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1 **Pre- and postnatal exposure to glyphosate-based herbicide causes behavioral and cognitive impairments**
2 **in adult mice: evidence of cortical and hippocampal dysfunction**

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27 for financial support.

28 **Abstract**

29 Glyphosate-based herbicides (GBH) are the most widely used pesticides worldwide. Despite considerable
30 progress in describing the neurotoxic potential of GBH, the harmful effects on brain cytoarchitecture and
31 behavior are still unclear. Here, we addressed the developmental impact of GBH by exposing female mice to 250
32 or 500 mg/kg doses of GBH during both pregnancy and lactation and then examined the downstream effects at
33 the behavioral, neurochemical and molecular levels. We show that pre- and neonatal exposure to GBH impairs
34 fertility and reproduction parameters as well as maternal behavior of exposed mothers. In offspring, GBH was
35 responsible for a global delay in innate reflexes and a deficit in motor development. At the adult age, exposed
36 animals showed a decrease of locomotor activity, sociability, learning and short and long-term memory
37 associated with alterations of cholinergic and dopaminergic systems. Furthermore, GBH activated microglia and
38 astrocytes, sign of neuroinflammation event in the medial prefrontal cortex and hippocampus. At the molecular
39 level, a down-regulation of brain-derived neurotrophic factor (BDNF) expression and an up-regulation of
40 tyrosine related kinase receptor (TrkB), NR1 subunit of NMDA receptor as well as tumor necrosis factor α
41 (TNF α) were found in the brain of GBH-exposed mice.

42 The present work demonstrates that GBH induces numerous behavioral and cognitive abnormalities closely
43 associated with significant histological, neurochemical and molecular impairments. It also raises fundamental
44 concerns about the ability of current safety testing to assess risks of pesticide exposure during developmental
45 periods of central nervous system.

46 **Key words:** Glyphosate, behavior, cognition, acetylcholinesterase, neuroinflammation, BDNF signaling.

47 **Introduction**

48 The American National Academy of Sciences estimated that 3% of brain developmental disorders, such as
49 autism spectrum disorders and learning disabilities, may be directly linked to exposure to environmental
50 chemicals (National Research Council 2000). Existing evidence supports the idea that lifelong susceptibility to
51 anxiety disorders can be partly determined by environmental factors during early development (Gross and Hen
52 2004). Furthermore, it is important to note that early perturbations of brain development may lead to
53 neurobehavioral disturbances expressed either in childhood or with delayed onset in adulthood (Olney, 2002). In
54 line with this, the potential of in utero or early postnatal pesticide exposure to affect brain development has been
55 shown and an emerging literature provides evidence for neurobehavioral consequences resulting from early
56 exposure to organophosphates (OPs) pesticides (for review, see Heyer and Meredith 2017).

57 Glyphosate (Gly) (N-[phosphonomethyl]glycine; CAS registry number 1071-83-6), the active ingredient present
58 in Roundup® (Monsanto Company, St. Louis, MO), is the most heavily OP herbicide used worldwide (Powles et
59 al. 1997). The herbicidal action of Gly is due to inhibition of a key plant enzyme (5-enolpyruvyl shikimate-3-
60 phosphate synthase) (Franz et al. 1997). Since this enzyme is not present in vertebrates, it has long been assumed
61 that Gly does not affect non-target species. In addition, experimental data showing that the blood Gly
62 concentrations peaked at around 1–2 h post ingestion and that Gly is poorly metabolized and generally
63 eliminated in the urine and feces (Chan and Mahler 1992; Brewster et al. 1991) supported the safety hypothesis
64 of Gly. However, over the last few years, several concerns have been raised by the scientific community and
65 regulatory agencies regarding the potential adverse effects of Gly and its adjuvants on the environment and
66 human health (Solomon et al. 2007). Evidence of exposure to Gly has been revealed by its detection in urine
67 samples of people living in farm and non-farm household (Conrad et al. 2017) and in maternal and umbilical
68 cord serum of pregnant women in Thailand (Kongtip et al. 2017). Clinical reports of intoxication with
69 commercial formulations of Gly described negative effects on the nervous system, including Parkinsonism
70 (Barbosa et al. 2001; Wang et al. 2011), anxiety and short-term memory impairments (Nishiyori et al. 2014).
71 Moreover, an epidemiological study highlighted a strong correlation between the increasing application of Gly in
72 agriculture and the occurrence of several neurological diseases, including autism, dementia and anxiety disorder
73 at different ages (Seneff et al. 2015). Concomitantly, experimental studies also revealed neurotoxic effects of
74 glyphosate-based herbicide (GBH). Indeed, oral administration of GBH to pregnant rats alters the activity of
75 brain enzymes in both mothers and offspring (Daruich et al. 2001). These data suggest that GBH could impact
76 maternal behavior and subsequently offspring's sociability given the crucial role of early maternal care on
77 offspring's abilities (Branchi et al. 2013). However, there have been no systematic studies evaluating the effects
78 of GBH on maternal care and social behavior of their offspring. At the behavioral level, it has been shown that
79 early exposure to GBH results later in life in depressive-like behavior (Cattani et al. 2017) and contradictory
80 effects on the anxiety-related behavior (Gallegos et al. 2016; Baier et al. 2017). Recently, we found that GBH
81 induces cognitive alterations in young male mice, supporting the idea that early exposure to GBH can interfere
82 with brain structures involved in learning and memory leading to cognitive deficits (Bali et al. 2019). However,
83 the effect of prenatal and neonatal exposure to GBH on learning and memory is still unexplored. In this context,
84 the present study was conducted using a multifaceted behavioral battery to assess the profile of gestational and
85 lactational GBH effects in a mouse model. Behavioral assays covering neonatal age and adulthood were selected
86 to measure a range of early reflex development as well as locomotor, affective, sociability and cognitive

87 functions. Mechanistic, cytoarchitectural, neurochemical and molecular mechanisms underlying of GBH-
88 induced neurobehavioral deficits were evaluated in this follow-up study, with the goal to provide benchmark
89 data for GBH risk assessment in the brain.

90 **Materials and Methods**

91 **Pesticide**

92 Roundup herbicide (glyphosate concentration: 360g/l in the form of glyphosate isopropylamine salt 486 g/l), was
93 used in the liquid commercial form supplied by Monsanto Company (St. Louis, MO, USA). The molecular
94 formula is $C_6H_{17}N_2O_5P$ (molecular weight: 228.183 g/mol; melting point: 200 °C; density: 1.218 g/cm³)

95 **Animals**

96 Male and female Swiss mice (3-months-old) were obtained from the animal husbandry of the Faculty of
97 Sciences, Cadi Ayyad University, Marrakech, Morocco. The animals were housed in Plexiglas cages (30 x 15 x
98 12 cm) under standard conditions of temperature ($22 \pm 2^\circ\text{C}$) and photoperiod (12h / 12h) with food and water *ad*
99 *libitum*. All procedures were approved by the Council Committee of Research Laboratories of the Faculty of
100 Sciences, Cadi Ayyad University (Marrakech, Morocco) and conducted in accordance with European Council
101 Directive: EU2010/63. All efforts were made to minimize animal suffering.

102 **Doses and protocol of exposure**

103 Females were mated with breeding males (2 females for 1 male) over a day, and were examined on the following
104 day by vaginal plug inspection to assess successful mating. If judged copulated, the female was removed from
105 the cage of the male and housed individually. This was considered as the day 0 of gestation (G0).

106 Female exposure to GBH through oral gavage occurred daily from G0 to postnatal day 21 (P21) (Fig. 1). Three
107 experimental groups were formed, each one including a minimum of six female mice: a group exposed to a
108 lower dose (250 mg/kg) of GBH, a group exposed to a higher dose (500 mg/kg) of GBH, and a control group
109 which received vehicle (tap water). These doses were selected based on the no-observed adverse effect level
110 (NOAEL) indications (i.e: 500 mg/kg/day) (EPA, 1993). The GBH doses used in the present study were higher
111 than the GBH levels to which the population is normally exposed (Solomon, 2016). However, as in other
112 toxicological studies, exposure to relatively high doses was used in order to demonstrate a plausible drug-
113 action (see for example Ford et al. 2017).

114 **Gestation outcomes, maternal behavior and body weight of pups**

115 In order to detect any signs of poisoning, all pregnant mice were observed daily from the first administration day
116 (G0) until parturition. In addition, several parameters of maternal behavior, fertility and reproduction were
117 evaluated according to Ema et al. (2007).

118 **Motor and sensory development assessment**

119 The behavioral testing (negative geotaxis, righting reflex, cliff avoidance and rotarod tests) was performed as
120 described by Ait bali et al. (2016). Animals (n = 10 for each group: control, 250 mg/kg and 500 mg/kg; two
121 males from each litter) were tested during morning sessions starting at 9 a.m. The tests for sensorimotor
122 development assessed during the same day were separated by an interval of 30 min.

123 **Adult behavior**

124 From P60, behavioral tests were performed in order to assess locomotor activity (open field, OF), levels of
125 anxiety (OF and elevated-plus maze, EPM), social interaction (three-chambered sociability test, TCS), working
126 memory (Y-maze), recognition memory (novel object recognition test, NOR), and learning and emotional
127 memory performances (passive avoidance test, PA). The behavior of a total number of 10 mice for each group
128 was evaluated between 9 a.m. and 13 p.m and recorded with Ethovision XT Noldus 8.5 video tracking program

129 (Noldus Information Technology b.v., Wageningen, The Netherlands), connected to a video camera (JVC,
130 Japan).

131 ***Open field***

132 This test was performed to assess the general locomotor activity (Walsh and Cummins 1976). Activity
133 monitoring was conducted in a square shaped, white arena, measuring 50 × 50 × 50 cm. Mice were placed
134 individually into the arena and monitored for 20 min. The assessed parameters were the total distance travelled,
135 the velocity and the time spent in the center.

136 ***Elevated-plus maze***

137 The elevated plus maze is a widely accepted paradigm used to assess anxiety-like behavior in rodents (Pellow et
138 al. 1985). The elevated plus-maze included two opposing open arms (OA) (50 × 5 cm) and two closed arms (CA)
139 (50 × 5 × 15 cm) joining at a square central area (5 × 5 cm) to form a plus sign. The entire apparatus was
140 elevated to a height of 45 cm above the floor. Each mouse was tested within a 5-min test session. At the
141 beginning, each mouse was placed individually in the central area facing one of the open arms and allowed to
142 freely explore the maze. The time spent in the OA and CA as well as the number of entries into each arm were
143 quantified. An anxiety index ($1 - \frac{[\text{open arm time}/\text{total time}] + [\text{open arm entries}/\text{total number of entries}]}{2}$)
144 was determined according to Cohen et al. (2013).

145 ***Three-chambered sociability test***

146 The social interaction test was run in a three-chambered arena made of clear glass. Retractable doorways, built
147 into the two dividing walls, controlled access to the side chambers. Each of the two outside chambers had an
148 inverted empty wire cup, one housing a male “stimulus” mouse age-matched to the “test” mouse, and the other
149 with a plastic object (“plastic mouse”). The test session began with a 5 min habituation session with the test
150 mouse free to explore the entire arena. This mouse was then briefly confined to the center chamber while the
151 plastic object was placed in the cup on one side and an adult male mouse on the other side. The “stimulus”
152 mouse and the “plastic mouse” sides were alternated, left and right, between tests. Once the stimuli were in
153 position, the two side doors were simultaneously raised and the test mouse could access all three chambers for 5
154 min. Automatic monitoring recorded and scored the time spent in contact with each wire cup as well as the
155 number of visits. The apparatus was cleaned between tests using a 70% ethanol/ water solution.

156 ***Y-maze***

157 Y-maze was used in order to assess working memory performances (Hughes, 2004). This 3 arms apparatus was
158 made of brown wood (60 cm × 15 cm × 30 cm), positioned at equal angles of 120°. Mice were placed at the end
159 of one arm and allowed to freely explore the maze over an 8 min session. The series of arm entries were
160 recorded, and alternation was defined as a triplet of explored arms. Alternation was considered as successful
161 when the 3 arms were different. The percentage of spontaneous alternation was calculated according to the
162 following equation: % alternation = $\frac{[\text{number of alternations}]}{(\text{total arm entries} - 2)} \times 100$ (Chen et al. 2016).

