

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

338

### 339 2.5. Statistical analyses

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

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

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

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

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

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

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

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

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

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

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

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

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

410

### 411 **3. Results**

412

#### 413 3.1. Overall mouse development

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

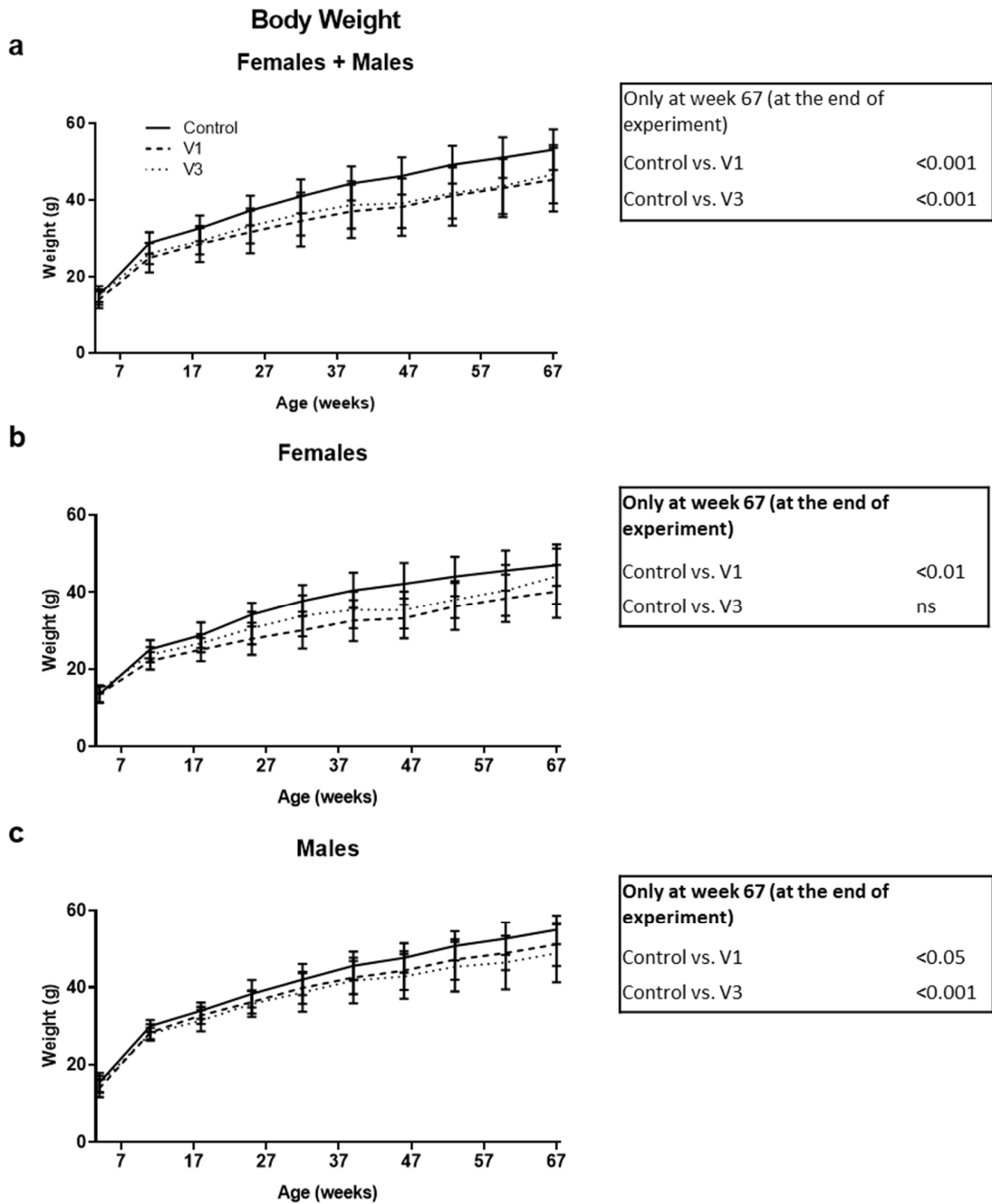
416

#### 417 3.2 Mouse weight

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

424

425



426

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

430

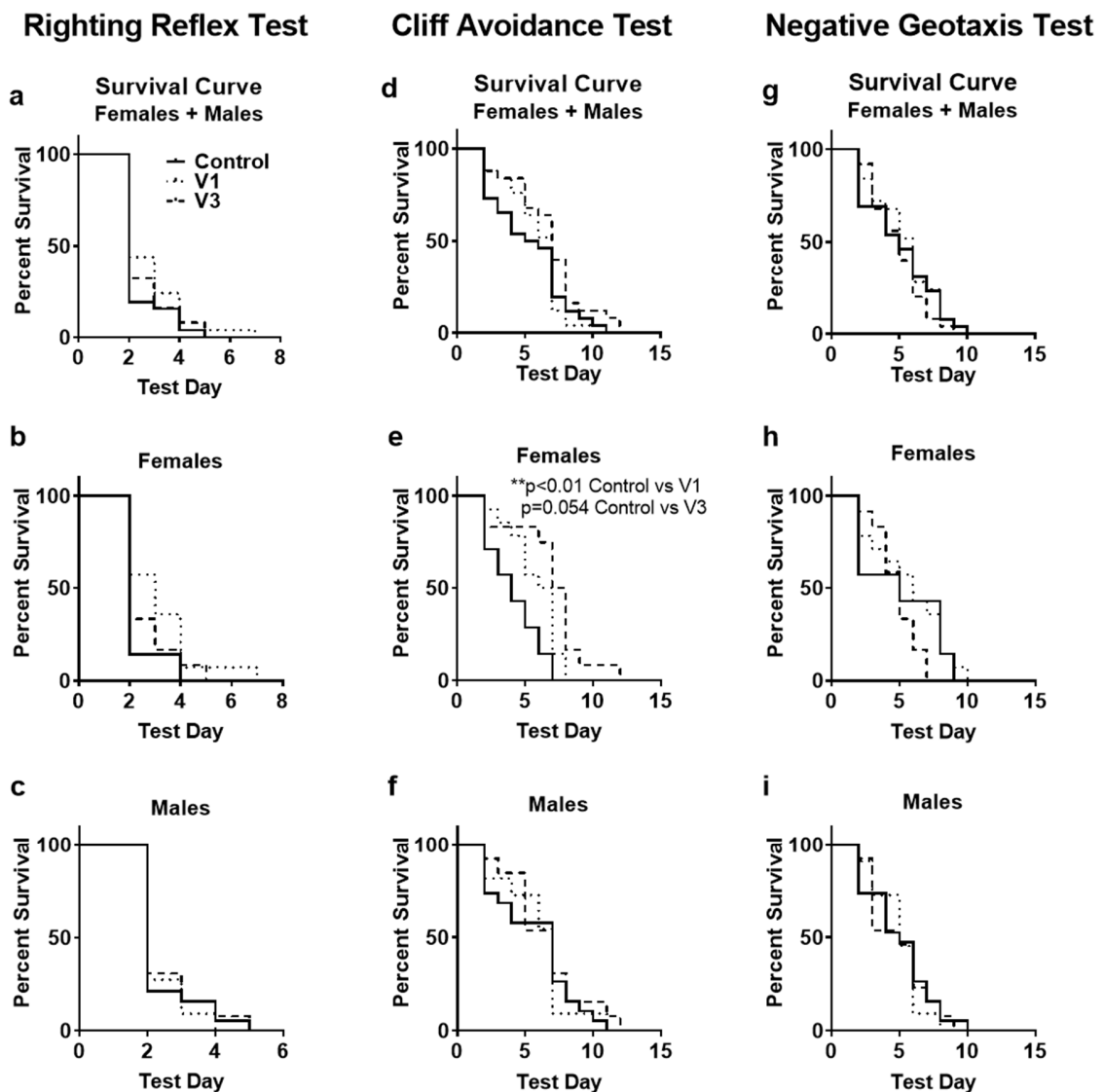
431 3.3. Behavioural tests

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

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### 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).



