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## Continuous low-level dietary exposure to glyphosate elicits dose and sex-dependent synaptic and microglial adaptations in the rodent brain.

Noemie Cresto, Margot Courret, Athénaïs Génin, Céline Marie Pauline Martin, Julie Bourret, Sophie Sakkaki, Frederic de Bock, Alicia Janvier, Arnaud Polizzi, Laurence Payraastre, et al.

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1           Continuous low-level dietary exposure to glyphosate  
2           elicits dose and sex-dependent synaptic and microglial  
3           adaptations in the rodent brain.

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43 **Abstract**

44

45 Prolonged exposure to low levels of dietary contaminants is a context in modern  
46 life that could alter organ physiology gradually. Here, our objective was to investigate  
47 the impact of continuous exposure to acceptable daily intake (ADI) and non-observable  
48 adverse effect level (NOAEL) of glyphosate from gestation to adulthood using  
49 C57BL/6J mice and incorporating these levels into their food pellets. From adulthood,  
50 we analyzed neurophysiological and neuro-glia cellular adaptations in male and female  
51 animals.

52

53 Using ex-vivo hippocampal slice electrophysiology, we found a reduced efficacy  
54 of Schaffer collateral-to-CA1 excitatory synapses in glyphosate-exposed dietary  
55 conditions, with ADI and NOAEL dose-dependent effects. Short-term facilitation of  
56 excitatory synaptic transmission was specifically increased in NOAEL conditions, with  
57 a predominant influence in males, suggesting a reduced probability of neurotransmitter  
58 release. Long-term synaptic potentiation (LTP) was decreased in NOAEL-exposed  
59 mice. Next, we explore whether these neurophysiological modifications are associated  
60 with neuro-glia changes in the somatosensory cortex and hippocampus. High-  
61 resolution confocal microscopy analyses unveil a dose-dependent increased density  
62 of excitatory Vglut1<sup>+</sup> Homer1<sup>+</sup> synapses. Microglial Iba1<sup>+</sup> cells displayed a shortening  
63 of their ramifications, a sign of cellular reactivity that was more pronounced in males at  
64 NOAEL levels. The morphology of GFAP<sup>+</sup> astrocytes was not modified. Finally, we  
65 asked whether mouse-specific cross-correlations exist among all data sets generated.  
66 This examination included the novel object recognition (NOR) test from an open-field  
67 screening to which all mice were exposed before ex vivo functional and  
68 immunohistochemical examinations. We report a negative linear regression between

69 the number of synapses and NOR or LTP maintenance when plotting ADI and NOAEL  
70 datasets.

71

72           These results outline synaptic and microglial cell adaptations resulting from  
73 prenatal and continuous dietary low levels of glyphosate, discernible in, but not limited  
74 to, adult males exposed to the NOAEL. We discuss the significance of these findings  
75 to real-world consumer situations and long-term brain resilience.

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94 **Highlights**

95 1) Dietary exposure to low glyphosate levels from prenatal to adulthood prompts  
96 neuro-glial adjustments.

97 2) In adults, synaptic function and number are modified mainly at NOAEL glyphosate  
98 compared to ADI.

99 3) Reduced microglial cell ramifications hint at activation, mainly at NOAEL dose.

100 4) These results hint at a dose-dependent vulnerability in the adult brain caused by  
101 dietary glyphosate.

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119 **Introduction**

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121 The possibility that environmental contaminants may pose risks to consumers'  
122 health is increasingly debated <sup>1</sup>. Given its extensive usage, glyphosate is under  
123 scrutiny due to its presence in numerous matrices, including human biofluids, making  
124 it a primary focus of investigation <sup>2-4</sup>. Regulatory bodies worldwide have granted  
125 glyphosate's approval for use; however, concerns regarding its impact on humans and  
126 the environment steadily mount <sup>5</sup>.

127

128 Glyphosate was originally employed as an inhibitor of 5-enolpyruvylshikimate-  
129 3-phosphate synthase in plants <sup>6</sup>. From this initial framework, glyphosate was shown  
130 to impact bacteria, including within the microbiota <sup>7</sup>, and other physiological functions,  
131 such as at the brain level <sup>8</sup>. Epidemiological studies have suggested a potential  
132 association between exposure to environmental contaminants, such as glyphosate,  
133 and neurodevelopmental risks <sup>8</sup>, although controversy exists <sup>9</sup>. The hypothesis exists  
134 that glyphosate, as an aminophosphonic analog of the natural amino acid glycine, may  
135 directly influence synaptic transmission. Experimentally, screenings for neurological  
136 adaptations have been performed in settings where high glyphosate or glyphosate-  
137 based herbicides are administered and tested or over short periods <sup>8 10-19</sup>. More  
138 recently, a paradigm shift has emerged, involving consumer-relevant modalities for  
139 exposure to contaminants <sup>20</sup>. The latter involves testing, at a multi-organ level, the  
140 acceptable daily intake (ADI) or non-observable adverse effect level (NOAEL) of  
141 contaminants over extended periods <sup>21-23</sup>.

142

143 To advance our understanding of this complex subject, and to specifically  
144 examine the impact of low-levels dietary glyphosate on the brain, we designed a  
145 protocol in which groups of C57BL/6J male and female mice were continuously  
146 exposed to glyphosate levels equivalent to ADI (0.5 mg/Kg body weight/day) and  
147 NOAEL (50 mg/Kg body weight/day) standards from the pre-natal to post-natal stages  
148 and compared to a control diet. We tested the hypothesis that dietary exposure to  
149 glyphosate could alter synaptic transmission in the mouse central nervous system. The  
150 selected read-outs represent fundamental biomarkers indicative of proper neuronal  
151 connectivity. Next, we used synaptic density and glial cell high-resolution imaging to  
152 study the existence of structural modifications. Finally, we correlate functional and  
153 histological read-outs with behavioral testing. Our results show the dose-dependent  
154 impacts induced by dietary exposure to glyphosate, particularly in male mice, where  
155 adaptations in synaptic transmission and density, as well as morphological changes in  
156 microglia, occurred. Whether these adjustments may determine a frail condition or  
157 impact brain resilience during adulthood is discussed.

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168 **Methods**

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170 *Animals*

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172 All procedures were conducted by the Directive of the Council of the European  
173 Communities, supervised by the local animal welfare units, and approved by the  
174 French Ministry of Research (APAFIS # 16136 and APAFIS # 2020021914472552 #  
175 24578 v3 at INRAE-Toxalim and IGF). Eight-week-old female and male C57BL/6J mice  
176 were purchased from Charles Rivers laboratories, allowed to acclimatize for one week,  
177 and fed a standard chow diet. Mice were housed in facilities under a 12 h light/12 h  
178 dark cycle (room temperature:  $22\pm 1^\circ\text{C}$ ), with *ad libitum* access to food and water. At  
179 Toxalim, nine male and eighteen female mice were randomly divided into 3 groups and  
180 fed control, ADI, or NOAEL diets for one week before mating. At mating, mice were  
181 housed 3 per cage (one male and two females) and fed the same diets as previously.  
182 After a mating period of 5 days (observation of a vaginal plug), the males were removed  
183 from the cages. At weaning, F1 males and females were separated and housed with  
184 3-4 mice per cage according to their experimental group as detailed: n=12 control  
185 (CTR) males in 3 cages, n=12 ADI males in 3 cages, n=9 NOAEL males in 3 cages,  
186 n=8 CTR females in 2 cages, n=11 ADI females in 3 cages, n=16 NOAEL females in  
187 5 cages. F1 mice were continuously fed the same diet as their parents. Body weight,  
188 general well-being, food, and water consumption were daily monitored. Tests (F1 from  
189 PN60, at IGF) were performed during the daily portion of the circadian rhythm (9 a.m.  
190 - 2 p.m.). To conform to the 3R rules, animals were used for consecutive exams (non-  
191 invasive behavioral explorations, ex-vivo hippocampal slice recording, and brain tissue  
192 histology; see Figure legends for specific numbers).



193 *Glyphosate-containing pellets, dosage, and exposure protocol.*

194

195         Glyphosate (Sigma-Aldrich) was solubilized in a mixture of methanol and  
196 acetone at a volume ratio of 9:1 (v/v). The glyphosate solution was evenly distributed  
197 onto the vitamin powder (PV 200, Scientific Animal Food Engineering; SAFE, Augy,  
198 France) and subsequently homogenized using a rotavapor (Laborota 4000™; BUCHI  
199 Switzerland). The homogenization process involved 30 minutes at 45 °C to evaporate  
200 the solvents, followed by an additional 50 minutes at room temperature. The control  
201 feed was prepared using the same procedure as described above, with the vitamin  
202 powder treated with a 9:1 mixture of methanol:acetone, excluding the addition of  
203 glyphosate. The vitamin powder, whether enriched with glyphosate or not, was sent to  
204 the Animal and Food Science Unit (SAAJ, Jouy en Josas, France) at the National  
205 Research Institute for Agriculture, Food and Environment (INRAE). They prepared  
206 control and glyphosate-enriched pellets by combining control or glyphosate-enriched  
207 vitamin powders (1%) with mineral supplements (7%) and other dietary constituents  
208 (63% carbohydrate, 5% fat, 22% protein, and 2% cellulose). Glyphosate quantification  
209 in the pellets was conducted by Eurofins (Nantes, France) using gas-chromatography–  
210 tandem mass spectrometry and liquid-chromatography–tandem mass spectrometry  
211 (ADI 1.7 mg/kg and NOAEL 170 mg/kg of pellet). Exposure levels were calculated  
212 based on the weekly annotations of food consumption and animal body weights  
213 (Supplemental Figure 1).