163 ***Novel object recognition***

164 This test was used to evaluate recognition memory. It was based on the natural preference of mice for a new
165 object with respect to a familiar one. The apparatus consisted of an OF made in Plexiglas (50 x 50 x 50 cm)
166 containing two identical or different objects according to the phase of the test. The objects to be discriminated
167 were three plastic objects. The task procedure consists of three phases: habituation, training, and retention phase,

168 according to the protocol described by Bevins and Besheer (2006) for a one trial nonmatching to sample learning
169 procedure. The habituation phase with the apparatus was conducted for 10 min without the presence of the
170 objects. The next day, during the training session, two identical objects were placed in the back corner of the
171 box. The experimental mouse was then placed midway at the front of the box and the total time spent exploring
172 the two objects was recorded for 5 min. During the retention session (1 h after), one of the two identical objects
173 was replaced by a new one and the mouse was allowed to explore the different objects for 10 min. The time
174 spent next to each of the two objects (the familiar one and the novel one) was recorded and discrimination
175 between them was calculated using a discrimination index [DI = (novel object exploration time/ total exploration
176 time of the two objects) – (familiar object exploration time/ total exploration time of two objects) x 100].

177 *Passive avoidance*

178 The test is based on the association formed between an aversive stimulus (a foot shock) and a specific
179 environmental context. The apparatus consisted of a two-compartment (light-dark) box. The light compartment
180 (10 × 13 × 15 cm) was illuminated while the dark one (10 × 13 × 15 cm) was equipped with energized grid floor,
181 separated by a guillotine door. The entrance of animals to the dark box was punished by an electric foot shock
182 (0.2 mA for 1 s duration). 24 h before the training session, the mice were allowed to explore freely the apparatus
183 for 3 min (habituation). On the training day, each mouse was placed in the center of the light compartment facing
184 away from the guillotine door. After 10 s of adaptation the guillotine door was opened exposing the dark
185 compartment. When the mouse entered the dark box with all four paws, the guillotine door was closed, and the
186 foot shock was delivered. On the test day, the mouse was returned to the illuminated compartment and the
187 procedure was repeated except that no shock was delivered. The test session was carried out 2 h (short-term
188 memory) or 24 h (long-term memory) after the training. Each time, the latency to enter the dark compartment
189 was recorded. Mice whose latency on the training session exceeded 60 s were excluded from the experiment in
190 order to minimize the deviation of baseline data. If the animal did not enter the dark compartment during the test
191 within 300 s, the trial was stopped and the final score was established as 300 s. After each session, the apparatus
192 was cleaned using 70% ethanol.

193 **Determination of acetylcholinesterase enzyme activity**

194 After the behavioral analyses, mice were killed by decapitation, their brains immediately removed from the skull
195 and the PFC and hippocampi were dissected for biochemical analyses. Acetylcholinesterase (AChE) activity was
196 determined in tissue homogenates as described previously (Bali et al. 2019). Briefly, acetylthiocholine (ASCh)
197 was used as a substrate of AChE with Thiocholine (SCh) as a reaction product. The activity of AChE was
198 determined according to the colorimetric method of Ellman et al. (1961) based on the reaction of SCh with
199 DTNB (5,5'-Dithiobis (2-nitrobenzoic acid)), which gives a yellow compound (TNB: S Thio-2-N nitro-benzoate)
200 absorbing light at 412 nm. The absorbance of the TNB measured with a spectrophotometer is proportional to the
201 enzymatic activity of AChE. The specific activity is calculated and presented as a percentage:

202
$$SA = \frac{\Delta OD / \text{min} \times 1000}{\varepsilon \times [\text{weight of brain tissue}]}$$

203 - $\Delta OD / \text{min}$: Variation of OD per min;

204 - $[\text{weight of brain tissue}]$: Weight of brain tissue of each mouse (g);

205 - ε : Molar extinction coefficient of TNB at 412 nm with $\varepsilon_{TNB} = 13,6 \times 10^{-6} M^{-1} \text{cm}$.

206 **Tissue sampling and immunofluorescence**

207 Upon conclusion of behavioral testing, control and treated mice were anesthetized with an intraperitoneal
208 injection of urethane 40% (1 g/kg, from Sigma–Aldrich, France) and transcardially perfused with saline solution
209 (0.9%), followed by ice-cold 4% formaldehyde in phosphate buffered saline (PBS; 0.1 M). The brains were then
210 removed, post-fixed in the same fixative for 12 h and cryoprotected overnight in 30% sucrose. They were then
211 cut on a freezing cryostat (Leica Microsystems, Germany) into 30 μm frontal sections. The sections containing
212 the substantia nigra pars compacta (SNc), the ventral tegmental area (VTA) and the striatum were used for
213 tyrosine hydroxylase (TH) immunofluorescence, while sections containing PFC and dorsal hippocampus stocked
214 for GFAP and Iba-1 immunofluorescence. The regions of interest were determined according to stereotaxic atlas
215 of Paxinos and Franklin (2001). Sections were kept in PBS containing 0.05% Triton X-100 and 10% normal
216 donkey serum (NDS) for 1 h. Thereafter, they were incubated with the appropriate primary antibodies (mouse
217 monoclonal anti-TH 1 :1000, Immunostar cat. 22941; rabbit polyclonal anti-GFAP; 1:500, Abcam, Ab7260;
218 rabbit polyclonal anti-Iba-1; 1 :1000; Wako, cat. 019-19741) diluted in PBS with 3% NDS and 0.05 Triton X-
219 100 with gentle stirring at 25°C. The following day, the sections were rinsed and incubated for 2h with the
220 adequate secondary antibodies (1:1000; Jackson ImmunoResearch, West Grove, PA, USA) at room temperature
221 for 2h. After PBS rinsing, the sections were mounted on gelatin-coated glass slides and coverslipped with Dako
222 fluorescence mounting medium (Dako Italia, Milan, Italy).

223 **Image analysis**

224 The TH-immunostained sections were used to assess the number of dopaminergic cells in the SNc and VTA
225 (Bregma: - 6.30 mm) and dopaminergic fibers in the striatum (Bregma: 0.48 mm), while the GFAP and Iba-1
226 immunostained sections were used to assess reactive astocytes and microglia, respectively, in the PFC (Bregma:
227 3.20 mm) and dorsal hippocampus (Bregma: - 3.80 mm). Three mice per group were used for these analyses. For
228 each brain region, three representative sections (0.5 μm Z-step size) from anterior to posterior were acquired
229 with a laser scanning confocal microscope (LSM5 Pascal; Zeiss, DE, Germany) using either a 20x objective (for
230 TH⁺, GFAP⁺ and Iba-1⁺ cells count) or a 40x objective (for measuring the density of TH⁺ innervation) with the
231 pinhole was set at 1 Airy unit. TH⁺, GFAP⁺, and Iba-1⁺ cells were manually counted using the point tool in
232 ImageJ software (Image processing and analysis in Java, NIH, USA). The ROI Manager tool in Image-J software
233 was employed to quantify integral optical density of TH, GFAP and Iba-1 expression. All analyses were carried
234 out by an operator blinded to the experimental groups.

235 **RNA isolation, cDNA preparation, and quantitative real-time PCR**

236 Twenty four hours following the completion of behavioral and cognitive tests, RNA was extracted from PFC and
237 hippocampus tissues from control and GBH 500 mg/kg exposed mice ($n = 3$ each). Isolation of the total RNA
238 was carried out using Trizol (Sigma-Aldrich, St. Louis, MO, USA) according to a previously described protocol
239 (Rio et al. 2010). RNA level was quantified by measuring absorbance at 260 and 280 nm. The final RNA
240 concentration and purity was determined using Nanodrop (Thermo Fisher Scientific, Waltham, MA, USA),
241 obtaining absorbance 260/280 ratios between 2 and 2.2 in all the samples. Retrotranscription of 1 μg total RNA
242 was carried out in a 25 μl reaction volume containing: 1x RT-Buffer, 0.1 $\mu\text{g}/\mu\text{l}$ bovine serum albumin (BSA),
243 0.05% Triton, 1 mM dNTPs, 7.5 μM Random Hexamer Primers, 40 U RIBOlock, and 200 U RevertAid®
244 Reverse Transcriptase (all RT ingredients were provided by Thermo Scientific). The reaction was performed 10
245 min at 25°C, 90 min at 42°C, 15 min at 70°C. Quantitative real-time PCR (qRT-PCR) was carried out using an
246 ABI Prism 7300 (Applied Biosystems) detection system. Analyses were performed in technical duplicate and
247 biological triplicate. Data from qRT-PCR experiments were analyzed using the delta cycle threshold (ΔCt)
248 method. The expression levels were normalized to reference gene: TBP (TATA box Binding Protein). For each
249 tissue (PFC, hippocampus), the ΔCt average of control samples was used as calibrator. Primers were designed
250 using Annhyb software (<http://www.bioinformatics.org/annhyb/>) and were synthesized by Invitrogen. Primer
251 sequences are reported in supplementary table 1.

252 **Statistical analysis**

253 Fertility and reproduction parameters as well as maternal behavior were analyzed by one-way ANOVA, while
254 body weight gain and early sensorimotor endpoint results were analyzed using the repeated measure two-way
255 ANOVA (GBH dose and age), followed by a Holm-Sidak's *post hoc* test for multiple comparisons. The dataset
256 of behavioral tests in adult mice, enzyme activity results and histological assays were compared between
257 different groups (treated and control) and analyzed using one-way ANOVA, followed by a Holm-Sidak's *post*
258 *hoc* for multiple comparisons. The biomolecular data were analyzed with t-test. The results are presented as
259 mean \pm S.E.M, and a value of $p < 0.05$ was considered statistically significant. All statistical analyses were
260 carried out using the software SigmaPlot 11.0 for Windows and all graphs were generated with Prism 7.0 for
261 Windows (GraphPad software).

262 **Results**

263 **Gestation outcome and maternal behavior following GBH exposure**

264 Administration of GBH to pregnant females affected fertility and reproduction parameters. Indeed, the fertility
 265 rate and the gestational index were lower in treated groups compared to control. Similarly, both the number of
 266 litters and the total number of mice per litter were significantly lower in the GBH exposed groups. Treatment
 267 with GBH 500 mg/kg, but not with the lower dosage, also affected retrieving and nesting index (table 2). In
 268 contrast, the statistical analysis did not reveal a significant difference in the gestation length between treated and
 269 control groups ($F_{(2,18)} = 1.08, p > 0.05$) (table 1).

270 **Table 1** Reproductive findings in mice given GBH during pregnancy and lactation

	Glyphosate-based herbicide		
	0 (Control)	250 mg/kg	500 mg/kg
No. of females copulated	8	10	24
No. of pregnant females	7	6	6
Fecundity Index (%)	87	60	25
No. of death during pregnancy	0	0	1
Gestation length (days)	19.8 ± 0.37	20 ± 0.54	19.2 ± 0.2
No. of females with live born	7	6	5
Gestation index (%)	100	100	83
No. of females with totally litter loss	0	0	1
No. of litters	7	6	5
Total no. of pups born	56	40	35
No. of pups born alive	51	34	33
No. of dead pups	5	6	2
Delivery index (%)	100	100	100
Lactation index (%)	88.2	79.4	69.6
Nest building index (%)	100	100	80
Retrieving index (%)	100	100	80
% of males	37	45	40

271 Fecundity Index (%) = (no. of pregnant females/ no. of females copulated) x 100.

272 Values are given as the mean ± S.D.

273 Gestation index (%) = (no. of females with live pups born / no. of pregnant females) x 100.

274 Delivery index (%) = (no. of females delivering / no. of pregnant females) x 100.

275 Lactation index (%) = (no. of living offspring on day 21 / no. of offspring born alive) x 100.

276 Nesting index = (no. of females building their nests for a maximum duration of 30 min/ no. of females delivering) x 100.

277 Retrieving index = (no. of females retrieving over pups for a maximum duration of 30 min/ no. of females delivering) x 100.

278 % of males = (no. of males / no. of pups) x 100.