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**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

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

451

### 452 3.3.2. Post-weaning tests

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

455

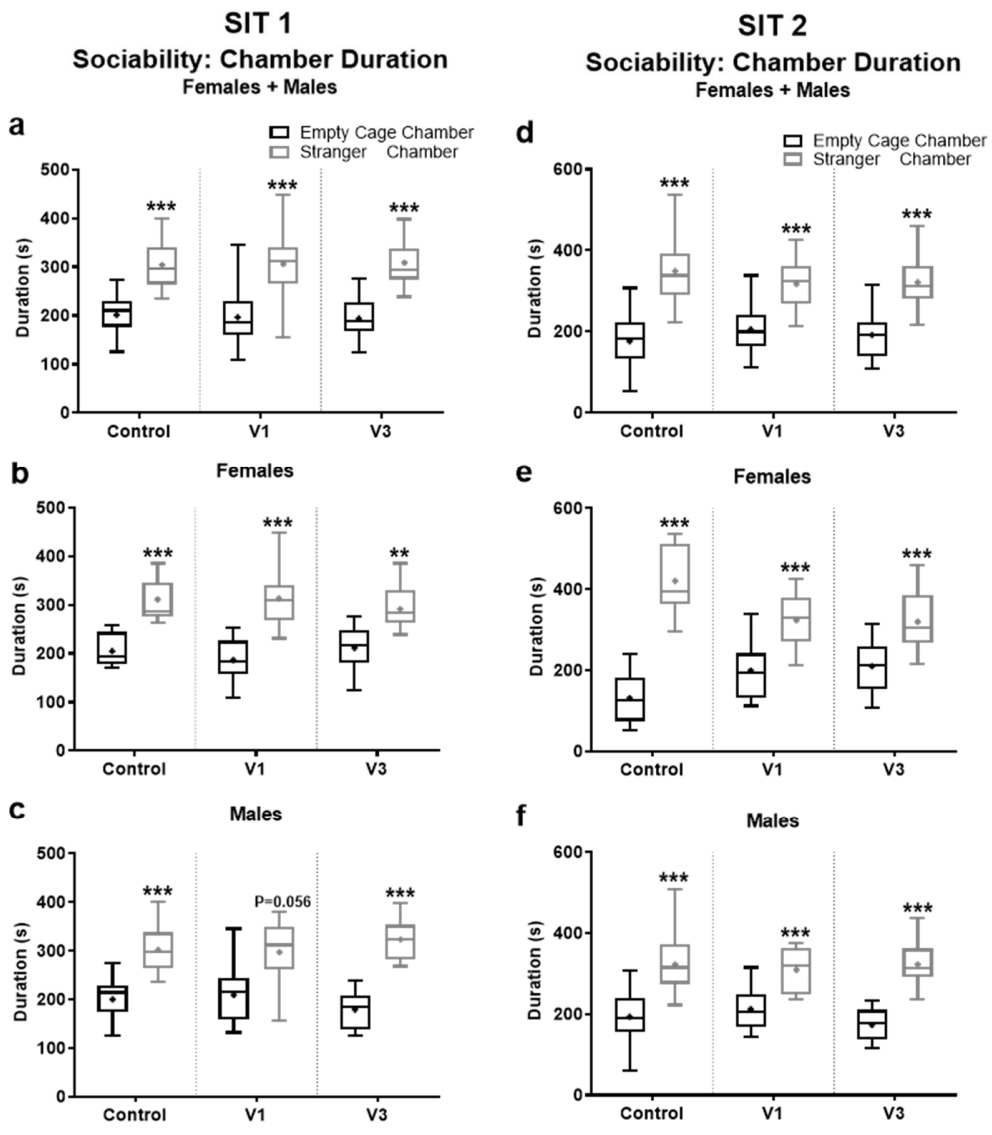
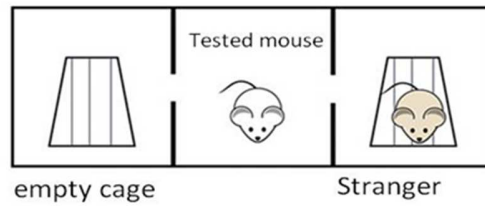
#### 456 3.3.2.1. Social interaction test