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216

217 *Behavioral screening.*

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219 We conducted a non-invasive open field (OF) assessment to measure  
220 locomotion (distance covered), time spent in the center arena (indicative of anxiety-like  
221 traits), and novel object recognition (working memory; NOR). Specifically, the animals  
222 were placed within a 50 cm x 45 cm open field (OF) arena. Locomotor activity in this  
223 setting provides both qualitative and quantitative biomarker indications of the animals'  
224 well-being and exploratory skills. The activity was captured and recorded over 10  
225 minutes using the EthoVision XT15 video tracking system (Noldus in Wageningen,  
226 Netherlands). Following a 24-hour interval, mice underwent NOR testing, which serves  
227 as a quantitative measure of working memory. Initially, the mice were placed in the  
228 testing arena for 10 minutes, with two identical objects (transparent cylindrical; A and  
229 A'). The re-test trial occurred 24 hours later, during which object A was reintroduced  
230 alongside a new object B (opaque cubic), both positioned within the arena. The time  
231 (tA and tB) spent exploring the two objects was recorded. A recognition index (RI) was  
232 calculated as  $RI = tB / (tA + tB)$ .

233

234 *Ex-vivo electrophysiological recordings.*

235

236 After rapid cervical dislocation, the right hippocampus was isolated and  
237 sectioned into 300  $\mu$ m-thick slices in an ice-cold solution of artificial cerebrospinal fluid  
238 containing sucrose (aCSF sucrose composed in mM of 87 NaCl, 25 NaHCO<sub>3</sub>, 75  
239 sucrose, 10 D-glucose, 2.5 KCl, 1 NaH<sub>2</sub>PO<sub>4</sub>, 7 MgCl<sub>2</sub> and oxygenated with carbogen  
240 (95% oxygen (O<sub>2</sub>) and 5% carbon dioxide (CO<sub>2</sub>)) using a vibratome (VT1200S, Leica,  
241 Bannockburn, IL, USA). The slices were stored at room temperature in a chamber

242 containing the cutting solution for 15 minutes before being transferred to a second  
243 chamber containing normal aCSF (119 NaCl, 26.2 NaHCO<sub>3</sub>, 11 D-glucose, 2.5 KCl, 1  
244 NaH<sub>2</sub>PO<sub>4</sub>, 1.3 MgSO<sub>4</sub>·7H<sub>2</sub>O in mM and saturated with carbogen) heated to 34°C, for  
245 at least one hour before the first recording.

246

247 Slices were transferred to an immersed chamber mounted on a BX61 Olympus  
248 microscope to record extracellular field potentials. The slices were perfused with aCSF  
249 containing picrotoxin (100 µM) at 30-32°C at a flow rate of 1.5 ml/min by a peristaltic  
250 pump. A cut between CA3 and CA1 was made to avoid epileptiform activity. Glass  
251 pipettes, drawn with a horizontal puller (Sutter Instrument, Novato, CA, USA) and filled  
252 with aCSF, were used to stimulate the Schaffer collaterals of CA1. They were placed  
253 approximately 200 µm from the area where the evoked field excitatory postsynaptic  
254 potentials (fEPSPs) were recorded by a second glass pipette filled with aCSF and  
255 connected to the recording system. The fEPSPs were recorded and filtered (low-pass  
256 at 1 kHz) with an Axopatch 200 A amplifier (Axon Instruments, Union City, CA, USA),  
257 digitized at 10 kHz with an A/D converter (Digidata 1322 A, Axon Instruments), then  
258 stored and analyzed on a computer using Pclamp9 software (Molecular Devices, San  
259 Jose, CA, USA). Baseline evoked responses were monitored for 10 minutes, and only  
260 slices with stable fEPSP amplitudes were included. Input-output (I-O) relationships for  
261 fEPSPs were measured at the start of each experiment by applying a series of stimuli  
262 of increasing intensity to Schaffer's collaterals and plotting the initial slope of the fEPSP  
263 against the amplitude of the fiber volley. Paired-pulse facilitation (PPF) was evoked by  
264 administering two stimuli at a 40 ms interval and was measured by dividing the  
265 maximum amplitude of the second response by that of the first. Long-term potentiation

266 (LTP) was induced by tetanic stimulation of Schaffer collaterals (two trains of 100 Hz  
267 for 1 s, 20 s apart).

268

269 *Synapse and glial cell analyses.*

270

271 After brain isolation, the left hemisphere was placed in a 4% paraformaldehyde  
272 (PFA) solution for tissue fixation for 24 hours at 4°C. The following day, the PFA was  
273 replaced with phosphate-buffered saline (PBS) containing 30% sucrose for  
274 cryoprotection for 24 hours at 4°C. The left hemisphere was cut into 20 µm-thick slices  
275 using a cryostat (Leica, Germany). Slices were stored in cryoprotection buffer (30%  
276 water, ethylene glycol, glycerol, and 10% 1M tris buffer) at -20°C.

277

278 After three washes in PBS, the slices were blocked with a PBS-horse serum-  
279 Triton solution (PBS with 20% horse serum and 0.25% Triton) for 1h at room  
280 temperature, then incubated overnight at 4°C with the primary antibodies in the  
281 blocking solution. Antibodies against the glial acidic fibrillary protein (chicken GFAP  
282 antibody ab4674) and ionized calcium-bound adaptor molecule 1 (rabbit antibody IBA1  
283 Wako 019-19741) were used to label astrocytes and microglia, respectively, for  
284 morphological analysis <sup>24</sup>. Antibodies against vesicular transporter 1 (Vglut1) and  
285 Homer protein homolog 1 (Homer1) were used to label pre- and post-synapses,  
286 respectively. The following day, slices were washed three times with PBS, incubated  
287 for 2h with secondary antibodies in blocking solution, and washed three times with PBS  
288 before mounting with Fluoromount containing DAPI (Invitrogen). For morphological  
289 analysis of microglia and astrocytes, z-stack images were acquired using an  
290 automated imaging system (Axioscan, Zeiss, Plateforme Montpellier Ressources

291 Imagerie) for morphological analysis at 20X. Astrocytes were selected based on GFAP  
292 and microglia on IBA1 labeling. After cell isolation, the 8-bit images were first filtered  
293 (unsharp mask process set to 2.0 radius and 0.9 mask weight) and adjusted using  
294 Triangle thresholding (min = 175 and max = 255). The ImageJ 'Sholl analysis' plugin  
295 was then used to assess cell branching <sup>25</sup>. It consists of applying concentric circles  
296 from the center of the cell soma spaced 5  $\mu\text{m}$  apart. Each intersection between the  
297 GFAP labeling for astrocytes or IBA1 for microglia and the circle is counted to assess  
298 the amount of cell extension. For synapse analyses, images were acquired with a  
299 confocal microscope (Zeiss Airyscan, Plateforme Montpellier Ressources Imagerie)  
300 using a 63X oil immersion objective (image size: 103.10 x 103.10  $\mu\text{m}$ , Resolution x,y:  
301 140 nm; z: 450 nm). Two regions of interest in two sections were acquired per animal.  
302 For each section, in both channels (488 nm and 633 nm), we imaged serial optical  
303 sections at 0.17  $\mu\text{m}$  intervals over a total of 5  $\mu\text{m}$  for 30 optical sections. For image  
304 analysis, automated quantification was performed using the Distance Analysis plugin  
305 (DiAna)<sup>26</sup>. The plugin performs automated object-based co-localization and distance  
306 analysis in 3D. As the first step, we used the global intensity thresholding (median) to  
307 select dots in each channel corresponding to Vglut1 and Homer1. This step created a  
308 new image for each channel representing each dot. Then, the plugin evaluates the co-  
309 localization between the dots of each channel. Co-localized signals were considered  
310 synapses, and the number of synapses was automatically calculated. Results were  
311 expressed in the number of synapses per 100  $\mu\text{m}^3$ .

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316 *Statistical analyses.*

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318 GraphPad Prism (La Jolla, California) was used. The normality of all the data  
319 was tested using the Shapiro-Wilk test. The one-factor or two-factor ANOVA mean  
320 comparison test was then performed for data that followed a normal distribution, and  
321 the Kruskal-Wallis test for data that did not follow a normal distribution. Multiple  
322 comparisons were computed using the Bonferonni posthoc test for ANOVAs and  
323 Dunn's posthoc test for Kruskal-Wallis. A group T-test was performed on the object  
324 recognition test data to check that the scores differed from the theoretical value, i.e., a  
325 discrimination ratio 0.5 corresponding to an identical exploration of the two objects.  
326 Multiple correlations and graphical representations were generated using R software.  
327 Symbols indicate statistical significance (\*  $p < 0.05$ ; \*\*  $p < 0.01$ ; \*\*\*  $p < 0.001$ ; \*\*\*\*  
328  $p < 0.0001$ ); \*: CTR vs NOAEL; \$: ADI vs NOAEL; #: CTR vs ADI; ns: not significant.  
329 Supplemental Table 1 provides details of statistical analyses computed for all  
330 experiments.

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341 **Results**

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343 *Dietary glyphosate is associated with adaptations in synaptic transmission and long-*

344 *term potentiation during adulthood.*

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346 In our experimental settings, mice did not exhibit significant differences in food  
347 consumption, except for isolated time points in males fed NOAEL glyphosate  
348 (Supplemental Figure 2C-D). Body weight was reduced (~10%) long-term in male mice  
349 fed with NOAEL glyphosate pellets (Supplemental Figure 2A-B). In these groups, and  
350 following behavioral testing (see below), we investigated whether synaptic-level  
351 modifications ensue due to dietary glyphosate. First, to interrogate synaptic strength,  
352 we evaluated basal evoked synaptic transmission at CA1 Schaffer collateral synapses  
353 in acute hippocampal slices from adult mice (Figure 1A). We confirm that non-exposed  
354 mice had normal excitatory synaptic transmission by comparing the amplitude of the  
355 presynaptic fiber volley (input) to the slope of the excitatory field potential (fEPSP;  
356 output) <sup>27</sup>. In contrast, in the glyphosate-exposed groups, the excitatory synaptic  
357 efficacy decreased significantly by ~50% and ~30% in the NOAEL and ADI conditions,  
358 respectively, and compared to control (Figure 1C). We segregated females (Figure 1D)  
359 and males (Figure 1E), confirming the effects of NOAEL glyphosate on both sexes. We  
360 further investigated the presence of presynaptic alterations by assessing paired-pulse  
361 facilitation (PPF), a form of short-term plasticity sensitive to changes in presynaptic  
362 release probability (Figure 1B). Consistent with the intact basal synaptic transmission,  
363 we found a paired-pulse ratio (PPR) approaching 1.5 in non-exposed mice. In ADI-  
364 exposed mice, the PPR was similar to the control, while it increased in NOAEL  
365 conditions (Figure 1F), suggesting a reduced probability of release at schaffer

366 collateral terminals. Figure 1G-H shows the patterns of sex-specific modifications, with  
367 effects significant in males.