279 **GBH decreased body weight of pregnant females and their offspring**

280 The two-way repeated measures ANOVA showed a significant effect of treatment ($F_{(2,19)} = 11.80, p < 0.01$) and
 281 period of treatment ($F_{(2,19)} = 211.62, p < 0.001$) on dam weights. However, the interaction between the two
 282 factors was not significant ($F_{(2,19)} = 0.95, p > 0.05$). The *post-hoc* comparisons showed that body weight gain was
 283 significantly reduced following GBH treatment at both 250 and 500 mg/kg with respect to the control ($p < 0.05$)
 284 (table 2). Likewise, our results indicated a significant effect of treatment and age on pup's body weight ($F_{(2,19)} =$
 285 $11.80, p < 0.01$; $F_{(2,19)} = 11.80, p < 0.01$, respectively) as well as for the interaction between the two factors
 286 ($F_{(2,19)} = 11.80, p < 0.01$). Furthermore, Holm-sidak comparisons revealed a significant reduction of body weight
 287 gain in offspring delivered from both GBH exposed dams groups ($p < 0.05$) (table 2).

Table 2 GBH affects body weight gain of mice mothers and their offspring

	Glyphosate-based herbicide			<i>Post-hoc</i>					
	0 (Control)	250 mg/kg	500 mg/kg	250 vs control		500 vs control		250 vs 500	
				<i>t</i>	<i>p</i>	<i>t</i>	<i>p</i>	<i>t</i>	<i>p</i>
Pregnancy									
G1	0.78 ± 0.34	-1.22 ± 0.48	-3.66 ± 0.14	3.59	ns	7.98	***	1.94	ns
G7	5.24 ± 0.55	-2.20 ± 0.66	0.24 ± 0.49	5.46	*	8.98	***	1.56	ns
G15	10.38 ± 0.67	8.12 ± 0.55	5.18 ± 0.56	4.06	ns	9.34	***	2.34	ns
G19	16.34 ± 0.65	14.16 ± 0.49	10.48 ± 0.78	3.91	ns	10.53	***	2.93	**
Lactation									
L1	9.22 ± 0.95	5.04 ± 1.19	3.80 ± 0.76	4.34	ns	5.63	*	0.98	ns
L7	10.00 ± 0.93	6.76 ± 1.28	5.52 ± 1.01	3.37	ns	4.66	ns	0.33	ns
L15	11.06 ± 0.66	7.54 ± 1.31	6.30 ± 0.83	3.66	ns	4.95	*	2.80	ns
L19	11.44 ± 0.83	8.44 ± 1.01	7.20 ± 0.51	3.12	ns	4.41	ns	0.33	ns
Litter									
P1	1.31 ± 0.02	1.15 ± 0.07	1.05 ± 0.88	1.02	ns	1.65	ns	0.51	ns
P7	2.51 ± 0.03	2.08 ± 0.14	1.91 ± 0.11	2.68	ns	3.79	ns	0.90	ns
P15	5.47 ± 0.23	4.06 ± 0.22	3.17 ± 0.13	8.92	***	14.52	***	4.59	***
P21	7.56 ± 0.18	6.57 ± 0.24	6.43 ± 0.20	6.23	**	7.10	***	0.71	ns

289 **G** gestation day; **L** lactation day; **P** postnatal day; * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$; ns (no significant) $p > 0.05$

290 **GBH delayed developmental skills of pre- and postnatally exposed offspring**

291 **Righting reflex:** We investigated whether pre- and post-natal exposure to GBH caused atypical sensorimotor
 292 skills development in offspring. Two-way repeated measure ANOVA showed a significant main effect of
 293 treatment ($F_{(2,23)} = 13.83, p < 0.001$) and the expected effect of age, indicating a reduction in righting time as the
 294 mice developed ($F_{(2,23)} = 309.10, p < 0.001$). Notably, GBH treatment delayed the development of the righting
 295 reflex as shown by a significant interaction between the two factors ($F_{(2,23)} = 8.38, p < 0.001$). *Post-hoc* analysis
 296 revealed that mice treated with 250 mg/kg or 500 mg/kg GBH had slower righting reflexes time than the controls
 297 only at P5 (250 vs control mg/kg: $q = 7.45, p < 0.001$; 500 mg/kg vs control: $q = 10.32, p < 0.001$) (Fig. 2a).

298 **Negative geotaxis:** The two-way ANOVA revealed a significant effect of treatment ($F_{(2,23)} = 83.84, p < 0.001$)
 299 and the expected maturation effect reflected by the significant effect of age ($F_{(2,23)} = 59.44, p < 0.001$). The
 300 interaction was also significant ($F_{(2,23)} = 23.41, p < 0.001$). *Post-hoc* comparisons confirmed that mice treated
 301 with 500 mg/kg had higher negative geotaxis time compared to the controls at P5, 7 and 9 ($q = 19.27, p < 0.001$;
 302 $q = 10.45, p < 0.001$ and $q = 5.97, p < 0.01$, respectively) (Fig. 2b).

303 **Cliff avoidance:** A main effect of treatment was found ($F_{(2,23)} = 4.05, p < 0.05$), as well as the expected effect of
 304 age ($F_{(2,23)} = 23.05, p < 0.001$). However, the interaction was not significant ($F_{(2,23)} = 2.40, p > 0.05$). *Post-hoc*
 305 comparisons confirmed that mice treated with 500 mg/kg of GBH had slower cliff avoidance time than controls
 306 only at P 5 ($q = 4.84, p < 0.05$) (Fig. 2c).

307 **Traction test:** One-way ANOVA analysis showed a significant difference between treated and control groups
 308 ($F_{(2,23)} = 21.38, p < 0.001$). *Post-hoc* analysis revealed that mice treated with 250 mg/kg or 500 mg/kg GBH
 309 showed shorter fall down latencies than the controls at P10 (250 mg/kg vs control: $q = 2.41, p < 0.05$; 500 mg/kg

310 vs control: $q = 6.47, p < 0.001$). The difference between the two doses of GBH was also statistically significant
311 (250 mg/kg vs 500 mg/kg: $q = 4.06, p < 0.01$) (Fig. 2d).

312 **Rotarod test:** The two-way ANOVA analysis revealed that the motor coordination was significantly affected by
313 the treatment ($F_{(2,23)} = 13.61, p < 0.001$), as well as by the age of the animals ($F_{(2,23)} = 64.04, p < 0.001$).
314 Likewise, the interaction was significant ($F_{(2,23)} = 4.76, p < 0.001$). Multiple comparisons confirmed that both
315 GBH-treated groups had lower fall down latency than the control group on P23 (250 mg/kg vs control: $q = 4.95,$
316 $p < 0.05$; 500 mg/kg vs control: $q = 8.01, p < 0.001$) and P24 (250 mg/kg vs control: $q = 4.83, p < 0.05$; 500
317 mg/kg vs control: $q = 5.42, p < 0.01$) (Fig. 2e).

318 **Effects of GBH on offspring lasting into adulthood**

319 In order to investigate whether GBH exposure during prenatal and postnatal developmental might translate into
320 long-lasting consequences on adult behavior, we tested the behavioral repertoire of adults' offspring by assaying
321 locomotor activity, anxiety-like phenotype, social interaction as well as different forms of memory.

322 **Open field test**

323 As indicators of locomotor activity and anxiety-like levels, we recorded the total distance traveled over the open
324 field as well as the velocity and the time spent in the central zone of the maze (anxiety index). One-way ANOVA
325 analysis of the total distance traveled and the percentage of the time spent in the central zone revealed significant
326 differences between treated and control groups ($F_{(2,17)} = 3.76, p < 0.05$; $F_{(2,17)} = 50.67, p < 0.001$, respectively).
327 However, no difference was found between groups for the velocity ($F_{(2,17)} = 3.76, p > 0.05$) (Fig. 3c), even
328 though treated mice seemed slower than controls. Multiple comparisons confirmed that the group of 500 mg/kg
329 exhibited significant decrease of distance traveled compared to the control group ($t = 2.69, p < 0.05$) (Fig. 3b).
330 Similarly, *post hoc* analysis revealed that both GBH-treated groups spent significantly less time in the center of
331 the open field compared to the control (250 mg/kg vs control: $t = 6.57, p < 0.001$; 500 mg/kg vs control: $t = 9.88,$
332 $p < 0.001$). In addition, the same analysis revealed a significant difference between treated groups ($t = 3.30, p <$
333 0.01) (Fig. 3d).

334 **Elevated-plus maze**

335 The open field result was confirmed by EPM data. Indeed, One-way ANOVA analysis of the ratio of time spent
336 in the OA and the anxiety index showed a significant difference between treated and control group ($F_{(2,17)} =$
337 $72.38, p < 0.001$; $F_{(2,17)} = 18.17, p < 0.001$, respectively). However, the analysis of the ratio of entries number
338 into the OA did not showed any statistical difference between groups ($F_{(2,17)} = 1.05, p > 0.05$) (Fig. 3g).
339 Multiples comparisons confirmed that the ratio of time spent in the OA was significantly lower in treated groups
340 compared to the control group (250 mg/kg vs control: $t = 9.29; p < 0.001$; 500 mg/kg vs control: $t = 11.26; p <$
341 0.001) (Fig. 3f). Moreover, the anxiety index was significantly higher in treated groups (250 mg/kg vs control: t
342 $= 4.36; p < 0.001$; 500 mg/kg vs control: $t = 5.78; p < 0.001$) (Fig. 3h).

343 **Three-chambered sociability**

344 This test was used to investigate the voluntary social interaction of animals. The data analysis revealed a
345 significant interaction between the wire cup (holding mouse vs object) and treatment ($F_{(2,17)} = 73.53, p < 0.001$).
346 However, there was no significant main effect of both treatment and wire cup ($F_{(2,17)} = 2.00, p > 0.05$; $F_{(2,17)} =$
347 $1.10, p > 0.05$, respectively). *Post-hoc* comparisons revealed that mice treated either with 250 or 500 mg/kg of
348 GBH spent less time with the wire cup holding another conspecific (250 mg/kg vs control: $t = 7.42, p < 0.001$;
349 500 mg/kg vs control: $t = 9.42, p < 0.001$; 250 mg/kg vs 500 mg/kg: $t = 1.99, p < 0.05$) and more time with the
350 wire cup holding the object (250 mg/kg vs control: $t = 4.89, p < 0.001$; 500 mg/kg vs control: $t = 7.06, p <$
351 0.001 ; 250 mg/kg vs 500 mg/kg: $t = 2.16, p < 0.05$) compared to the controls (Fig. 4b). We also found a
352 significant effect of the wire cup factor on the visit number as well as its interaction with the treatment factor
353 ($F_{(2,17)} = 26.25, p < 0.001$; $F_{(2,17)} = 17.89, p < 0.001$, respectively). Multiple comparisons confirmed that only
354 mice exposed to 250 mg/kg showed less visit number of wire cup holding another conspecific ($t = 4.89, p <$
355 0.001) while both GBH-exposed groups showed higher visit number of the wire cup holding the object compared
356 to the controls (250 mg/kg vs control: $t = 4.39, p < 0.001$; 500 mg/kg vs control: $t = 6.32, p < 0.001$) (Fig. 4c).

357 **Passive avoidance**

358 We further examined mice by step-through avoidance learning, an experimental paradigm used for assessing
359 learning, short and long-term memory. The change in the latency to enter into the dark compartment was
360 compared among the three treatment groups and was found to be affected by both treatment and time of test
361 ($F_{(2,17)} = 13.96, p < 0.01$; $F_{(2,17)} = 31.00, p < 0.01$, respectively). The analysis revealed also a significant
362 interaction between the two factors ($F_{(2,17)} = 7.46, p < 0.05$). *Post-hoc* analysis indicated that only the group
363 exposed to 500 mg/kg of GBH showed a significant decrease of latency after 2h (500 mg/kg vs control: $t = 6.32,$
364 $p < 0.001$) while both treated groups exhibited a significant decrease of latency 24 h of after the electrical shock
365 administration (250 mg/kg vs control: $t = 4.39, p < 0.001$; 500 mg/kg vs control: $t = 6.32, p < 0.001$) (Fig. 4d).