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

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

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





465  
 466 **Figure 3.** Social skills assessment was conducted twice in mice by the Social Interaction  
 467 Test (SIT): SIT1 (2 - 4 months of age) and SIT2 (9 - 10 months of age). Sociability  
 468 differences were expressed by the chamber duration within mouse groups and no  
 469 comparison was done between treated groups and control. (**a, b and d - f**) No sociability  
 470 anomaly was observed in any mouse groups and no differences were seen between  
 471 males and females as all mice spent significantly more time with the stranger *vs.* the

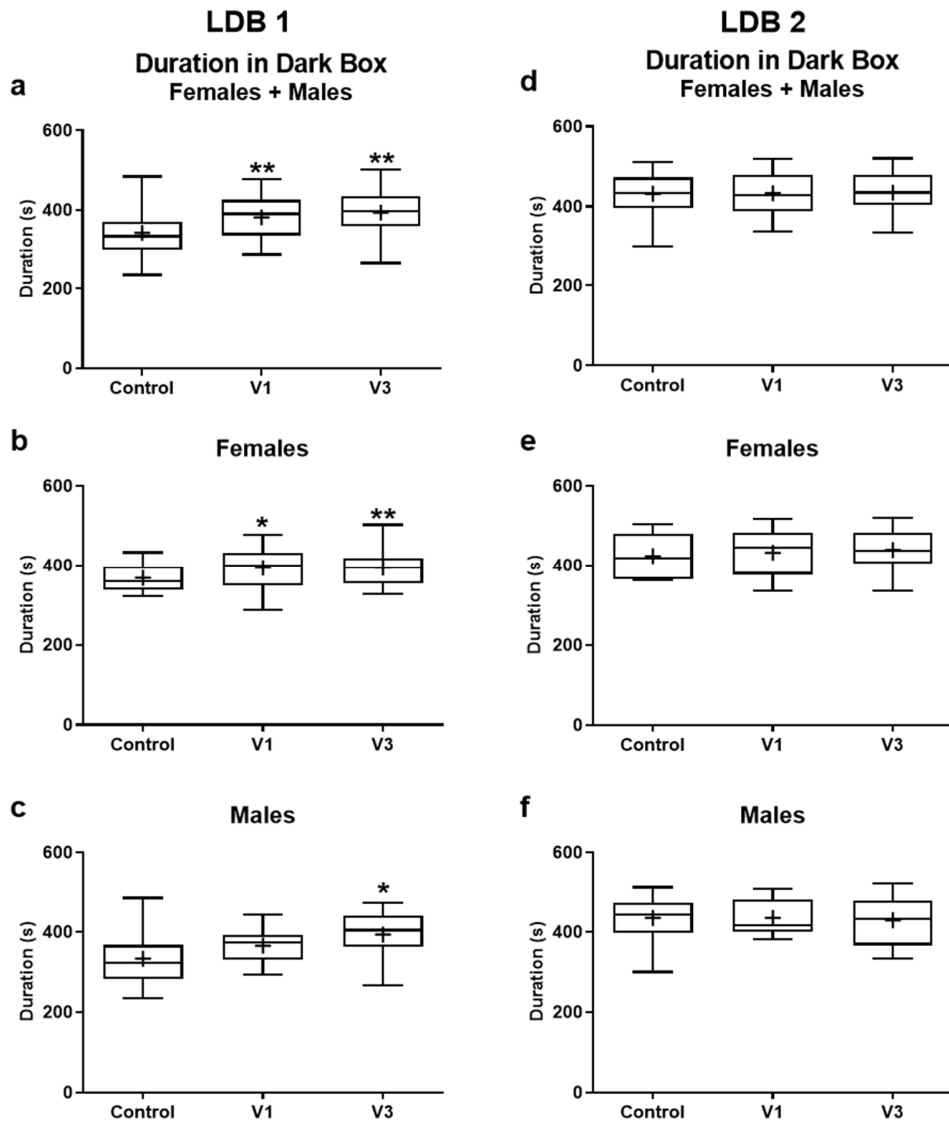
472 empty cage. (c) Males from the V1 group showed no significant difference between the  
473 time spent with the stranger 1 vs the empty cage. \*\* $p < 0.01$  and \*\*\* $p < 0.001$ .