368

369 Because glyphosate exposure reduces the efficacy of CA1 Schaffer collateral  
370 synapses in adult mice, we next investigated its impact on long-term synaptic plasticity  
371 (Figure 2A). We found that the magnitude of LTP, induced by brief tetanic stimulation  
372 of Schaffer collaterals, was reduced by ~30% in NOAEL-exposed mice (Figure 2B and  
373 2E) compared to non-exposed condition. The ADI exposition did not alter the LTP.  
374 Figure 2C-D and Figure 2F-G show the patterns of sex-specific modifications. Male  
375 mice generally presented the most significant modifications (Figures 1 and 2). See  
376 Supplemental Table 1 for complete statistical analyses. Together, these results  
377 indicate the existence of synaptic defects, particularly in male mice continuously  
378 exposed to dietary NOAEL glyphosate.

379

380 *Dietary glyphosate is associated with increased synaptic density.*

381

382 Next, we evaluate whether an alteration in the number of synapses  
383 accompanies the observed electrophysiological modifications. Using high-resolution  
384 imaging, we quantified the number of excitatory synapses in the hippocampus of adult  
385 mice exposed to glyphosate. Synapses were labeled with pre- and post-synaptic  
386 markers, Vglut1 and Homer1 (Figure 3A). The co-localization between Vglut1 and  
387 Homer1 was measured, with two co-localized dots indicating one synapse. The density  
388 of excitatory synapses increased in the glyphosate-exposed groups (Figure 3B). Sex-  
389 dependent data are shown in Figure 3C-D. Taken together, these data indicate that



390 continuous glyphosate exposure leads to functional and histological synapse  
391 modifications in a dose and sex-dependent manner.

392

393 *Impact of glyphosate exposure on the morphology of astrocytes and microglial cells.*

394

395         Accumulating evidence indicates the participation of glial cells in synaptic  
396 maturation and remodeling in health and disease <sup>28</sup>. Previous evidence showed that,  
397 in mice, glyphosate ingestion triggers neuroinflammatory changes, although evidence  
398 refers to high-level exposures <sup>29,30</sup>. Here, we examined the microglia and astrocyte  
399 histological alterations possibly associated with ADI and NOAEL glyphosate in males  
400 and females. We utilized Sholl's analysis and found that exposure to NOAEL-  
401 glyphosate was associated with decreased ramifications of Iba1+ microglia in the  
402 somatosensory parietal cortex and hippocampus (Figure 4C-D). This modification,  
403 observed in female and male mice (Figure 4F-G and I-J), hints at cellular activation.  
404 ADI-glyphosate had a negligible impact on microglial cells (Figure 4F-G and I-J). The  
405 analysis of GFAP+ astrocytes (Figure 4B) did not reveal significant morphological  
406 modifications associated with glyphosate exposure (Figure 4E-H), except for moderate  
407 cell remodeling in male mice under ADI conditions (Figure 4K). See Supplemental  
408 Table 1 for complete statistical analyses.

409

410 *Cross-correlations between individual-specific read-outs.*

411

412         Because functional synaptic and histological examinations were performed in a  
413 mouse-specific manner, we tested whether correlations exist among the data sets. In  
414 this analysis, we also integrated mouse-specific results from OF and NOR tests

415 conducted before the electrophysiological assessments. With this specific behavioral  
416 test setting, male and female mice did not show locomotor deficits (Supplemental  
417 Figure 3A-D) or anxiety-like traits (Supplemental Figure 3E-G) as the time spent at the  
418 center of the OM was unchanged after dietary glyphosate exposure. Mice exposed to  
419 NOAEL glyphosate exhibited a significant decrease in discrimination ratio (Figure 5A-  
420 D), a biomarker of spatial memory changes. When cross-correlating all results, we  
421 found a negative linear regression between the number of synapses and NOR or LTP  
422 maintenance in ADI and NOAEL-exposed animals (Figure 5E-F). These results  
423 suggest the presence of an increased number of non-fully functional synapses under  
424 NOAEL conditions. Furthermore, a trend correlation existed between NOR score and  
425 basal synaptic transmission in NOAEL-exposed animals (Figure 5G), although two  
426 outliers were present. A correlation matrix summarises trends and significant  
427 correlations between histological and functional hippocampal-associated read-outs  
428 obtained in a mouse-specific manner (Figure 5H).

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## 440 **Discussion**

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442           There is considerable debate regarding whether glyphosate adversely affects  
443 brain health and physiological function. Our findings indicate that continuous dietary  
444 exposure to low-level glyphosate levels from prenatal stages through adulthood can  
445 adjust synaptic transmission performance in a mouse model. At the cellular level, the  
446 increased synaptic density and histological indicators of microglial cell reactivity were  
447 concomitant to the electrophysiological changes. The NOAEL dose elicited most  
448 adaptations, predominantly in, but not limited to, adult males. The effect of ADI levels  
449 was more constrained, albeit discernable for selected read-outs. Taken together, these  
450 data may imply the potential for brain vulnerability associated with persistent low-level  
451 glyphosate exposure <sup>23</sup>. We should further investigate the functional relevance of the  
452 observed neuronal and microglial changes, especially considering their potential role  
453 across brain diseases. <sup>31-33</sup>.

454

455 *Glyphosate dosages and exposure duration: existing evidence.*

456

457           The majority of existing experimental evidence is based on exposure protocols  
458 involving high doses of glyphosate and its commercially available formulations, such  
459 as glyphosate-based herbicides (GBH). Because GBH contain polyethylene tallow  
460 amine (POEA)-based surfactants and heavy metals, a current question is whether  
461 glyphosate alone or in combinations with adjuvants display similar or diverse patterns  
462 of toxicity <sup>34</sup>. In rodents, GBH negatively impacted neuronal transmission and  
463 behavioral outputs <sup>14,35</sup>. Maternal exposure to GBH at high levels induced autistic-like  
464 deficiencies in male offspring <sup>36</sup>. Glyphosate or GBH exposure has been reported to

465 cause perturbations in neurodevelopmental processes, leading to long-term  
466 neurophysiological changes in animal models<sup>37-40</sup>. At doses of 250 or 500 mg/kg GBH,  
467 offspring showed behavioral changes<sup>12</sup>. Both glyphosate and GBH were shown to  
468 cause disruption of neurogenesis accompanied by compensatory responses,  
469 modifying synaptic plasticity in the hippocampus<sup>40</sup>. Exposure to GBH leads to  
470 glutamate excitotoxicity, oxidative damage, and astrocyte dysfunction in offspring  
471 hippocampus<sup>14</sup>. TNF-alpha increased brain levels were reported in mice pre-natally  
472 exposed to GBH 0.3 mg/Kg<sup>30</sup>.

473

474         Recent studies have started unveiling the impact of low-level dietary glyphosate  
475 on neurophysiological functions. At NOAEL levels, varying anxiety-like outcomes were  
476 reported<sup>21,23</sup>. We did not find changes in the time spent in the center of an OF arena,  
477 while others reported anxiety-like traits in females with NOAEL continuous exposure  
478<sup>23</sup>. Sex-dependent modifications in social preference were found to result from NOAEL  
479 dietary exposure<sup>21</sup>. Assessing memory using novel object recognition, we previously  
480 found that male mice are not affected by NOAEL glyphosate exposure<sup>41</sup>. For this  
481 present study, we changed the experimental paradigm by increasing the time before  
482 each test session, thus exploring long-term memory. Here, a negative correlation  
483 existed between the number of synapses and NOR or LTP maintenance in NOAEL  
484 glyphosate-exposed animals (Fig. 5E-F), suggesting synaptic dysfunction. In  
485 accordance with our results, NOAEL glyphosate exposure during a critical period of  
486 neurodevelopment negatively impacted synaptic organization in the hippocampus with  
487 learning and memory deficits<sup>12</sup>.

488

489 *How can glyphosate impact the brain?*

490

491 Varying glyphosate levels can be found in human body fluids, such as urine or  
492 amniotic fluids <sup>42</sup>, and high-exposure intoxications can occur <sup>43,44</sup>. Glyphosate was  
493 reported to cross the placental and blood-brain barrier, events that could favor  
494 neurodevelopmental modifications <sup>45–47</sup>. Perinatal glyphosate exposure was linked with  
495 a risk of developing attention deficit and hyperactivity disorders in children, with a  
496 possible negative impact if parents were previously exposed <sup>48 49</sup>.

497

498 As working hypotheses, exposure to glyphosate could trigger peripherally-  
499 mediated (e.g., immune responses <sup>45</sup> or modified microbiota within a gut-brain axis  
500 <sup>50,51</sup>) or direct damage to the brain borders and cerebrovascular permeability <sup>22</sup>.  
501 Importantly, microbiota adaptations were reported in mice continuously exposed to  
502 NOAEL and ADI dietary glyphosate, suggesting a scenario of a dysregulated gut-brain  
503 axis <sup>23</sup>. Our results revealed cellular adjustments in the brain, although we did not delve  
504 into specific mechanisms <sup>5,8</sup>. We identify microglial cells as sensitive to glyphosate,  
505 showing histological signs of cell reactivity in adults, along with an increased density  
506 and decreased efficacy of excitatory synapses. It is essential to understand the causal  
507 relationship between these multi-cellular events, including tracking how these changes  
508 may unfold. Microglial cells contribute to brain synapse remodeling during  
509 development, health, and disease <sup>52–55</sup>. Their function is fundamental for synaptic  
510 maturation and establishing functional circuits <sup>56</sup>. Our data, including the observed sex-  
511 dependent effects of glyphosate exposure, align with this framework. Future studies  
512 should test the hypothesis that microglia reactivity due to glyphosate exposure drives  
513 abnormal trajectories of synapse development.