366 **Y-maze**

367 The effect of GBH on working memory was evaluated by Y-maze task. One-way ANOVA analysis showed a
368 significant difference in spontaneous activity between treated and control groups ($F_{(2,17)} = 11.50, p < 0.001$).
369 *Post-hoc* comparisons confirmed that the spontaneous alternation in GBH-treated mice was lower than that in
370 control mice (250 mg/kg vs control: $t = 2.14, p < 0.05$; 500 mg/kg vs control: $t = 4.78, p < 0.001$) and this effect
371 was significantly more pronounced in 500 mg/kg than in 250 mg/kg ($t = 2.64, p < 0.001$) (Fig. 4e).

372 **Novel object recognition**

373 This test was used to assess the potential effects of GBH on recognition memory. One-way ANOVA analysis
374 showed a significant difference in both the ratio of time spent beside the new object and the discrimination index
375 ($F_{(2,17)} = 37.70, p < 0.001$). Multiple comparisons revealed that GBH-exposed mice spent less time exploring the
376 novel object and had low discrimination index compared to the controls (250 mg/kg vs control: $t = 5.43, p <$
377 0.001 ; 500 mg/kg vs control: $t = 8.58, p < 0.001$). In addition, the same analysis revealed a significant difference
378 between treated groups ($t = 3.15, p < 0.01$) (Fig. 4f-g).

379 **Biochemical, histological and molecular changes within brain of GBH-exposed mice**

380 ***GBH effects on AChE activity***

381 Because the cholinergic system is closely associated with anxious and cognitive functions (Mineur et al. 2013;
382 Coyle et al. 1983), we sought to assess the impact of GBH exposure on AChE in the supernatants of specific
383 brain areas homogenates. ANOVA analysis showed significant differences between groups in PFC and whole
384 brain ($F_{(2,2)} = 12.78; p < 0.05$; $F_{(2,2)} = 13.93; p < 0.05$, respectively), while no statistical difference was found in
385 the hippocampus ($F_{(2,2)} = 5.79; p > 0.05$) (Fig. 5c). *Post-hoc* comparisons revealed that only the group exposed
386 to 500 mg/kg of GBH showed a significant decrease of AChE activity in the whole brain (500 mg/kg vs control:
387 $t = 5.24, p < 0.05$) (Fig. 5a), while the activity of this enzyme was significantly decreased in PFC for both doses
388 250 and 500 mg/kg doses (250 mg/kg vs control: $t = 4.85, p < 0.05$; 500 mg/kg vs control: $t = 3.65, p < 0.05$)
389 (Fig. 5b).

390 ***GBH effect on dopaminergic system***

391 A growing number of data correlate movement control abnormalities with dopaminergic systems dysfunction
392 (Rodríguez et al. 2013; Gallo et al. 2015). To examine the impact of GBH exposure on dopaminergic neurons,
393 we evaluated the expression of tyrosine hydroxylase (TH), a key enzyme involved in dopamine synthesis.
394 Indeed, the quantitative analysis of TH-immunolabeled cells number in the SNc, in the VTA and in the striatum
395 indicated a significant difference between control and treated groups ($F_{(2,17)} = 17.89, p < 0.001$; $F_{(2,17)} = 14.78, p$
396 < 0.01 ; $F_{(2,17)} = 8.41, p < 0.05$, respectively). *Post-hoc* comparisons confirmed a significant reduction in the
397 number of TH positive cell bodies (TH⁺) in the SNc (250 mg/kg vs control: 3.66, $p < 0.05$; 500 mg/kg vs control:
398 $t = 5.92, p < 0.01$) (Fig. 6b) and in the VTA (250 mg/kg vs control: 4.47, $p < 0.01$; 500 mg/kg vs control: $t =$
399 $4.91, p < 0.01$) of GBH-exposed animals compared to the controls (Fig. 6c). Moreover, the same analysis
400 showed that 500 mg/kg treated group showed a significant decrease in the integral optical density of the TH⁺
401 fibers immunofluorescence in the striatum compared to the control and 250 mg/kg groups (500 mg/kg vs control:
402 $t = 4.07, p < 0.01$; 250 mg/kg vs 500 mg/kg: $t = 2.46, p < 0.05$) (Fig. 6d).

403 ***GBH causes neuroinflammation***

404 Clear evidence showed that organophosphate OPs intoxication is associated with inflammatory responses as
405 revealed the activation of both astrocytes and microglial cells (Banks and Lin 2012). In this perspective, we
406 evaluated neuroinflammation by assessing the expression of both GFAP and Iba-1 proteins in astrocytes and
407 microglial cells, respectively, in different regions of the PFC and the dorsal hippocampus. Interestingly, the
408 quantitative analysis of GFAP and Iba-1 immunofluorescence showed a significant difference among groups.
409 The treated groups showed a significant increase in the number, integral optical density and area occupied by
410 GFAP⁺ and Iba-1⁺ cells in both PFC and hippocampus (Fig. 7-8 and supplementary table 2-3).

411 ***GBH affects the expression of genes associated with neuroinflammation, synaptic plasticity and cell survival***

412 Since one major hallmarks of the inflammatory response is the release of cytokines and chemokines from
413 activated glial cells, we measured the mRNA expression of tumor necrosis factor alpha (TNF α) by quantitative
414 real time PCR (qRT-PCR). The statistical analysis showed significant increased levels of TNF α mRNA
415 expression in the hippocampus of exposed mice compared to controls ($t = 5.26, p < 0.05$) while no statistical
416 difference was found in the PFC ($t = 0.41, p > 0.05$) (Fig. 9a).

417 It is well reported that the activation of NMDA glutamate receptors (NMDARs) is crucial for synaptic plasticity
418 underlying both short- and long-term memory storage, and that the disruption of these receptors function can
419 cause profound cognitive deficits (Lewis 1997; Malenka and Nicoll 1999). Moreover, the interaction between
420 proinflammatory cytokines and the glutamatergic neurotransmission has been previously described (Fogal and
421 Hewett 2008). Indeed, excessive activation of NMDA receptors leads to elevated calcium influx into cells, which
422 perturbs mitochondria and increases generation of reactive oxygen species, inflammation leading ultimately to
423 neurodegeneration. To test whether GBH could also impact glutamatergic signaling, we assessed the expression
424 level of genes encoding for different NMDA receptors subunits. Our results showed that GBH induced
425 significant increase in the mRNA expression of NR1 subunit in the PFC ($t = 4.92, p < 0.01$) (Fig. 9b) while no
426 significant differences could be observed for both NR2A and NR2B subunits in either the PFC or the
427 hippocampus ($p > 0.05$) (Fig. 9c-d).

428 The role of various memory associated neurotrophins such as brain derived neurotrophic factor (BDNF), has
429 also been suggested in memory dysfunction (Alonso et al. 2005). Based on our data related to cognitive deficits,
430 we assessed the impact of GBH exposure on the expression of brain-derived neurotrophic factor (BDNF) and its
431 receptor tyrosine regulated kinase B (TrkB) within the PFC and hippocampus. The analysis showed a
432 statistically significant decreasing effect of GBH on the expression of BDNF ($t = 3.76, p < 0.05$) and a
433 significant increasing effect on the expression of its receptor TrkB ($t = 3.08, p < 0.05$) in the cortices of treated
434 mice (Fig. 9 e-f). However, no statistical significant differences were found in the hippocampi ($t = 1.65, p >$
435 $0.05; t = 0.05, p > 0.05$, respectively) (figure 9 e-f).

436 **Discussion**

437 In the present study we characterized the long-term effects of GBH exposure, which may interfere with brain
438 development and lead to permanent abnormalities. Thus, we developed an experimental murine model of chronic
439 pre- and postnatal exposure to GBH and examined the downstream effects at the behavioral, histological and
440 molecular levels in pups and adult mice.

441 ***GBH affects reproduction parameters***

442 Our results showed that continuous exposure to GBH during pregnancy and lactation affected fertility and
443 reproduction in mouse females. Furthermore, GBH exposure exerted negative effects also on maternal behavior.
444 Reproduction and fertility toxicity observed in this work are in agreement with the results published in a report
445 of the World Health Organization (WHO, 1994). Indeed, exposure of 3500 mg/kg of Gly in rats from day 6 to 19
446 of pregnancy caused increased maternal mortality, augmented incidence of early resorptions, and decreased
447 number of implantations and viable fetuses (WHO, 1994). Although the precise reprotoxicity mechanism is yet
448 to be clarified, it has been previously reported that Gly targets two crucial steps of steroidogenesis in mammals:
449 at the first rate-limiting level of mitochondrial cholesterol transport (Walsh et al. 2000), and at the last
450 irreversible conversion of sexual androgens into estrogens, via a direct action on the aromatase enzyme
451 (Richard et al. 2005).

452 ***GBH delayed sensorimotor development***

453 So far, it has been shown that GBH is able to cross the placental barrier which could possibly alter the
454 developmental process of the fetus (Mose et al. 2008). More recently, the report published by the non-
455 governmental american organization "Moms Across America" suggested that detection of relatively high levels
456 of Gly in breast milk in three out of 10 women raised a bioaccumulation problem (Honeycutt and Rowlands
457 2014) and therefore posed potential risks for breastfed offspring. Our results support these hypotheses: early
458 after birth, we found that GBH-exposed offspring displayed delayed developmental reflexes likely related to
459 abnormal maturity of sensorimotor, vestibular and/or proprioceptive functions (Secher et al. 2006; Santillán et al.
460 2010). In the present work, we also highlighted that offspring prenatally exposed to GBH show a significantly
461 shorter latency to fall from the rotarod than controls which could be to alterations in the cerebellar function,
462 since this center is heavily involved in the rotarod performance (Hamm et al. 1994). The reduction in the motor
463 coordination could also be due to a failure in neuromuscular maturity as mentioned by Perez-Reyes *et al.* (1998).
464 This suggestion is further reinforced by our results obtained through the suspension test showing reduced muscle
465 strength in exposed animals. In sum, the pattern of developmental deficits emerged from this study is in
466 agreement with previous human and animal studies showing similar reflexes defects (Engel et al. 2011;
467 Laugeray et al. 2014; Lan et al. 2017).

468 ***GBH reduces locomotor activity and affects dopaminergic system***

469 Normal development of neonatal reflexes can be considered as an index of brain maturation, and late acquisition
470 of these milestones represent a predictive factor of other behavioral changes in adulthood. In line with this idea,
471 our results show that adult progeny from GBH treated groups presented locomotor hypoactivity which is in
472 agreement with Gallegos et al. (2016), showing that rats exposed to 200 mg/kg of GBH during pregnancy and
473 lactation are hypoactive, and with our previous observations in juvenile mice (Ait bali et al. 2017). Albeit
474 locomotor hypoactivity was previously reported following prenatal exposure to GBH (Gallegos et al. (2016), the
475 neuronal basis still unclear. Thus, to generate a mechanistic understanding whereby GBH produces locomotor

476 hypoactivity, we assessed the outcomes of GBH exposure on dopaminergic system. The nigrostriatal pathway,
477 crucial in movement control, has been shown to be vulnerable to herbicides (Thiruchelvam et al. 2000;
478 Rodríguez et al. 2013). Considering the idea that reduction in locomotor activity is positively correlated with loss
479 of dopaminergic neurons in the SNc (Bano et al. 2014, Gallo et al. 2015), one of the most intriguing findings of
480 our work is that GBH-exposed mice show a robust decrease in the number of dopaminergic neurons, both in the
481 SNc and VTA, and fibers, in the striatum. It should be noted that since pesticide is used in commercial
482 formulation which combine an active ingredient with adjuvants, the toxicity exerted by GBH cannot therefore be
483 exclusively due to the active ingredient but either to the toxicity of adjuvants or to the possible synergy between
484 Gly and the other formulation ingredients (El-Shenawy, 2009, Mesnage et al., 2013). The decrease of
485 dopaminergic cells we observed may be due to apoptotic events triggered by elevated oxidative stress observed
486 after Gly exposure (Astiz et al. 2009). Our results are in agreement with Hernandez-Plata et al. (2015) showing
487 that intraperitoneal exposure to 150 mg/kg of Gly for 2 weeks produces a decrease of DA level in the striatum of
488 adult rats that is associated with hypoactivity. Interestingly, human reports support the central effects of GBH on
489 basal ganglia circuits following intoxication with commercial formulations of Gly, described as alterations in the
490 GP and SNc closely related to Parkinsonian syndrome (Barbosa et al. 2001). Finally, although the hypoactivity
491 induced by Gly administration is similar to that observed after administration of a DA antagonist (Hernandez-
492 Plata et al. 2015), Gly can affect other neurotransmitter systems involved in motor control (Martinez et al. 2018).
493 Quantitative analysis of serotonin, dopamine and norepinephrine levels confirmed a dramatic loss of these
494 neurotransmitters mainly in the striatum, PFC and hippocampus of rats exposed to 35, 75, 150 or 800 mg/kg of
495 Gly (Martinez et al. 2018), strongly indicating that further studies are needed to decipher the contribution of
496 these systems to GBH-produced hypoactivity.