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.



492  
 493 **Figure 4.** Anxiety and exploratory behaviour evaluation by the light dark box (LDB)  
 494 test. The LDB test was conducted twice: at 4-5 and 8-9 months of age (LDB1 and LDB2,  
 495 respectively). (a) LDB1: All treated groups showed a significant increase in the spent  
 496 time in the dark chamber. (b and c) Females from all treated groups and only males  
 497 from the V3 groups spent more time in the dark chamber compared to controls. (d,e,f)  
 498 LDB2: No significant difference was observed between treated groups and control  
 499 when the data were stratified by sex. \* $p < 0.05$  and \*\* $p < 0.01$  vs. control.

500

501 3.3.2.3. Novel object recognition test

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

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

507

#### 508 3.3.2.4. Barnes maze test

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

510

##### 511 3.3.2.4.1. Acquisition phase

###### 512 *Primary latency and primary errors*

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

520 Primary errors in control and both the treatment groups showed a significant  
521 decrease ( $p \leq 0.001$ ) when both sexes combined (Figure S.4d) on day 4 compared with  
522 day 1 which was similarly observed in all the groups of male mice ( $p \leq 0.01$ ; Figure S.4f).  
523 During the 4-day acquisition trial in female mice, only control and V1 group showed a  
524 significant reduction in primary errors on day 4 compared with day 1 ( $p < 0.001$ ), while  
525 V3 females showed no significant reduction (Figure S.4e). No significant difference was  
526 observed in both treatment groups compared to control on day 4 (Figure S.4d, S.4e and  
527 S.4f).

528

###### 529 *Total latency and total errors*

530 The total latency was significantly decreased ( $p < 0.001$ ) on day 4 compared with day  
531 1 in control and in the two treatment groups in both sexes combined over the 4 days of  
532 the acquisition training period (Figure S.5a). Similarly, when separated by sex, all the  
533 groups of female (Figure S.5b) and male mice (Figure S.5c) showed a significant  
534 reduction in total latency ( $p \leq 0.001$ ). Total errors were likewise reduced significantly in  
535 control and all the treatment groups on day 4 compared with day 1 during 4 days of the  
536 acquisition training in both sexes combined ( $p \leq 0.01$ ) (Figure S.5d). When stratified by  
537 sex, control and V1 groups of female mice made significant improvements in total  
538 errors ( $p < 0.001$ ; (Figure S.5e) on day 4. During the 4 days of acquisition training, control  
539 and V3 groups in male mice showed a significant reduction in total errors on day 4  
540 compared with day 1 ( $p \leq 0.01$ ) (Figure S.5f). Only female mice from V1 group made  
541 significantly more total errors on day 4 compared to control ( $p < 0.001$ ; Figure S.5e). No  
542 significant difference in total errors between the treatment groups and the control group  
543 was observed on day 4 when both sexes combined and in male mice.