514 *Study Limitations and Conclusions.*

515

516 Here, it is important to highlight that the majority of adaptations were observed  
517 in males subjected to continuous, daily exposure to NOAEL glyphosate levels, a  
518 scenario that exaggerates most consumer settings. Importantly, at ADI glyphosate  
519 levels, we observed some alterations in specific synaptic transmission measures and  
520 density. Drawing precise conclusions about the actual pathological significance of  
521 these changes presents a challenge. We suggest the hypothesis of a vulnerable, frail  
522 brain condition associated with this contaminant. This notion may gain significance in  
523 the context of pre-existing or acute pathological settings, such as genetic brain  
524 disorders or head trauma, leading to a dual-hit framework. Furthermore, the presented  
525 study leaves several unanswered questions and the possibility for further investigation.  
526 For instance, regional and temporal transcript analyses could unveil signatures  
527 indicating a frail condition, supported by the notion that glial cells critically contribute to  
528 controlling neuronal transmission in health and disease <sup>28</sup>. Similar reasoning applies  
529 to neurons, as we found synaptic adaptations using *ex-vivo* electrophysiology.  
530 Importantly, glyphosate and its major metabolite, aminomethylphosphonic acid  
531 (AMPA), have structural similarities to glycine and glutamate, respectively. The  
532 possibility exists for glyphosate to bind to the glycine or glutamate NMDA receptor  
533 binding pockets, impacting learning and memory controlled by this receptor.  
534 Glyphosate could reduce glutamate uptake and metabolism from glial cells <sup>13</sup> and  
535 modify brain monoaminergic neurotransmitter levels in rodents <sup>19</sup>. These scenarios  
536 need to be investigated. Next, *in vivo* electrophysiology should be performed primarily  
537 based on the *ex-vivo* analysis and modifications presented herein <sup>57</sup>. Finally, we  
538 examined only a limited number of behavioral parameters. This choice was dictated by

539 the high number of groups and the subsequence performance of *ex-vivo*  
540 neurophysiology and cell examinations on the same mice.

541

542 In summary, low-level glyphosate, when delivered through the diet and daily  
543 from pre-natal stages to adulthood, modifies distinct parameters of synaptic  
544 transmission and neuro-microglial cell structures. These adjustments support the  
545 notion of a frail, sex-specific condition that may unfavorably impact brain resilience  
546 over time<sup>22 38</sup>.

547

548

#### 549 **Declaration of interests**

550 The authors declare that they have no known competing financial interests or personal  
551 relationships that could have appeared to influence the work reported in this paper.

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565 **Bibliography**

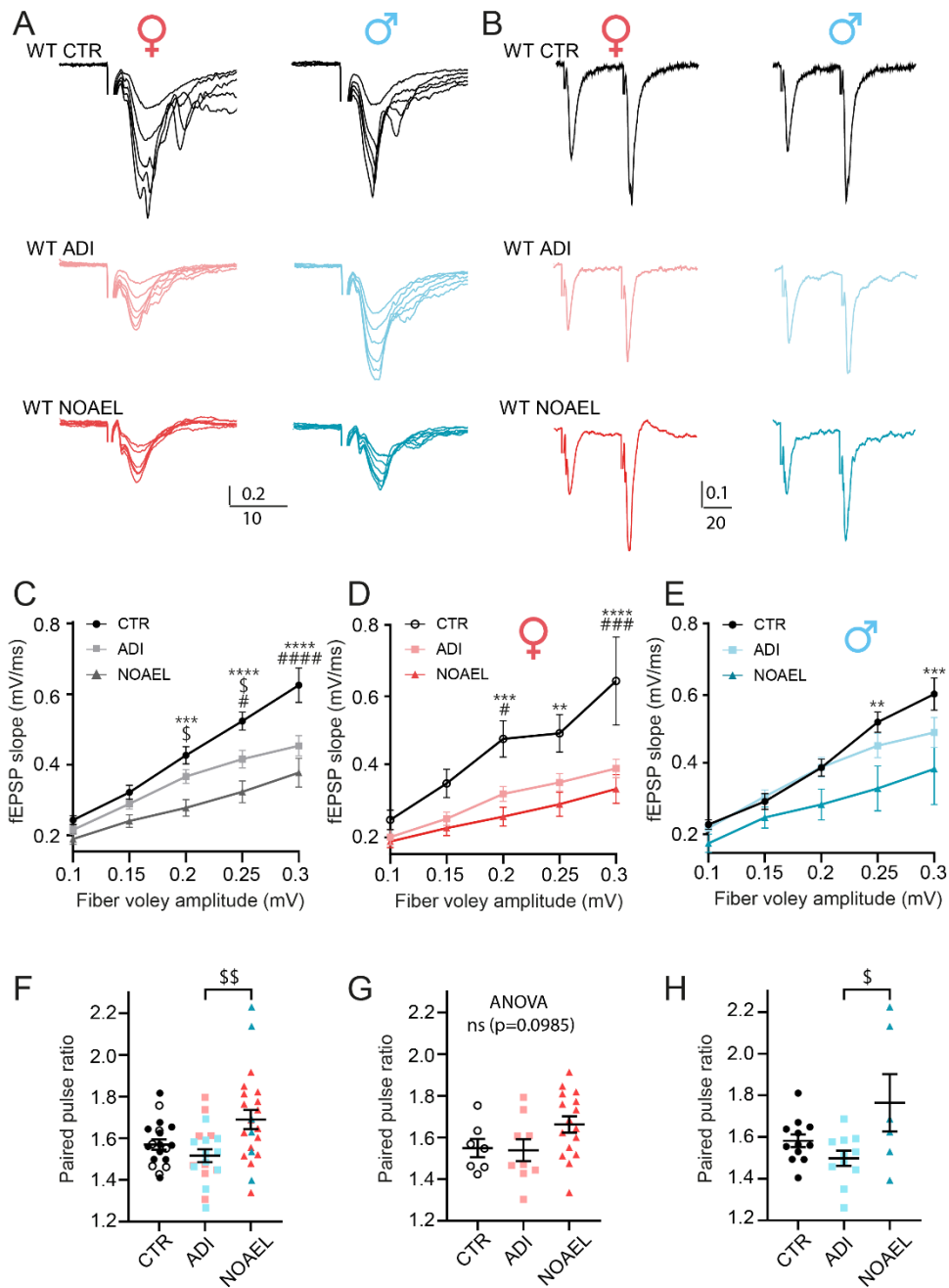
- 566 1. Kim, K.-H., Kabir, E. & Jahan, S. A. Exposure to pesticides and the associated  
567 human health effects. *Sci. Total Environ.* **575**, 525–535 (2017).
- 568 2. Myers, J. P. *et al.* Concerns over use of glyphosate-based herbicides and risks  
569 associated with exposures: a consensus statement. *Environ. Health Glob. Access*  
570 *Sci. Source* **15**, 19 (2016).
- 571 3. Niemann, L., Sieke, C., Pfeil, R. & Solecki, R. A critical review of glyphosate  
572 findings in human urine samples and comparison with the exposure of operators  
573 and consumers. *J. Für Verbraucherschutz Leb.* **10**, 3–12 (2015).
- 574 4. Van Bruggen, A. H. C. *et al.* Environmental and health effects of the herbicide  
575 glyphosate. *Sci. Total Environ.* **616–617**, 255–268 (2018).
- 576 5. Madani, N. A. & Carpenter, D. O. Effects of glyphosate and glyphosate-based  
577 herbicides like Roundup™ on the mammalian nervous system: A review. *Environ.*  
578 *Res.* **214**, 113933 (2022).
- 579 6. Schönbrunn, E. *et al.* Interaction of the herbicide glyphosate with its target  
580 enzyme 5-enolpyruvylshikimate 3-phosphate synthase in atomic detail. *Proc.*  
581 *Natl. Acad. Sci. U. S. A.* **98**, 1376–1380 (2001).
- 582 7. Mao, Q. *et al.* The Ramazzini Institute 13-week pilot study on glyphosate and  
583 Roundup administered at human-equivalent dose to Sprague Dawley rats: effects  
584 on the microbiome. *Environ. Health* **17**, 50 (2018).
- 585 8. Costas-Ferreira, C., Durán, R. & Faro, L. R. F. Toxic Effects of Glyphosate on the  
586 Nervous System: A Systematic Review. *Int. J. Mol. Sci.* **23**, 4605 (2022).
- 587 9. Chang, E. T., Odo, N. U. & Acquavella, J. F. Systematic literature review of the  
588 epidemiology of glyphosate and neurological outcomes. *Int. Arch. Occup.*  
589 *Environ. Health* **96**, 1–26 (2023).
- 590 10. Bali, Y. A., Kaikai, N.-E., Ba-M'hamed, S. & Bennis, M. Learning and memory  
591 impairments associated to acetylcholinesterase inhibition and oxidative stress  
592 following glyphosate based-herbicide exposure in mice. *Toxicology* **415**, 18–25  
593 (2019).
- 594 11. Ait Bali, Y., Ba-Mhamed, S. & Bennis, M. Behavioral and Immunohistochemical  
595 Study of the Effects of Subchronic and Chronic Exposure to Glyphosate in Mice.  
596 *Front. Behav. Neurosci.* **11**, 146 (2017).
- 597 12. Ait-Bali, Y. *et al.* Pre- and postnatal exposure to glyphosate-based herbicide  
598 causes behavioral and cognitive impairments in adult mice: evidence of cortical  
599 ad hippocampal dysfunction. *Arch. Toxicol.* **94**, 1703–1723 (2020).
- 600 13. Cattani, D. *et al.* Mechanisms underlying the neurotoxicity induced by glyphosate-  
601 based herbicide in immature rat hippocampus: involvement of glutamate  
602 excitotoxicity. *Toxicology* **320**, 34–45 (2014).
- 603 14. Cattani, D. *et al.* Developmental exposure to glyphosate-based herbicide and  
604 depressive-like behavior in adult offspring: Implication of glutamate excitotoxicity  
605 and oxidative stress. *Toxicology* **387**, 67–80 (2017).
- 606 15. Coullery, R., Pacchioni, A. M. & Rosso, S. B. Exposure to glyphosate during  
607 pregnancy induces neurobehavioral alterations and downregulation of Wnt5a-  
608 CaMKII pathway. *Reprod. Toxicol. Elmsford N* **96**, 390–398 (2020).
- 609 16. Hernández-Plata, I., Giordano, M., Díaz-Muñoz, M. & Rodríguez, V. M. The  
610 herbicide glyphosate causes behavioral changes and alterations in dopaminergic  
611 markers in male Sprague-Dawley rat. *Neurotoxicology* **46**, 79–91 (2015).
- 612 17. Ji, H., Xu, L., Wang, Z., Fan, X. & Wu, L. Differential microRNA expression in the  
613 prefrontal cortex of mouse offspring induced by glyphosate exposure during  
614 pregnancy and lactation. *Exp. Ther. Med.* **15**, 2457–2467 (2018).