497 ***GBH increase anxiety level and reduces social behavior***

498 Our data clearly showed that early exposure to GBH increases anxiety levels, which is consistent with another
499 study (Baier et al. 2017) in which Gly was administered to adult mice by intranasal irrigation. In contrast,
500 Gallegos et al. (2016) reported that rats exposed to 100 or 200 mg/kg of GBH (Glifloglex®) during pregnancy
501 and lactation experienced low levels of anxiety in adulthood. The difference observed between our results and
502 those of Gallegos et al. (2016) could be explained by the diversity of the commercial formula of Gly
503 administered in the two studies – i.e.: different chemical composition in terms of adjuvants. Indeed, it has been
504 pointed out that the herbicidal activity of Gly is potentiated by the presence of adjuvants (Mitchell et al. 1987),
505 thus supporting their potential role in the differential effects of Gly on anxiety levels.

506 It is well established that neuroinflammation expressed by overproduction of proinflammatory cytokines and
507 excessive activation of glia plays an important role in the etiology of many psychiatric disorders including
508 anxiety (Holloway-Erickson et al. 2012). Accordingly, the neuroinflammatory condition that we report in GBH-
509 treated mice, associated with increased anxiety levels, supports this idea. The PFC is strongly involved in the
510 expression of behavioral and autonomic responses to emotionally relevant stimuli, and imaging studies highlight
511 abnormalities in the structure and function of this region in patients with mood disorder (Kennedy et al. 2001;
512 Drevets et al. 2008). We hypothesized that GBH-induced neuroinflammation of this cortical area could lead to
513 cellular disorganization within the PFC since we already observed that GBH exposure induced loss of
514 serotonergic fibers within this structure in young mice (Ait bali et al. 2017). Nevertheless, the outcomes of
515 neuroinflammation within the PFC was not investigated in the present work and future studies will be necessary

516 to completely understand the mechanism whereby GBH-induced neuroinflammation produces functional
517 alteration of the PFC.

518 Our results show that perinatal exposure to GBH has a strong effect on the social skills of adult mice, as reflected
519 by significant alterations in the three-chamber test, an analysis conventionally used to test sociability also in
520 murine models of autism (Silverman et al. 2010). Despite that mice are a social species, engaging in high levels
521 of social interactions and sexual/parenting behaviors (Arakawa et al. 2008), perinatally GBH-exposed mice are
522 unable to distinguish a social partner from a new object in adulthood, indicating that Gly effects on social
523 behavior are long-lasting. Our results are in agreement with those of Laugeray et al. (2014) who found that
524 exposure to another OP, glufosinate, during pregnancy and suckling periods reduced sociability in mice. There is
525 evidence that the early social environment (e.g.: maternal care and interactions) has a profound impact on the
526 offspring ability to develop normal social skills (Branchi et al. 2013). Our results showing that GBH
527 significantly alters maternal weight gain, nest building capacity, and retrieving behavior suggest that atypical
528 sociability observed in GBN-exposed mice could be, at least in part, due to physiological or behavioral deficits
529 induced in the mothers. However, the cellular origins and circuit mechanisms contributing to these phenotypes
530 have yet to be identified, a future goal that will shed light into the pathogenic mechanisms that underlie social
531 interaction impairment after GBH exposure.

532 ***GBH induces cognitive and cholinergic system impairment and triggers neuroinflammation***

533 The current study showed that GBH affects recognition, working and contextual memory. These results are
534 similar to those recently published by our laboratory in juvenile mice exposed to the same doses of GBH (Bali et
535 al. 2019), and support those of Gallegos et al. (2018) revealing a significant impairment of recognition memory
536 in adult rat prenatally exposed to 100 or 200 mg/kg of GBH. These results are in agreement with clinical reports
537 showing that populations accidentally exposed to the Gly have developed short and long-term memory
538 impairments accompanied with hippocampal lesion (Barbosa et al. 2001, Nishiyori et al. 2014). Human and
539 animal studies have implicated the central cholinergic system as an important regulator of cognitive functions
540 such as learning and memory. Indeed, brain cholinergic hypofunction associated with memory deficits was
541 previously observed in patients with Alzheimer's disease (Coyle et al. 1983). Similarly, learning and cognitive
542 defects paralleled with cholinergic alteration were recorded in different animal models (for review, see Voorhees
543 et al. 2017). In agreement with these studies, our results showed that GBH triggers cholinergic crisis expressed
544 by significant decrease of AChE activity in the PFC and the whole brain. Although Gly is a weak inhibitor of the
545 AChE activity (Larsen et al., 2016), the results of the present work corroborate the previous data from our
546 laboratory showing that GBH reduced AchE activity in young mice (Bali et al. 2019). These findings support the
547 previous evidence showing that the commercial formulation is more toxic than Gly (Folmar et al., 1979; Richard
548 et al., 2005). It is well known that in addition to its involvement in adult neurotransmission by its catalytic
549 functions (hydrolysis of acetylcholine), AChE also plays a morphogenic role during the development of the
550 nervous system (Grisaru et al. 1999). Therefore, inhibition of AChE activity observed following GBH exposure
551 could interfere with the morphogenic role of this enzyme and would be the main mechanism inherent to the
552 neurobehavioral alterations induced by these compounds. Furthermore, the cognitive impairments associated
553 with the AChE inhibition following exposure to GBH could be also explained by over-stimulation and persistent
554 activation of both muscarinic and nicotinic receptors due to the accumulation of acetylcholine in the synaptic
555 cleft (Scheffel et al. 2018). This finding might offer a partial explanation for the complaints on memory loss

556 reported by workers chronically exposed to OPs compounds even though the neurotoxicity of Gly or GBH may
557 not be entirely due to disturbances of the cholinergic system.

558 It is recognized that the co-operation of the PFC and the hippocampus is vital for fundamental cognitive
559 functions and that disconnection or damage to either one of these two brain regions induces impaired cognitive
560 behaviors (Yoon et al. 2008). Furthermore, evidence linking inflammation to neurodegeneration and cognitive
561 defects (Dziedzic, 2006) suggests the possibility that induction of inflammation by chronic exposure to OPs may
562 be mechanistically related to deficits in cognitive ability. Based on these, our morphological analysis showed an
563 increase in the GFAP expression in the PFC and hippocampus of adult progeny from GBH treated groups.
564 Likewise, GBH elevated the expression of Iba-1 in PFC and hippocampus indicating that microglia, as the most
565 sensitive immune cells in the brain, was activated after GBH exposure. In addition, our study demonstrated a
566 significant increase in the expression of proinflammatory cytokines TNF α in the PFC and hippocampus after
567 GBH exposure. These results indicate that repeated exposure to GBH can trigger neuroinflammation response in
568 PFC and hippocampus of exposed mice. In agreement with our results, several previous studies elaborated that
569 exposure to OPs induced glial cells activation as well as cytokine and chemokine elevation contributing to
570 neuronal damage in the hippocampus and piriform cortex. Indeed, soman (an OP nerve agent) intoxication
571 upregulates the GFAP expression and activates microglia in many regions of the brain, including the
572 hippocampus (Angoa-Perez et al. 2010). Similarly, chronic administration of the chlorpyrifos increases GFAP
573 expression in rat hippocampus (Lim et al. 2011).

574 ***GBH increase the expression of glutamatergic receptor and affects cell survival pathway***

575 There is evidence that neuroinflammation exacerbates neuronal damage due to excitotoxicity via interactions
576 between proinflammatory cytokines and glutamatergic pathways (Fogal and Hewett 2008). Indeed, excessive
577 activation of NMDA receptors leads to elevated calcium influx into cells, which perturbs mitochondria and
578 increases generation of reactive oxygen species ultimately leading to cells death. Our biomolecular analysis
579 corroborates these findings showing that, in addition to neuroinflammation event, GBH increases the transcript
580 level of NR1 subunit of NMDA receptors in the PFC. Then, it would seem that GBH could facilitates and
581 enhances NMDA activation either through: 1) increase of NMDA receptors expression, 2) excessive release of
582 glutamate by activated astrocytes or 3) reduction of glutamate uptake and metabolism within glial cells,
583 associated with an increased release of this neurotransmitter in the synaptic cleft (Cattani et al. 2014). Taken
584 together, our data present additional mechanistic bases to explain excitotoxic condition associated with cognitive
585 impairments observed following GBH exposure.

586 In the PFC and hippocampus, signaling through the BDNF pathway plays an important role in the survival,
587 maintenance and growth and promotes neuronal plasticity and neurogenesis, processes that have been described
588 as potential cellular mechanisms for learning and memory (Sakata et al. 2013). Our results showed that mice
589 exposed to GBH display decreased BDNF levels in PFC, which is coupled with impaired cognitive impairments.
590 In the brain, BDNF binds to TrkB receptor causing its phosphorylation and subsequent activation of the
591 intracellular signaling cascade to promote synaptic plasticity (Cunha et al. 2010). Our study demonstrated that
592 the GBH increased TrkB transcript in the PFC. Therefore, the increased TrkB mRNA level we observed in the
593 PFC after exposure to GBH may reflect a compensatory mechanism to balance reduction of BDNF expression.
594 In agreement with our results, Jain et al. (2013) demonstrated that chronic exposure to Trizophos, another OP,

595 significantly reduced the mRNA expression and protein levels of BDNF in rat hippocampi correlated with
596 learning and memory deficits (Jain et al. 2013).

597 In summary, our results show that GBH has pervasive harmful effects when administered during the highly
598 sensitive pre- and postnatal periods. Our results indicate that GBH exposure induces multiple behavioral
599 abnormalities involving motor, emotional, social and cognitive functions and targets CNS integrity, affecting
600 cholinergic, dopaminergic and glutamatergic systems as well as neuroinflammation and cellular stress induction.
601 These results strongly shed light on additional (noncholinergic) mechanisms mediating neurological injury
602 induced by this OP pesticide. All data presented about the behavioral changes as well as brain abnormalities
603 induced by GBH pave the way for further analyses: brain microarray analysis is needed to gain a better
604 understanding regarding the molecular and cellular mechanisms involved in the neurodevelopmental effects of
605 GBH. Such analysis will, with no doubt, contribute to our knowledge of the constellations of genes involved in
606 pathological processes mediated by pre- and postnatal exposure to GBH.