544

#### 545 *Search strategies*

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

554

#### 555 3.3.2.4.2. Short- and long-term memory retention

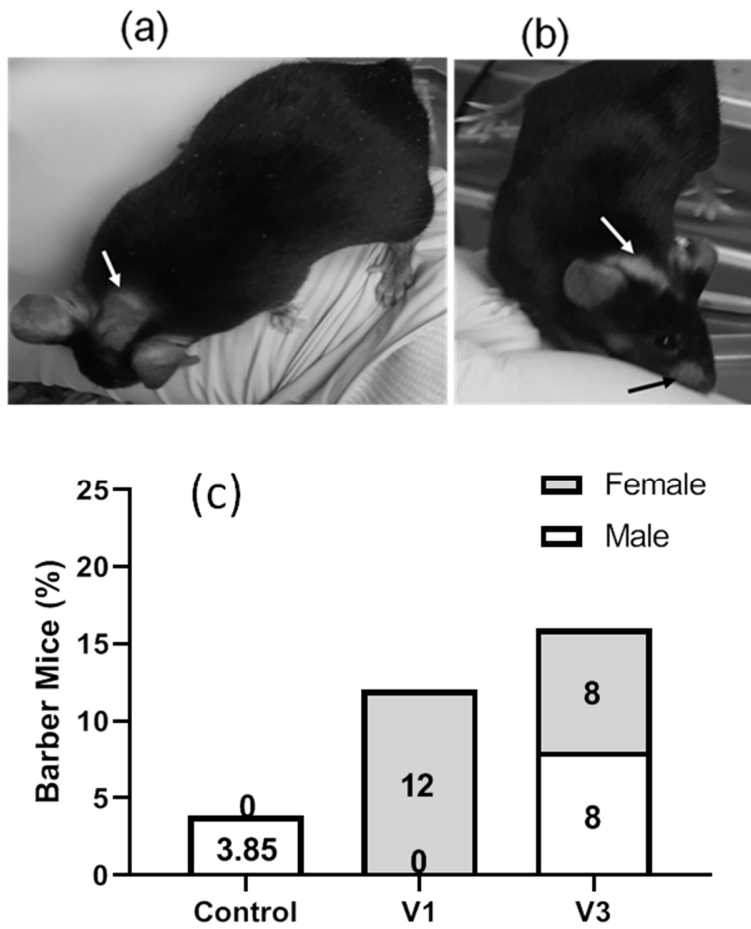
556 During the 1<sup>st</sup> (short-term) and 2<sup>nd</sup> (long-term) probe trial (day 5 and 12 after the last  
557 acquisition training, respectively), mice in both vaccine groups when both sexes  
558 combined (Figure S.7a) as well as females separately from males (Figure S.7b) did not  
559 show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

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

573

#### 574 3.4. Repetitive/aggressive-like behaviours

575 Barbering, a fur and whiskers tearing behaviour, was clearly observed by the end of  
576 the 3<sup>rd</sup> month of age of vaccinated mice compared to control (Figure 5a-b). Mice from V3  
577 showed the highest barbering percentage (16%) followed by V1 group (12%) at 10  
578 months of age (Figure 5c). However, the statistical analysis of these data showed that  
579 there was no significant difference between the two treated groups compared to control.  
580 Unlike the treated mice, all mice showing barbering behaviour in the control group  
581 were males, representing only 3.85% of this group. Females seemed to be more affected  
582 by barbering as the percentage of female barber mice from V1 groups was higher than  
583 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%).

591

592 **Table 2.**

593 Table 2a. Qualitative summary of the overall vaccine impacts on body weight, reflex development, neuromotor  
594 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 <sup>1</sup>	S	NS		Normal		S					
	M	NS <sup>2</sup>	NS			Abnormal	Normal	NS					
	F+M	S	NS			Normal		S					
V3	F	NS	S			Normal		S					NS
	M	S	NS										
	F+M	S	NS										

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

596 1: a statistically significant effect compared to control ( $p \leq 0.05$ ).

597 2: no statistically significant effect compared with control ( $p > 0.05$ ).

598

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

608

609

BM (visual-spatial learning and memory)									
Group	Sex	Acquisition phase trial <sup>1</sup>					1 <sup>st</sup> and 2 <sup>nd</sup> 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*						1	
	F+M	Decreased*						Increased* <sup>1</sup>	2
V3	F	Normal			Normal	Normal	Normal	2	
	M						Normal†	2	
	F+M						Normal	Increased* <sup>2</sup> †	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 <sup>1</sup> V1 males and females on day 12 compared to control male and females on day 12.