- 615 18. Luna, S., Neila, L. P., Vena, R., Borgatello, C. & Rosso, S. B. Glyphosate  
616 exposure induces synaptic impairment in hippocampal neurons and cognitive  
617 deficits in developing rats. *Arch. Toxicol.* **95**, 2137–2150 (2021).
- 618 19. Martínez, M.-A. *et al.* Neurotransmitter changes in rat brain regions following  
619 glyphosate exposure. *Environ. Res.* **161**, 212–219 (2018).
- 620 20. Bicca, D. F., Spiazzi, C. C., Ramalho, J. B., Soares, M. B. & Cibin, F. W. S. A  
621 subchronic low-dose exposure of a glyphosate-based herbicide induces  
622 depressive and anxious-like behavior in mice: quercetin therapeutic approach.  
623 *Environ. Sci. Pollut. Res. Int.* **28**, 67394–67403 (2021).
- 624 21. Del Castilo, I. *et al.* Lifelong Exposure to a Low-Dose of the Glyphosate-Based  
625 Herbicide RoundUp® Causes Intestinal Damage, Gut Dysbiosis, and Behavioral  
626 Changes in Mice. *Int. J. Mol. Sci.* **23**, 5583 (2022).
- 627 22. Cresto, N. *et al.* Pesticides at brain borders: Impact on the blood-brain barrier,  
628 neuroinflammation, and neurological risk trajectories. *Chemosphere* **324**, 138251  
629 (2023).
- 630 23. Buchenauer, L. *et al.* Maternal exposure of mice to glyphosate induces  
631 depression- and anxiety-like behavior in the offspring via alterations of the gut-  
632 brain axis. *Sci. Total Environ.* **905**, 167034 (2023).
- 633 24. Dincã, D. M. *et al.* Myotonic dystrophy RNA toxicity alters morphology, adhesion  
634 and migration of mouse and human astrocytes. *Nat. Commun.* **13**, 3841 (2022).
- 635 25. Ferreira, T. A. *et al.* Neuronal morphometry directly from bitmap images. *Nat.*  
636 *Methods* **11**, 982–984 (2014).
- 637 26. Gilles, J.-F., Dos Santos, M., Boudier, T., Bolte, S. & Heck, N. DiAna, an ImageJ  
638 tool for object-based 3D co-localization and distance analysis. *Methods San*  
639 *Diego Calif* **115**, 55–64 (2017).
- 640 27. Cresto, N. *et al.* Hippocampal Excitatory Synaptic Transmission and Plasticity Are  
641 Differentially Altered during Postnatal Development by Loss of the X-Linked  
642 Intellectual Disability Protein Oligophrenin-1. *Cells* **11**, 1545 (2022).
- 643 28. Rasband, M. N. Glial Contributions to Neural Function and Disease \*. *Mol. Cell.*  
644 *Proteomics* **15**, 355–361 (2016).
- 645 29. Winstone, J. K. *et al.* Glyphosate infiltrates the brain and increases pro-  
646 inflammatory cytokine TNF $\alpha$ : implications for neurodegenerative disorders. *J.*  
647 *Neuroinflammation* **19**, 193 (2022).
- 648 30. de Castro Vieira Carneiro, C. L. *et al.* Behavioral and neuroinflammatory changes  
649 caused by glyphosate: Base herbicide in mice offspring. *Birth Defects Res.* **115**,  
650 488–497 (2023).
- 651 31. Muzio, L., Viotti, A. & Martino, G. Microglia in Neuroinflammation and  
652 Neurodegeneration: From Understanding to Therapy. *Front. Neurosci.* **15**, (2021).
- 653 32. Klement, W. *et al.* Seizure progression and inflammatory mediators promote  
654 pericytosis and pericyte-microglia clustering at the cerebrovasculature. *Neurobiol.*  
655 *Dis.* **113**, 70–81 (2018).
- 656 33. Di Nunzio, M. *et al.* Microglia proliferation plays distinct roles in acquired epilepsy  
657 depending on disease stages. *Epilepsia* **62**, 1931–1945 (2021).
- 658 34. Mesnage, R., Bernay, B. & Séralini, G.-E. Ethoxylated adjuvants of glyphosate-  
659 based herbicides are active principles of human cell toxicity. *Toxicology* **313**,  
660 122–128 (2013).
- 661 35. Gallegos, C. E. *et al.* Exposure to a glyphosate-based herbicide during pregnancy  
662 and lactation induces neurobehavioral alterations in rat offspring. *Neurotoxicology*  
663 **53**, 20–28 (2016).

- 664 36. Pu, Y. *et al.* Glyphosate exposure exacerbates the dopaminergic neurotoxicity in  
665 the mouse brain after repeated administration of MPTP. *Neurosci. Lett.* **730**,  
666 135032 (2020).
- 667 37. Ruuskanen, S., Rainio, M. J., Uusitalo, M., Saikkonen, K. & Helander, M. Effects  
668 of parental exposure to glyphosate-based herbicides on embryonic development  
669 and oxidative status: a long-term experiment in a bird model. *Sci. Rep.* **10**, 6349  
670 (2020).
- 671 38. Forner-Piquer, I. *et al.* Differential impact of dose-range glyphosate on locomotor  
672 behavior, neuronal activity, glio-cerebrovascular structures, and transcript  
673 regulations in zebrafish larvae. *Chemosphere* **267**, 128986 (2021).
- 674 39. de Oliveira, M. A. L. *et al.* Perinatal exposure to glyphosate-based herbicides  
675 induced neurodevelopmental behaviors impairments and increased oxidative  
676 stress in the prefrontal cortex and hippocampus in offspring. *Int. J. Dev. Neurosci.*  
677 *Off. J. Int. Soc. Dev. Neurosci.* **82**, 528–538 (2022).
- 678 40. Ojiro, R. *et al.* Comparison of the effect of glyphosate and glyphosate-based  
679 herbicide on hippocampal neurogenesis after developmental exposure in rats.  
680 *Toxicology* **483**, 153369 (2023).
- 681 41. Sakkaki, S. *et al.* Dual-Hit: Glyphosate exposure at NOAEL level negatively  
682 impacts birth and glia-behavioural measures in heterozygous shank3 mutants.  
683 *Environ. Int.* **180**, 108201 (2023).
- 684 42. Ongono, J. S., Béranger, R., Baghdadli, A. & Mortamais, M. Pesticides used in  
685 Europe and autism spectrum disorder risk: can novel exposure hypotheses be  
686 formulated beyond organophosphates, organochlorines, pyrethroids and  
687 carbamates? - A systematic review. *Environ. Res.* **187**, 109646 (2020).
- 688 43. Gillezeau, C. *et al.* The evidence of human exposure to glyphosate: a review.  
689 *Environ. Health Glob. Access Sci. Source* **18**, 2 (2019).
- 690 44. Soukup, S. T. *et al.* Glyphosate and AMPA levels in human urine samples and  
691 their correlation with food consumption: results of the cross-sectional KarMeN  
692 study in Germany. *Arch. Toxicol.* **94**, 1575–1584 (2020).
- 693 45. von Ehrenstein, O. S. *et al.* Prenatal and infant exposure to ambient pesticides  
694 and autism spectrum disorder in children: population based case-control study.  
695 *BMJ* **364**, I962 (2019).
- 696 46. Martinez, A. & Al-Ahmad, A. J. Effects of glyphosate and aminomethylphosphonic  
697 acid on an isogenic model of the human blood-brain barrier. *Toxicol. Lett.* **304**,  
698 39–49 (2019).
- 699 47. Poulsen, M. S., Rytting, E., Mose, T. & Knudsen, L. E. Modeling placental  
700 transport: correlation of in vitro BeWo cell permeability and ex vivo human  
701 placental perfusion. *Toxicol. Vitro Int. J. Publ. Assoc. BIBRA* **23**, 1380–1386  
702 (2009).
- 703 48. de Araujo, J. S. A., Delgado, I. F. & Paumgarten, F. J. R. Glyphosate and  
704 adverse pregnancy outcomes, a systematic review of observational studies. *BMC*  
705 *Public Health* **16**, 472 (2016).
- 706 49. Arcury, T. A. *et al.* Pesticide Exposure among Latinx Children in Rural  
707 Farmworker and Urban Non-Farmworker Communities: Associations with Locality  
708 and Season. *Int. J. Environ. Res. Public Health* **20**, 5647 (2023).
- 709 50. Rueda-Ruzafa, L., Cruz, F., Roman, P. & Cardona, D. Gut microbiota and  
710 neurological effects of glyphosate. *Neurotoxicology* **75**, 1–8 (2019).
- 711 51. Walsh, L., Hill, C. & Ross, R. P. Impact of glyphosate (Roundup™) on the  
712 composition and functionality of the gut microbiome. *Gut Microbes* **15**, 2263935.