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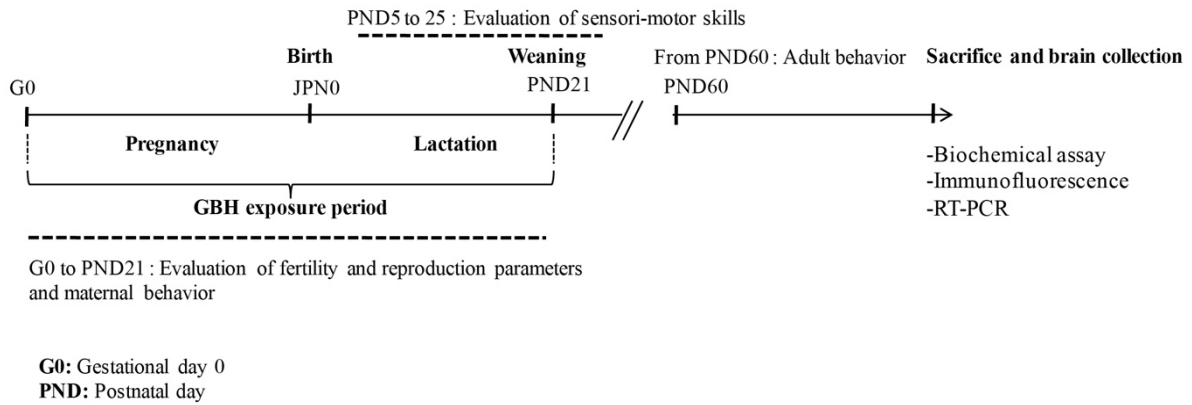
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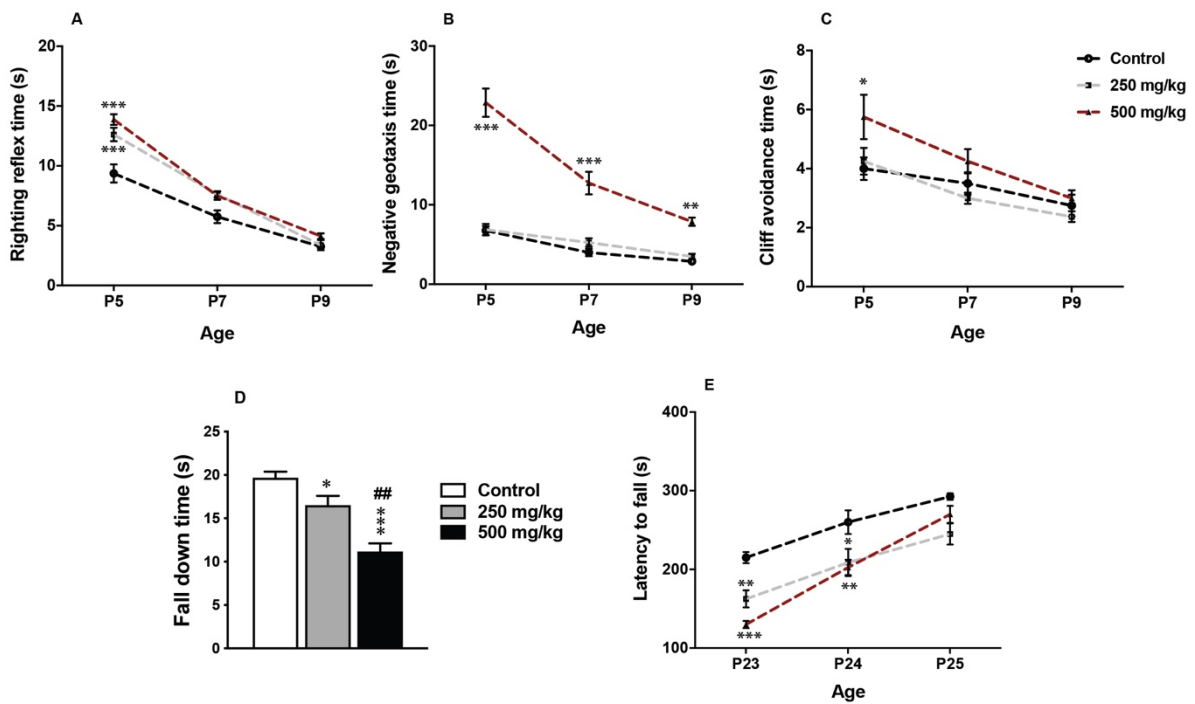
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890 **Figures**



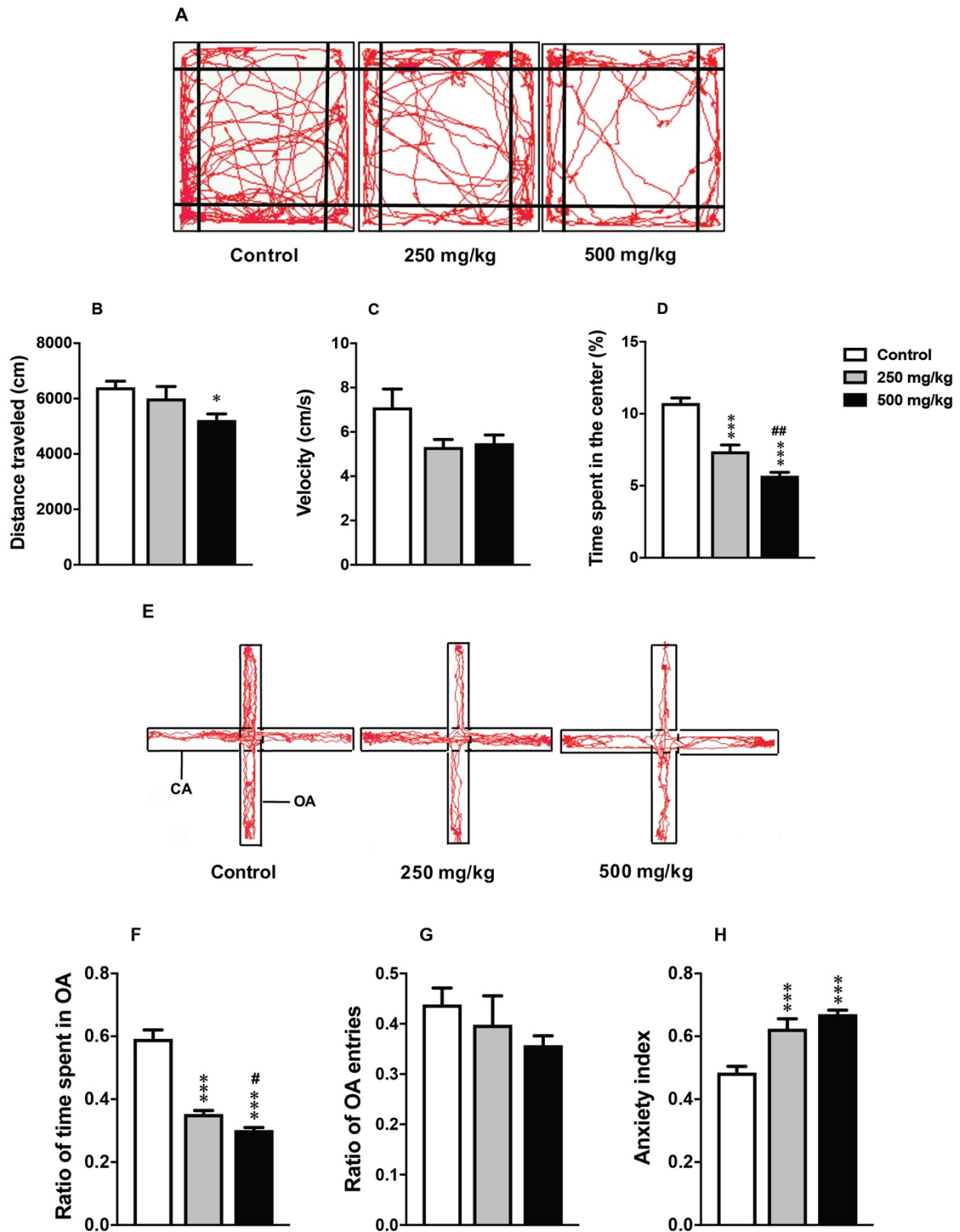
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892 **Fig. 1** Experimental protocol



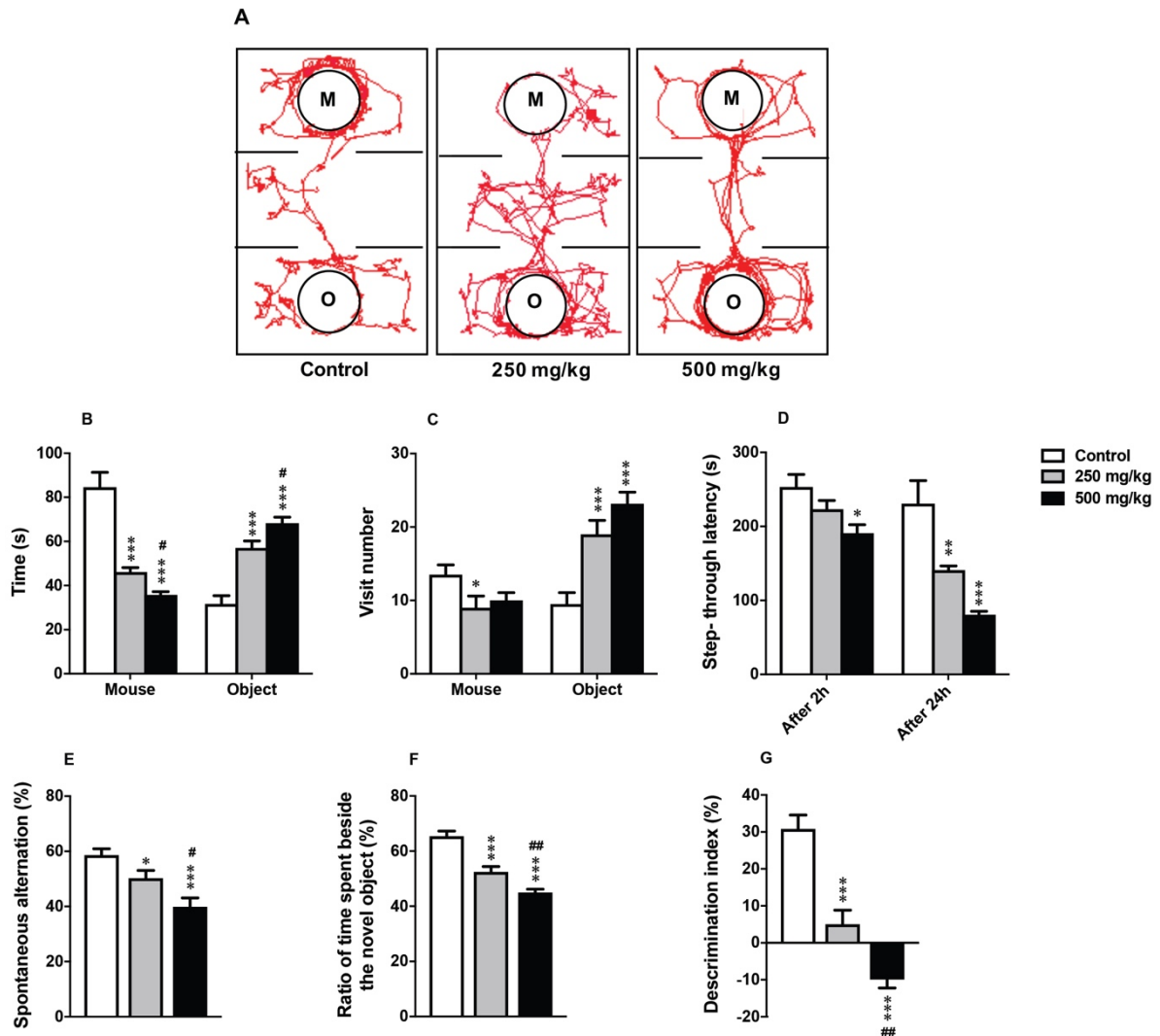
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894 **Fig. 2** Pre-and postnatal GBH exposure resulted in neurodevelopmental endpoints changes. **a** Righting reflex test. **b** Negative
895 geotaxis test. **c** Cliff avoidance test. **d** Traction test. **e** Rotarod test. Results are presented as mean ± SEM. * $p < 0.05$; ** $p <$
896 0.01 ; *** $p < 0.001$. The “*” refers to 250 mg/kg or 500 mg/kg vs control groups comparison