614 <sup>2</sup> V3 males and females on day 12 compared to control males and females on day 12.



615 **4. Discussion**

616

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

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

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

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

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

648 Although the mechanism behind the post-vaccination alterations of body weight is  
649 still unclear, various studies have suggested that body weight decrease can be due to  
650 nutritional changes caused by changes in animal water consumption after vaccination  
651 [79]. Other studies suggested that body weight reduction occurred as a result of changes  
652 in the expression of several genes in vaccinated animals [79,80].

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

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

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

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

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

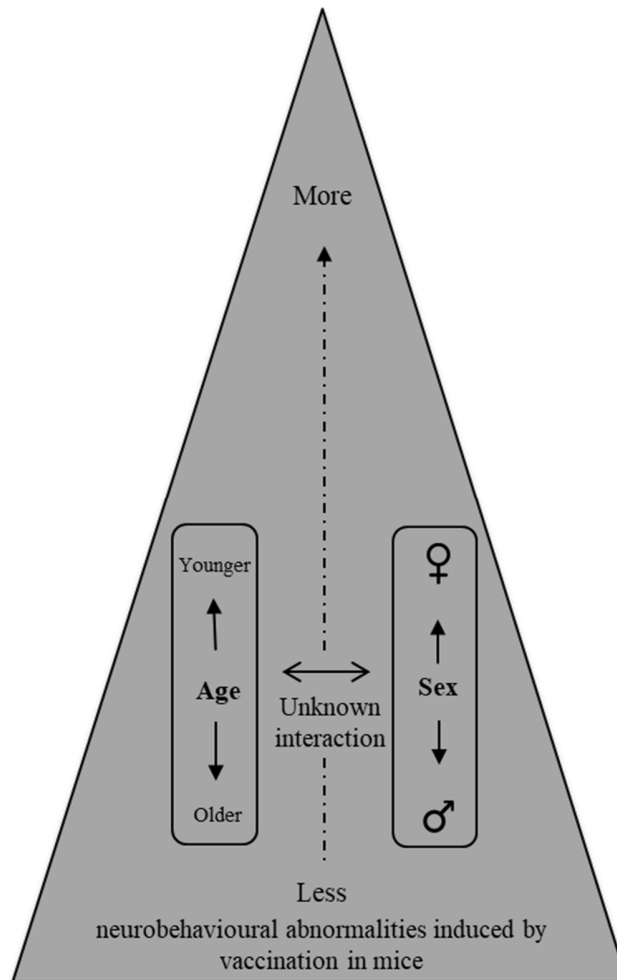
693 The improvement in the early neurobehavioural impairments observed by Curtis *et*  
694 *al* in primates [82] and those obtained from our SIT and LDB outcomes in mice mirror

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

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

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

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



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**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 AI adjuvant type.

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741

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.

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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 AI 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 toxicodynamics 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.

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

801 Overall, these concerns reflect general caveats to any model system approach to  
802 human diseases [93]. Nevertheless, any such model approach is the best that can be done  
803 in place of any invasive studies on humans. Clearly, the latter is neither ethical nor  
804 feasible. In regard to primate studies such as those performed by Curtis *et al* [82], the  
805 likely broad genetic variations in a wild type population would render the number of  
806 animals used insufficient to see small population effects, at least unless the individual  
807 animals were 'deconvolved' to see individual variations across the overall test groups  
808 compared to humans. However, small numbers in such individual analyses would  
809 almost certainly not be sufficient to perform statistical analysis. In our work, we have  
810 tried to address this issue by looking at individual animals over time as shown in Table  
811 2, recognizing that these represent qualitative data only.

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

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

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

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

840

## 841 **5. Conclusions**

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

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

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

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

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

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**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.

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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 AI adjuvant type.