- 713 52. Kettenmann, H., Kirchhoff, F. & Verkhratsky, A. Microglia: New Roles for the  
714 Synaptic Stripper. *Neuron* **77**, 10–18 (2013).
- 715 53. Frost, J. L. & Schafer, D. P. Microglia: Architects of the Developing Nervous  
716 System. *Trends Cell Biol.* **26**, 587–597 (2016).
- 717 54. Mosser, C.-A., Baptista, S., Arnoux, I. & Audinat, E. Microglia in CNS  
718 development: Shaping the brain for the future. *Prog. Neurobiol.* **149–150**, 1–20  
719 (2017).
- 720 55. Thion, M. S. & Garel, S. Microglial ontogeny, diversity and neurodevelopmental  
721 functions. *Curr. Opin. Genet. Dev.* **65**, 186–194 (2020).
- 722 56. Thion, M. S. *et al.* Microbiome Influences Prenatal and Adult Microglia in a Sex-  
723 Specific Manner. *Cell* **172**, 500-516.e16 (2018).
- 724 57. Forner-Piquer, I. *et al.* Varying modalities of perinatal exposure to a pesticide  
725 cocktail elicit neurological adaptations in mice and zebrafish. *Environ. Pollut.* **278**,  
726 116755 (2021).
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- 729
- 730
- 731

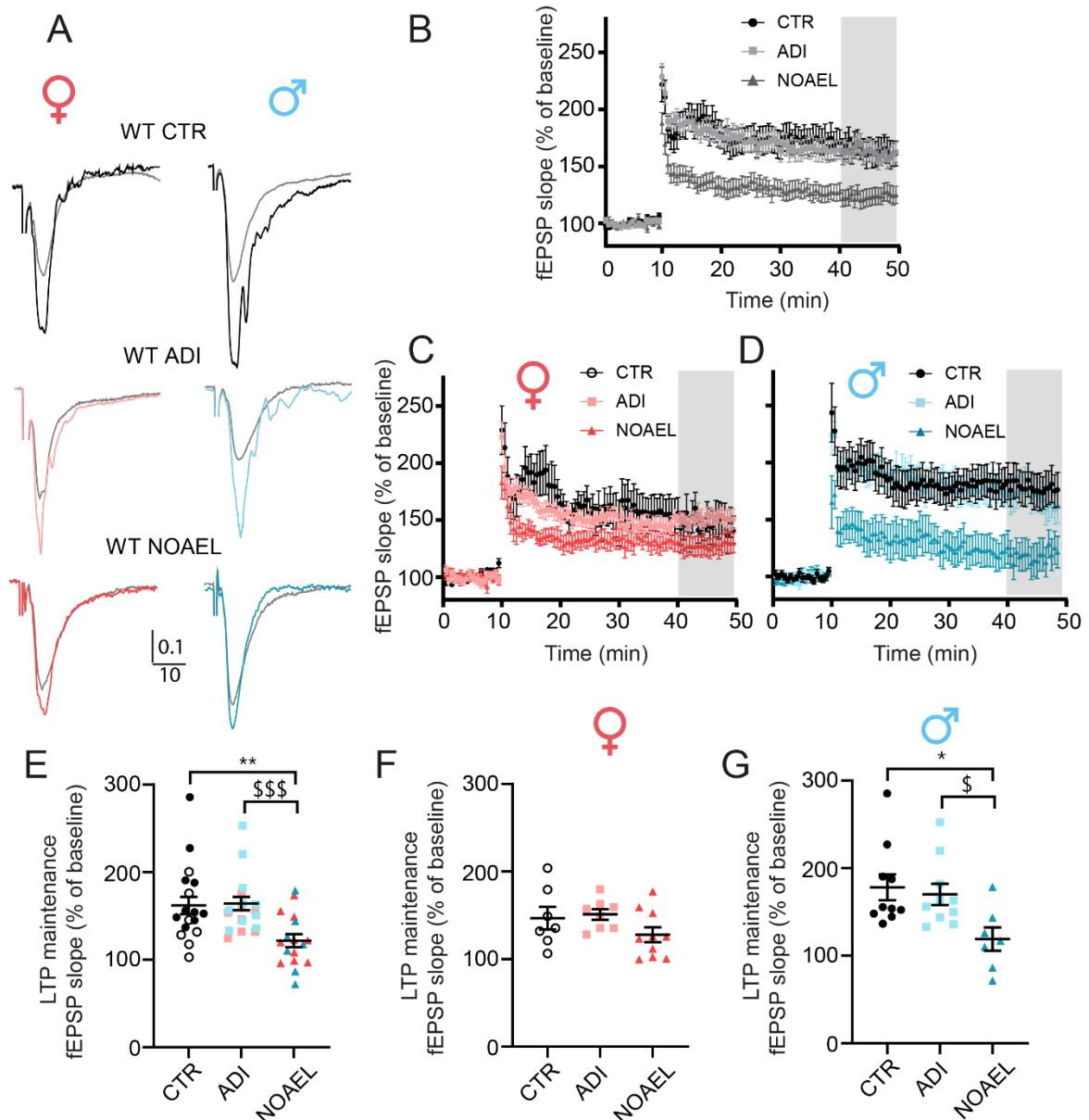


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733 **Figure 1. Basal excitatory synaptic transmission is reduced in the adult brain of ADI and**  
 734 **NOAEL glyphosate-exposed mice. (A, C to E)** The input/output curve shows that basal  
 735 excitatory synaptic transmission at CA1 Schaffer collateral synapses is reduced in glyphosate-  
 736 treated groups, males and females pooled (C). Males CTR, n=14 ; ADI, n=11 ; NOAEL, n=6;  
 737 Females CTR, n=6 ; ADI, n=8 ; NOAEL, n=13. Two-way ANOVA test (Males: p<0.0001;  
 738 Females: p<0.0001; Males and Females: p<0.0001). (B, F to H) Paired-pulse facilitation (PPF)  
 739 is increased in NOAEL glyphosate-treated mice, males, and females pooled (F), females (G),  
 740 and males (H). Males CTR, n=12 ; ADI, n=11 ; NOAEL, n=6 ; Females CTR, n=7 ; ADI, n=9 ;  
 741 NOAEL, n=16. One-way ANOVA test (Males: p=0.0236; Females: p=0.0985; Males and  
 742 Females: p=0.0033). Asterisks indicate statistical significance (\* p < 0.05; \*\* p < 0.01; \*\*\* p <  
 743 0.001; \*\*\*\* p < 0.0001) ; \* : CTR vs NOAEL ; \$ : ADI vs NOAEL ; # : CTR vs ADI ; ns : non-  
 744 significant.

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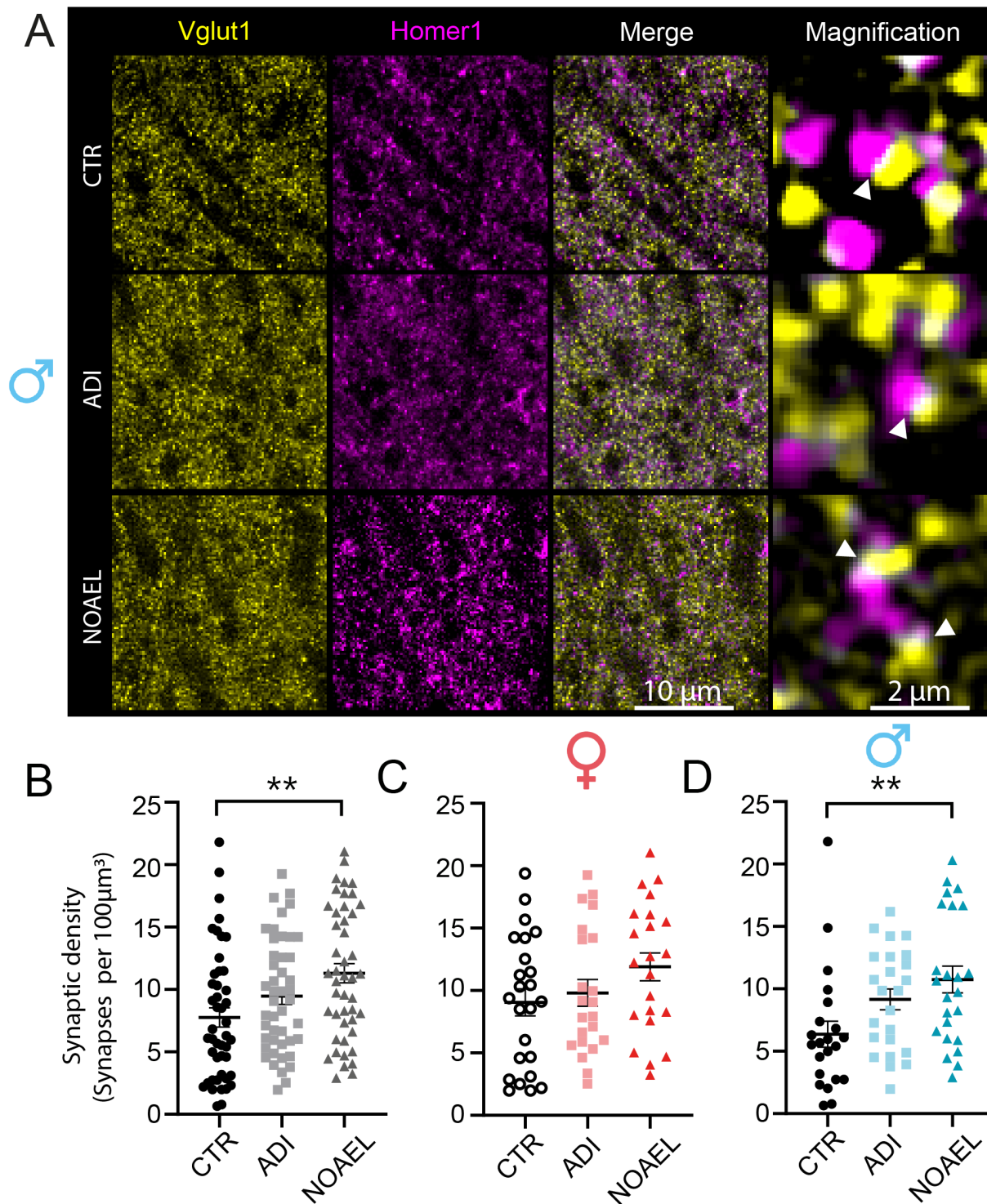
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748 **Figure 2. Long-term potentiation in the adult brain is reduced in NOAEL glyphosate**  
 749 **exposure conditions.** In all mice groups, LTP was measured by field potential recordings  
 750 (fEPSPs) at CA1 Schaffer collateral synapses. The light gray box highlights the last 10 min of  
 751 the recording used to calculate the LTP maintenance (50–60 min). **(A–D).** Average fEPSPs  
 752 after the tetanus were normalized to baseline values before high-frequency stimulation.  
 753 Reduced LTP was observed in the NOAEL-exposed group. **(E–G)** The histograms represent  
 754 the mean normalized fEPSP slope measured during the last 10 min of the recording, as  
 755 indicated by the light gray box in B. LTP was affected in NOAEL groups compared to non-  
 756 exposed mice. Males CTR, n=10 slices from 10 mice; ADI, n=10 slices from 10 mice; NOAEL,  
 757 n=7 slices from 6 mice; Females CTR, n=7 slices from 5 mice; ADI, n=8 slices from 8 mice;  
 758 NOAEL, n=10 slices from 9 mice. Kruskal-Wallis test (Males: p=0.0109); One-way ANOVA  
 759 (Females: p=0.1657) and Kruskal wallis test (Males and Females: p=0.0003). Sample traces  
 760 represent averaged field potentials before (light blue and light red) and 50–60 min after  
 761 tetanization (dark blue and dark red). Asterisks indicate statistical significance (\* p < 0.05; \*\* p  
 762 < 0.01; \*\*\* p < 0.001; \*\*\*\* p < 0.0001) ; \*: CTR vs NOAEL ; \$: ADI vs NOAEL ; #: CTR vs ADI  
 763 ; ns: non-significative.