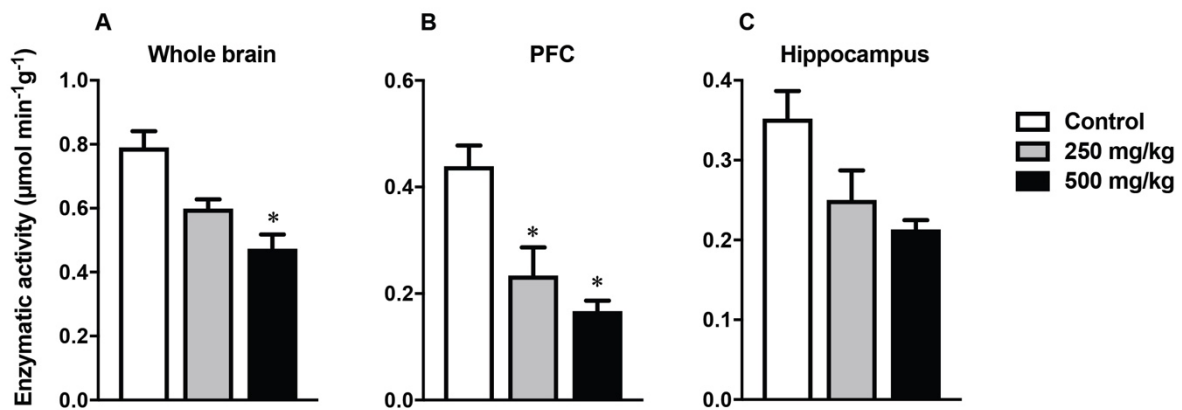


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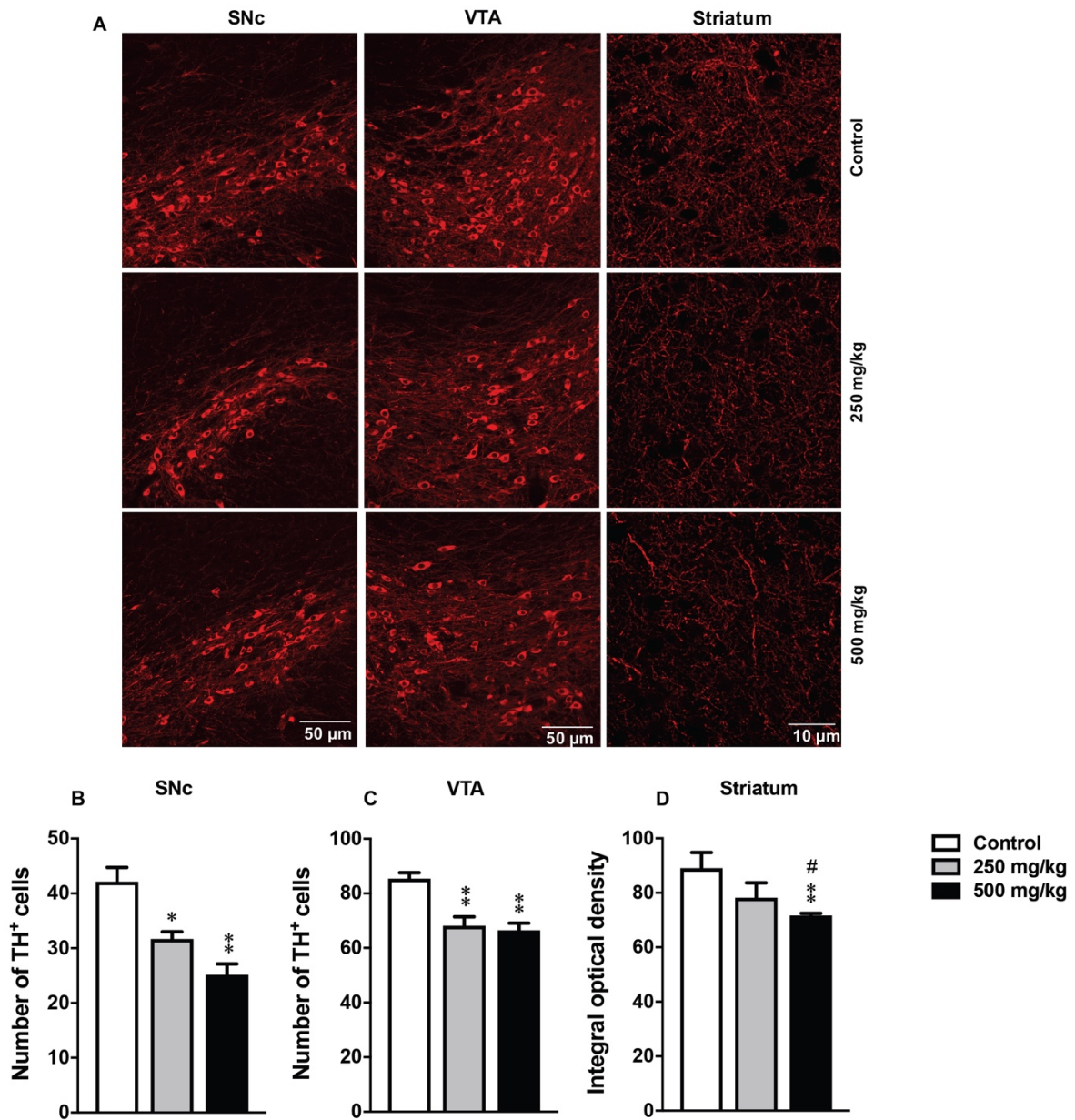
Fig. 3 Pre- and postnatal GBH exposure resulted in behavioral alterations in the offspring during adulthood. **a** Recording of the trajectory of in the OF test. **b-d** Effect of GBH on locomotor activity and anxiety-like phenotype in the open field test. **e** Recording of the trajectory of mice in the EPM test. **f-h** Effect of GBH on anxiety-like phenotype in EPM test. Results are presented as mean \pm SEM. * $p < 0.05$; *** $p < 0.001$; # $p < 0.05$; ## $p < 0.01$. The “*” refers to 250 mg/kg or 500 mg/kg vs control groups comparison and the “#” refers to the 250 mg/kg vs 500 mg/kg groups comparison. OA open arm; CA closed arm



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 905 **Fig. 4** Pre- and postnatal GBH exposure resulted in sociability and cognitive alterations in the offspring during adulthood. **a**
 906 Recording of the trajectory in the TCS test. **b-c** Effect of GBH on social interaction in the TCS test. **d** Effect of GBH on short
 907 and long-term memory in the PA test. **e** Effect of GBH on working memory in the Y-maze test. **f-g** Effect of GBH exposures
 908 on recognition memory in the NOR test. Results are presented as mean \pm SEM. * p < 0.05; ** p < 0.01; *** p < 0.001; # p <
 909 0.05; ## p < 0.01. The “*” refers to 250 mg/kg or 500 mg/kg vs control groups comparison and the “#” refers to the 250 mg/kg
 910 vs 500 mg/kg groups comparison. **M** mouse; **O** object

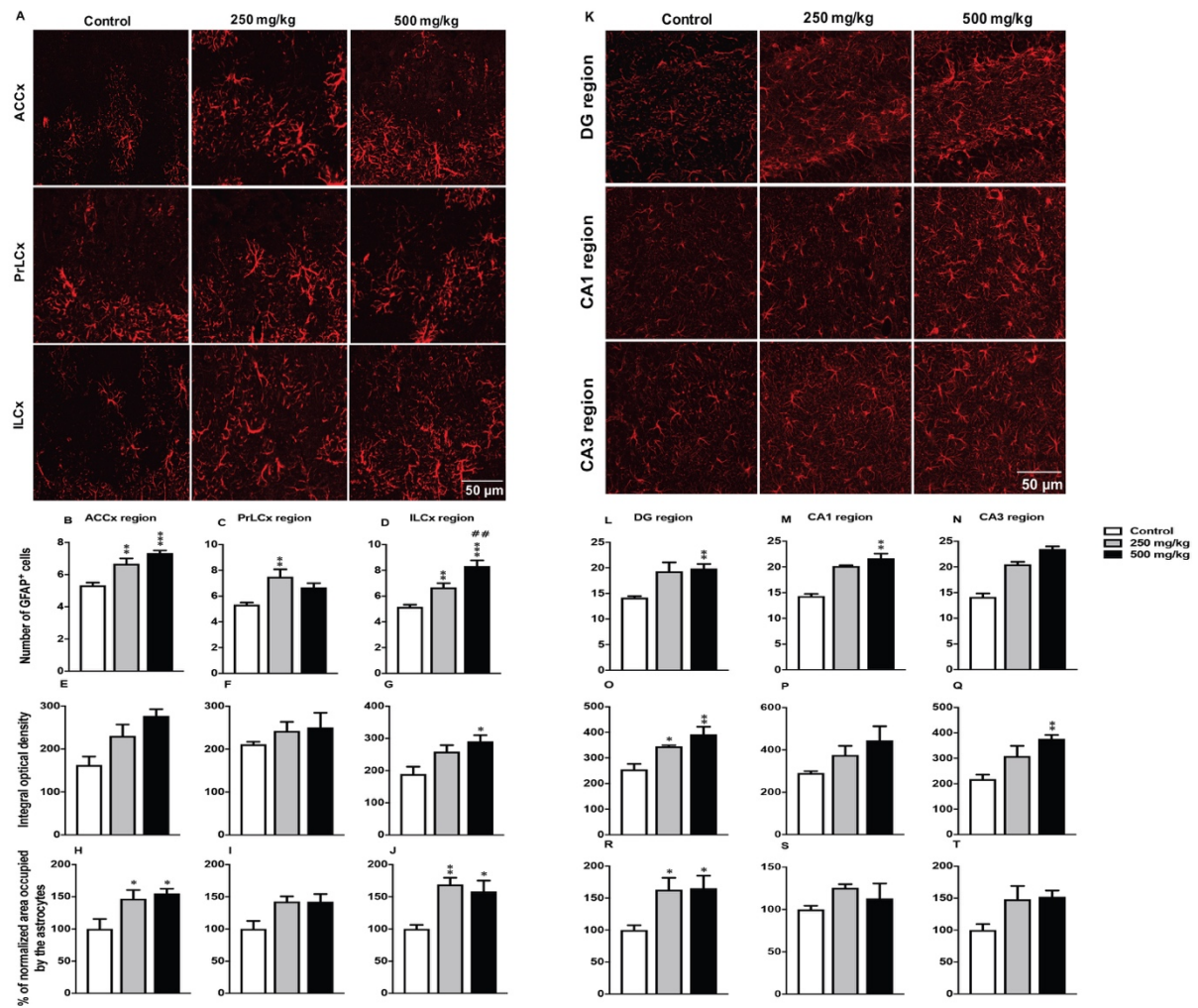


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 912 **Fig. 5** Pre- and postnatal GBH exposure resulted in AChE inhibition. **a** whole brain. **b** PFC. **c** hippocampus. Results are
 913 presented as mean \pm SEM. * p < 0.05. The “*” refers to 250 mg/kg or 500 mg/kg vs control group comparison