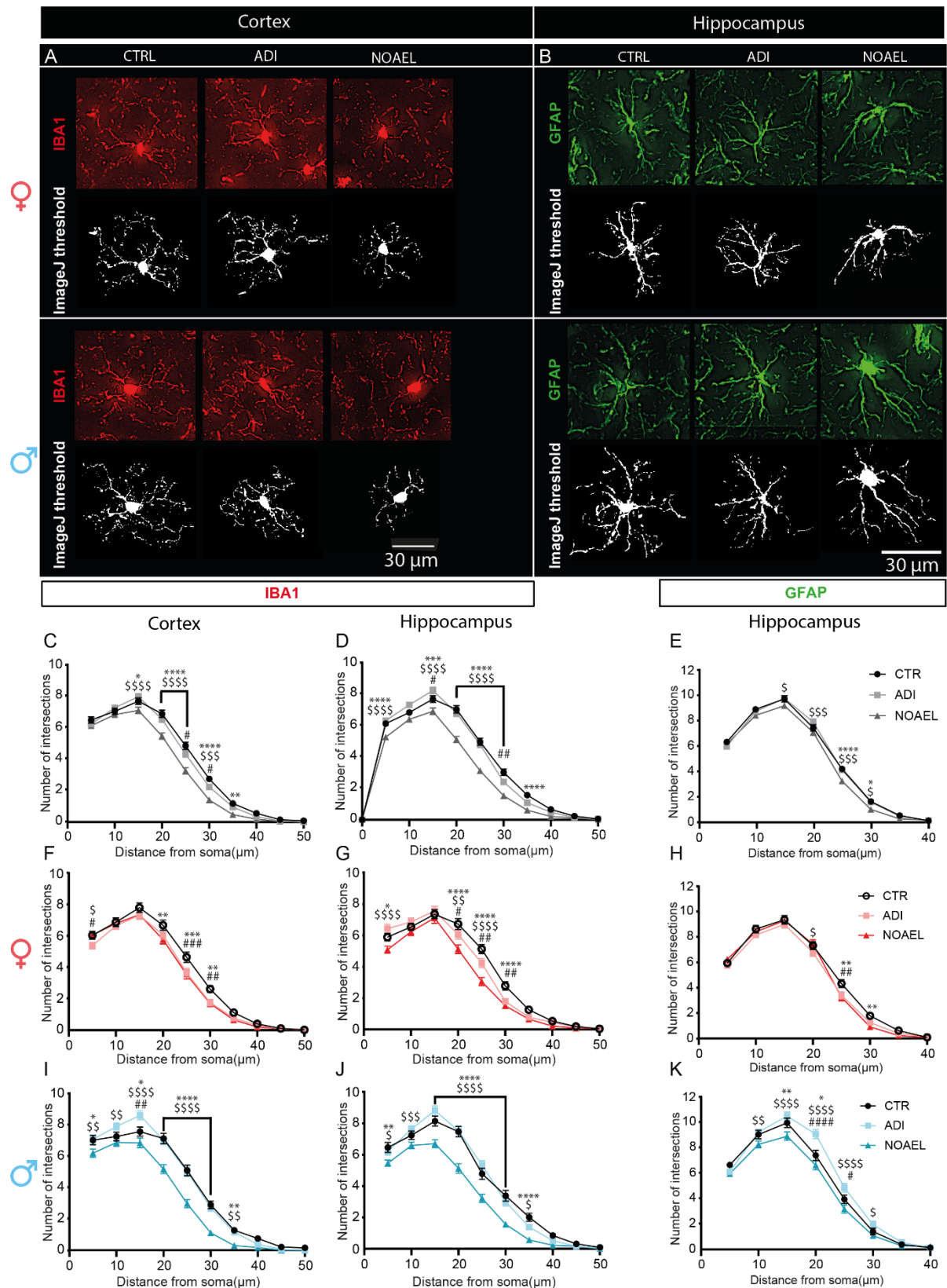
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766 **Figure 3. NOAEL exposure induces an increase in excitatory synapses in adult mice.**  
 767 **(A)** Representative images (males) of excitatory synapses labeled with Vglut1 (yellow) and  
 768 Homer1 (magenta), co-localized dots (white, marked with arrows) identified one synapse. **(B)**  
 769 Synaptic density, **(C)** synaptic density in females, and **(D)** in males (CTR: n = 22 ROI from n =  
 770 6 mice, DJA: n = 24 ROI from n = 6 mice, NOAEL: n = 24 ROI from n = 6). Asterisks indicate  
 771 statistical significance (\*\* p < 0.01).





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773 **Figure 4. NOAEL glyphosate exposure associates with histological microglia**  
 774 **modifications in the adult brain. (A)** Representative images showing IBA1  
 775 immunofluorescence in the parietal cortex in female and male mice. **(B)** Representative  
 776 images showing GFAP immunofluorescence in the hippocampus in female and male mice.  
 777 The morphology of microglia (IBA1 staining and ImageJ threshold) was assessed using Sholl  
 778 analysis in the parietal cortex and the hippocampus of CTRL, ADI, and NOAEL groups.

779 Quantification of the sholl analysis in the parietal cortex (**C, F, and I**) and the hippocampus (**D,**  
780 **G, and J**) of CTR, ADI, and NOAEL females and males. Cortex: Males CTR, n=105 cells from  
781 7 mice; ADI, n=120 cells from 8 mice; NOAEL, n=120 cells from 8 mice; Females CTR, n=120  
782 cells from 8 mice; ADI, n=117 cells from 8 mice; NOAEL, n=120 cells from 8 mice. Two-way  
783 ANOVA (Males:  $p < 0.0001$ ; Females:  $p < 0.0001$ ; Males and Females:  $p < 0.0001$ ).  
784 Hippocampus: Males CTR, n=90 cells from 6 mice; ADI, n=119 cells from 8 mice; NOAEL,  
785 n=115 cells from 8 mice; Females CTR, n=120 cells from 8 mice; ADI, n=113 cells from 8 mice;  
786 NOAEL, n=120 cells from 8 mice. Two-way ANOVA (Males:  $p < 0.0001$ ; Females:  $p < 0.0001$ ;  
787 Males and Females:  $p < 0.0001$ ). The morphology of astrocytes (GFAP staining and ImageJ  
788 threshold) was assessed using Sholl analysis in the hippocampus of CTR, ADI, and NOAEL  
789 groups. Quantification of the sholl analysis in the hippocampus (**E**) of CTR, ADI, and NOAEL  
790 females (**H**) and males (**K**). Hippocampus: Males CTR, n=75 cells from 4 mice; ADI, n=90 cells  
791 from 5 mice; NOAEL, n=90 cells from 5 mice; Females CTR, n=93 cells from 5 mice; ADI,  
792 n=113 cells from 6 mice; NOAEL, n=94 cells from 5 mice. Two-way ANOVA (Males:  $p < 0.0001$ ;  
793 Females:  $p = 0.0022$ ; Males and Females:  $p < 0.0001$ ). Asterisks indicate statistical significance  
794 (\*  $p < 0.05$ ; \*\*  $p < 0.01$ ; \*\*\*  $p < 0.001$ ; \*\*\*\*  $p < 0.0001$ ); \*: CTR vs NOAEL; \$: ADI vs NOAEL;  
795 #: CTR vs ADI; ns: non-significant. Statistical significance was reported between genotype  
796 comparisons under each graphic.