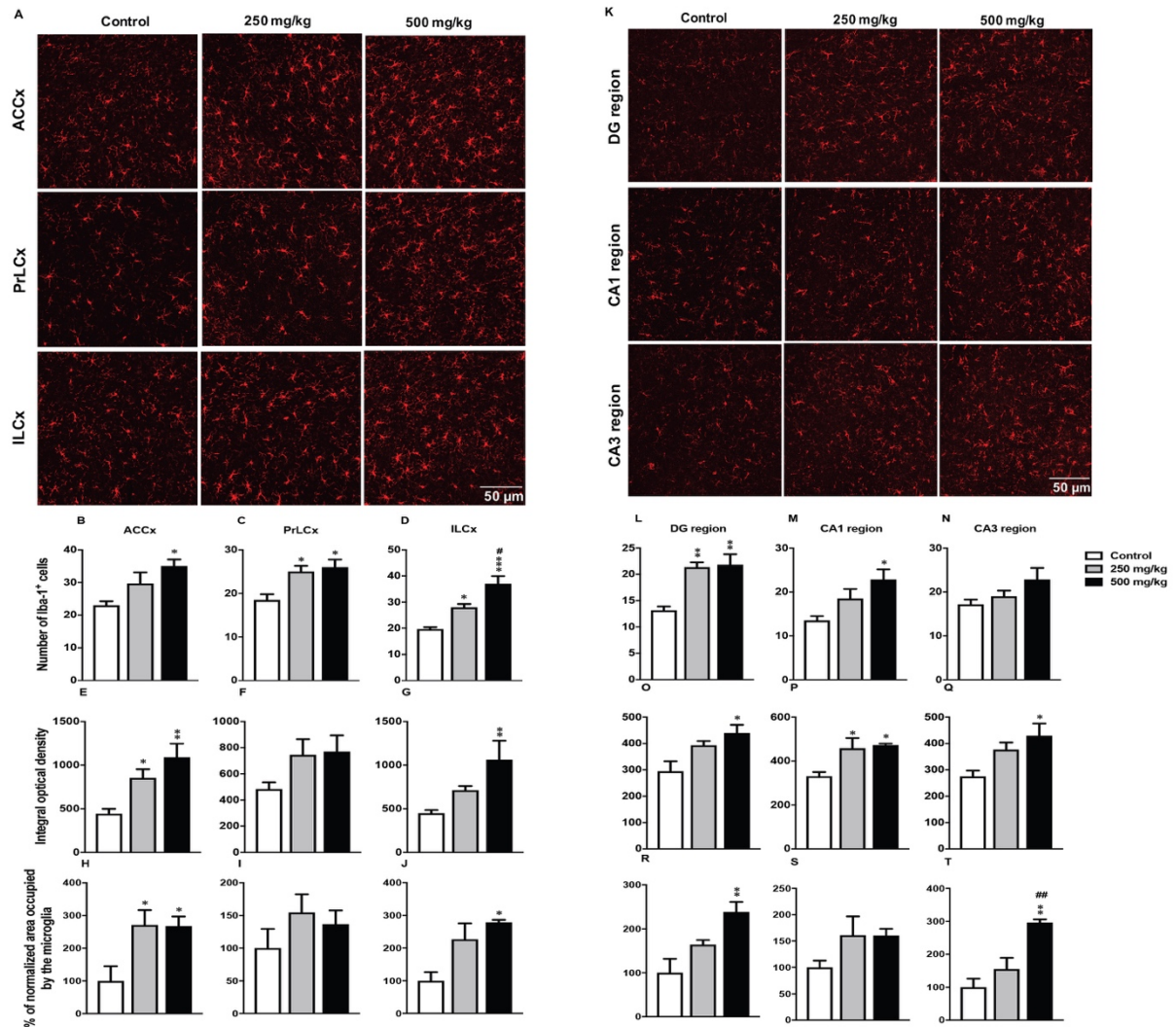


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Fig. 6 Pre- and postnatal GBH exposure resulted in dopaminergic circuit defects. **a** Photomicrographs of mice brain cross sections showing the tyrosine hydroxylase (TH)-immunoreactive neurons. **b** Count of TH positive cells in the SNc and **c** in the VTA. **d** The density of TH Immunoreactivity in the striatum. Results are presented as mean \pm SEM. * $p < 0.05$; ** $p < 0.01$; # $p < 0.05$. The “*” refers to 250 mg/kg or 500 mg/kg vs control groups comparison and the “#” refers to the 250 mg/kg vs 500 mg/kg groups comparison. SNc substantia nigra pars compacta; VTA ventral tegmental area

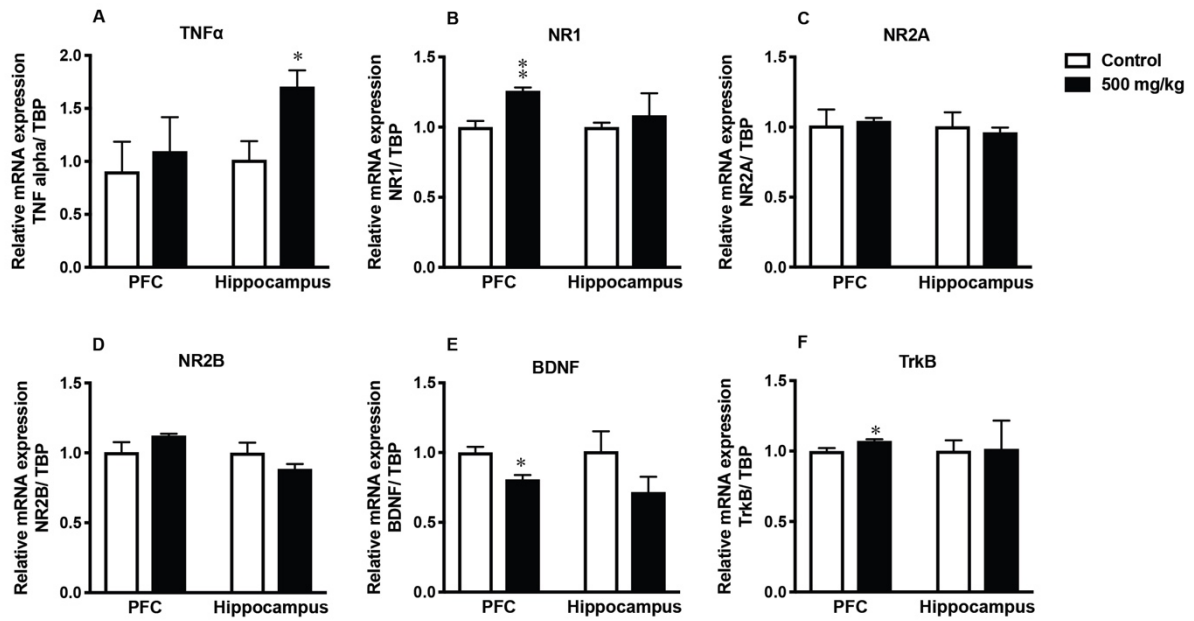


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 921 **Fig. 7** Pre- and postnatal GBH exposure resulted in reactive astrocytes. **a** and **k** Micrographs showing the expression of
 922 GFAP by immunofluorescence in the PFC and hippocampus. **b-d** Count of GFAP positive cells in the PFC and **l-n** in the
 923 hippocampus. **e-g** The integral optical density of GFAP positive cells in the PFC and **o-q** in the hippocampus. **h-j** Area
 924 occupied by GFAP positive cells in the PFC and **r-t** in the hippocampus. Results are presented as mean \pm SEM. * $p < 0.05$;
 925 ** $p < 0.01$; *** $p < 0.001$; ### $p < 0.01$. The “*” refers to 250 mg/kg or 500 mg/kg vs control groups comparison and the “#”
 926 refers to the 250 mg/kg vs 500 mg/kg groups comparison. ACCx anterior cingulate cortex; PrLCx prelimbic cortex; ILCx
 927 infralimbic cortex; DG dentate gyrus; CA1 Ammon’s horn 1; CA3 Ammon’s horn 3



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Fig. 8 Pre- and postnatal GBH exposure resulted in reactive microglia. **a** and **k** Micrographs showing the expression of Iba-1 by immunofluorescence in the PFC and hippocampus. **b-d** Count of Iba-1 positive cells in the PFC and **l-n** in the hippocampus. **e-g** The integral optical density of Iba-1 positive cells in the PFC and **o-q** in the hippocampus. **h-j** Area occupied by Iba-1 positive cells in the PFC and **r-t** in the hippocampus. Results are presented as mean \pm SEM. * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$; # $p < 0.05$; ## $p < 0.01$. The “*” refers to 250 mg/kg or 500 mg/kg vs control groups comparison and the “#” refers to the 250 mg/kg vs 500 mg/kg groups comparison. ACCx anterior cingulate cortex; PrLCx prelimbic cortex; ILCx infralimbic cortex; DG dentate gyrus; CA1 Ammon’s horn 1; CA3 Ammon’s horn 3



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Fig. 9 Pre- and postnatal GBH exposure resulted in genes expression in the PFC and hippocampus of adult progeny. **a** qRT-PCR analysis of TNF α transcript. **b-d** qRT-PCR analysis of NMDA receptor subunits transcripts. **e** qRT-PCR analysis of BDNF and **f** its receptor TrkB transcripts. For each tissue (PFC, Hippocampus), the gene expression is shown relatively to its control samples. Results are presented as mean \pm SEM. * p < 0.05; ** p < 0.01. The “*” refers to 250 mg/kg or 500 mg/kg vs control groups comparison

944 **Supplementary data**945 **Table 1** Primers for quantitative real time PCR analysis

Gene	N° Genbank	Forward primer (5'-3')	Reverse primer (5'-3')	(Size)
BDNF	NM_001285416.1	GCGTGTGTGACAGTATTAGCGAGTG	CAGTTGGCCTTTGGATACCGGG	116
TrkB	NM_001282961.1	GAAAAACAGCAACCTGCGGCAC	GAACGGATTACCCGTCAGGATCAGG	115
NR1	NM_001177657.2	CGTCCTGGGGCTGACTACCC	GCTGGACTGGTGGGAGTAGGG	97
NR2A	NM_008170.2	GACCCACTGACTGAGACCTGCG	CCCCTTGCAGCACTTCTTCACATTC	108
NR2B	NM_008171.3	GAACAAGGAGAGGAAGTGGGAGAGG	CAGTCTCAGGACACATTTCGAGGCC	95
TNFα	NM_013693.3	CTCAGCCTCTTCTCATTCTGCTTG	GGCCATTTGGGAACCTTCTCATCCC	106
TBP	NM_013684.3	GATCAAACCCAGAATTGTTCTCC	GGGGTAGATGTTTTCAAATGCTTC	106

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948 **Table 2** Statistical analysis of GFAP data

		ANOVA		Post-hoc					
				250 vs control		500 vs control		250 vs 500	
		F(2,2)	p	t	p	t	p	t	p
PFC	Number of GFAP⁺ cells								
	ACCx	18.66	**	4.00	**	6.00	***	2.00	Ns
	PrLCx	7.58	*	3.86	**	2.37	Ns	1.48	Ns
	ILCx	22.58	**	3.18	*	6.71	***	3.53	*
	Integral optical density								
	ACCx	7.39	*	2.27	Ns	3.82	**	1.55	Ns
	PrLCx	0.78	Ns	--	--	--	--	--	--
	ILCx	6.27	*	2.36	Ns	3.44	*	1.08	Ns
	Occupied surface								
	ACCx	5.58	*	2.63	*	3.09	*	0.46	Ns
	PrLCx	4.98	Ns	--	--	--	--	--	--
	ILCx	9.38	*	4.02	**	3.39	*	0.63	Ns
Hippocampus	Number of GFAP⁺ cells								
	DG	7.38	*	3.16	*	3.47	*	0.30	Ns
	CA1	41.56	***	6.86	***	8.62	***	1.76	Ns
	CA3	72.11	***	7.98	***	11.7	***	3.78	**
						6			
	Integral optical density								
	DG	10.96	*	3.01	*	4.61	**	1.60	Ns
	CA1	2.81	Ns	--	--	--	--	--	--
	CA3	8.76	*	2.37	Ns	4.17	**	1.79	Ns
	Occupied surface								
	DG	5.27	*	2.76	*	2.86	*	0.10	Ns
	CA1	1.44	Ns	--	--	--	--	--	--
CA3	4.04	Ns	--	--	--	--	--	--	

949 * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$; ns: no significant ($p > 0.05$).

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951 **Table 3** Statistical analysis of Iba-1 data

		ANOVA		<i>Post hoc</i>					
				<i>250 vs control</i>		<i>500 vs control</i>		<i>250 vs 500</i>	
		<i>F</i> _(2,2)	<i>p</i>	<i>t</i>	<i>p</i>	<i>t</i>	<i>p</i>	<i>t</i>	<i>p</i>
PFC	Number of Iba-1⁺ cells								
	ACCx	6.24	*	1.96	Ns	3.52	*	1.56	Ns
	PrLCx	7.55	*	3.10	*	3.58	*	0.47	Ns
	ILCx	20.57	**	3.09	*	6.44	***	3.34	*
	Integral optical density								
	ACCx	8.41	*	2.58	*	4.05	**	1.47	Ns
	PrLCx	2.28	Ns	--	--	--	--	--	--
	ILCx	9.67	*	2.11	Ns	4.39	**	2.50	Ns
	Occupied surface								
	ACCx	5.76	*	2.96	*	2.91	*	0.05	Ns
	PrLCx	1.10	Ns	--	--	--	--	--	--
	ILCx	5.91	*	2.54	Ns	3.20	*	0.93	Ns
Hippocampus	Number of Iba-1⁺ cells								
	DG	13.52	**	4.36	**	4.63	**	0.26	Ns
	CA1	5.89	*	1.83	Ns	3.42	*	1.59	Ns
	CA3	2.55	Ns	--	--	--	--	--	--
	Integral optical density								
	DG	6.19	*	2.33	Ns	3.44	*	1.10	Ns
	CA1	6.70	Ns	2.97	*	3.33	*	0.36	Ns
	CA3	5.43	*	2.13	Ns	3.24	*	1.11	Ns
	Occupied surface								
	DG	8.80	*	1.93	Ns	4.19	**	2.25	Ns
	CA1	2.33	Ns	--	--	--	--	--	--
	CA3	15.65	**	1.51	Ns	5.42	Ns	3.90	**

952 **p* < 0.05; ***p* < 0.01; ****p* < 0.001; ns: no significant (*p* > 0.05).