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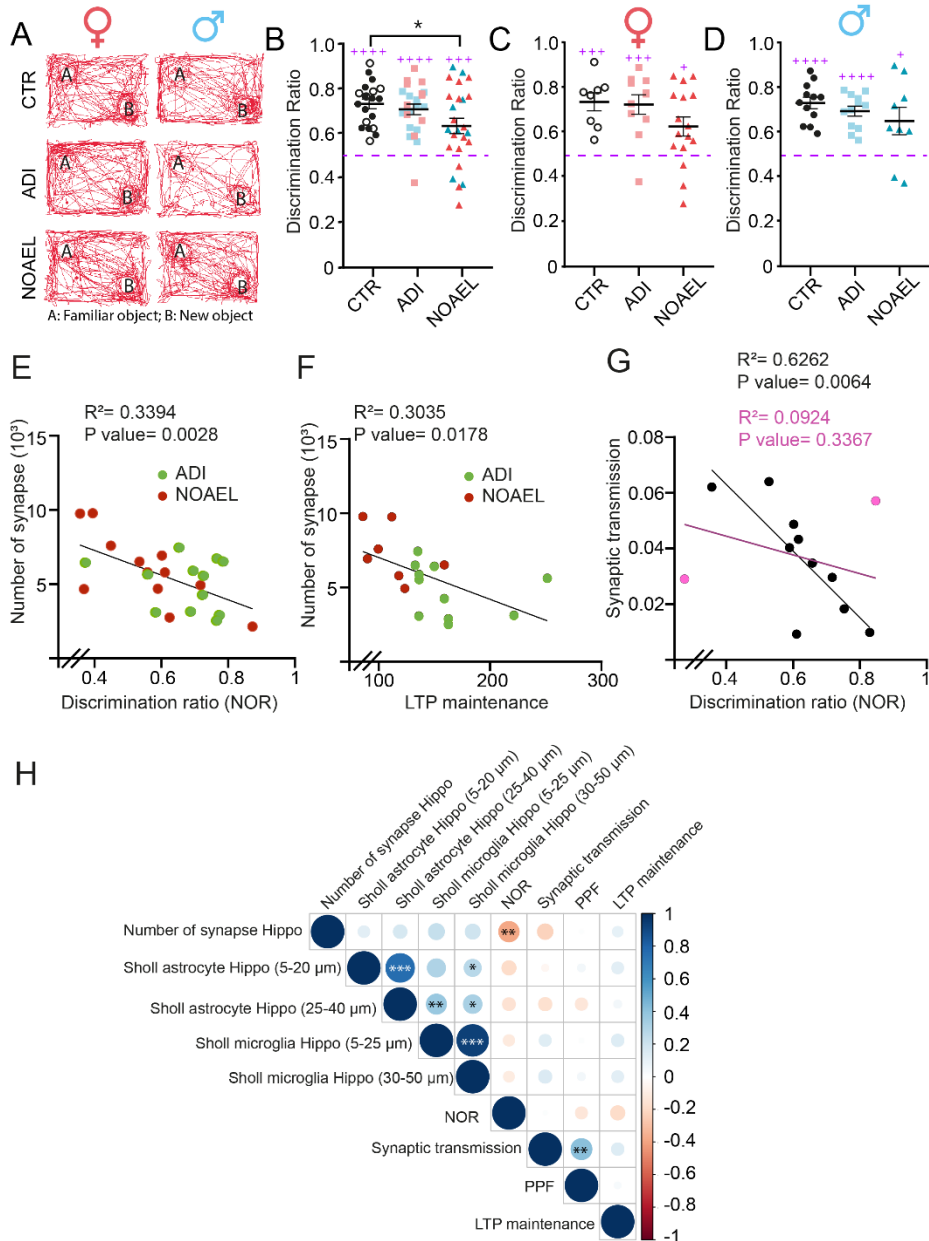
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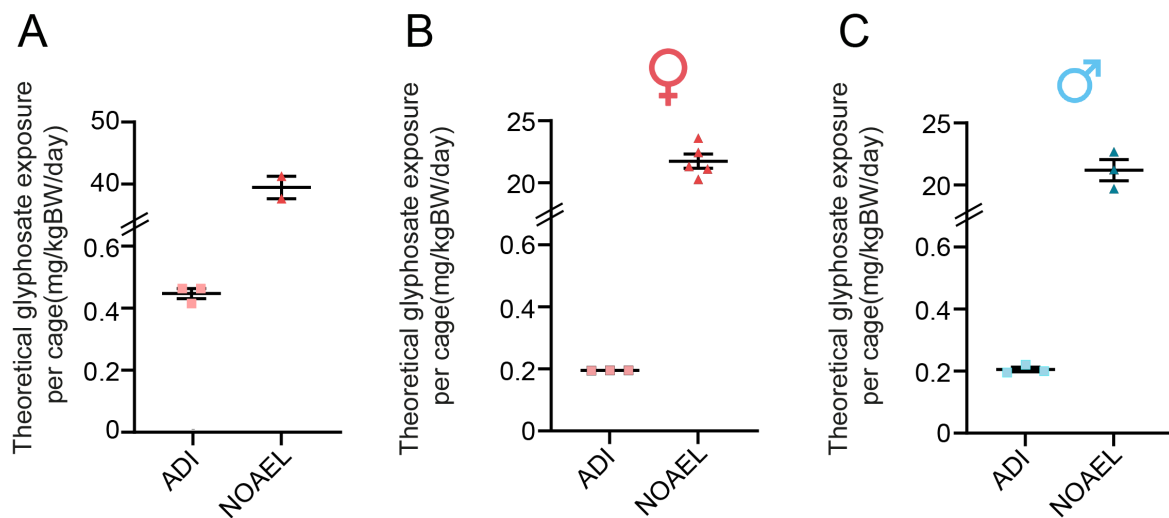




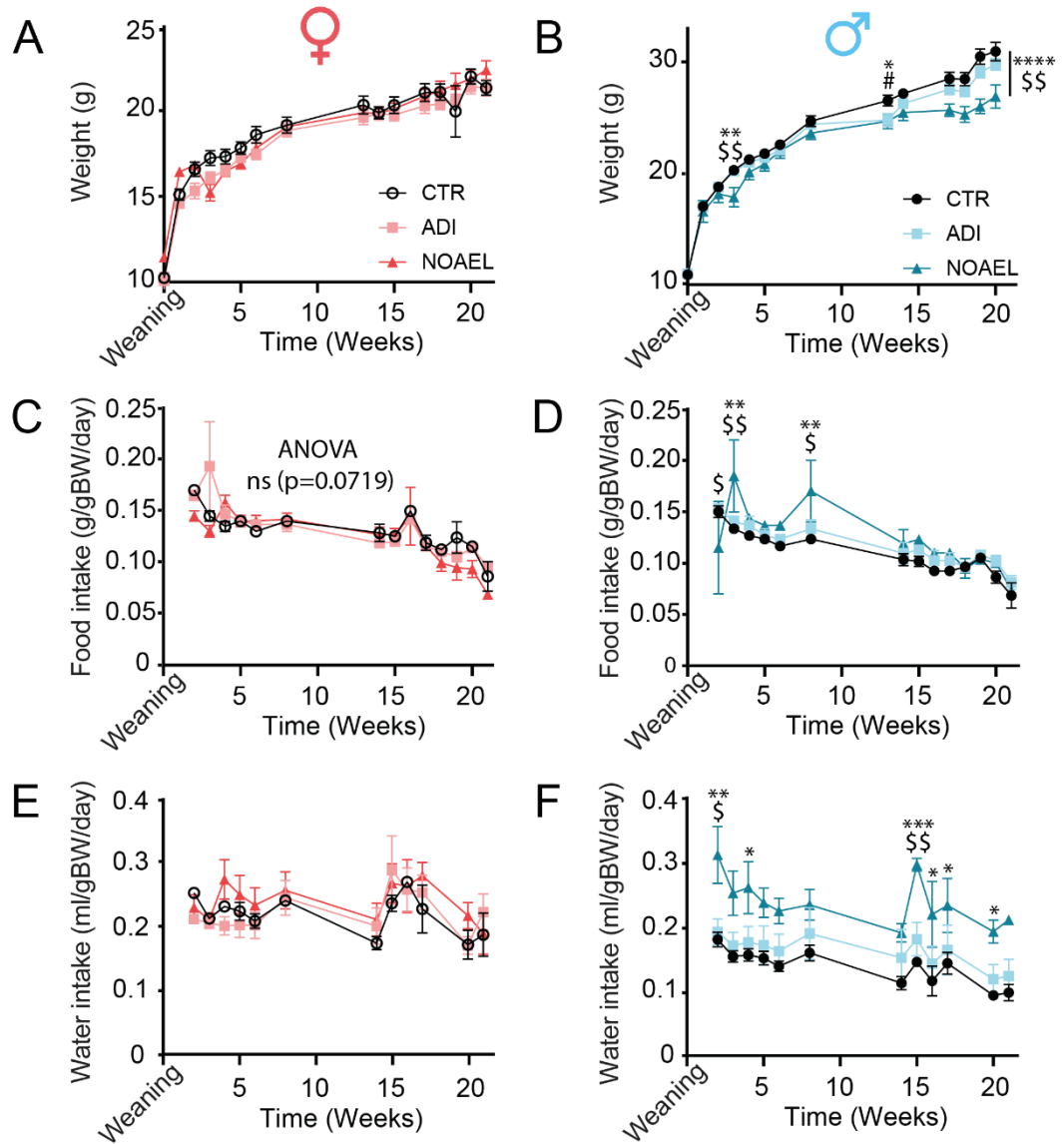
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**Figure 5. Mouse-specific cross-correlation of neurophysiological and histological data set.** (A-D) 24 hours after the open field test, mice were exposed to two similar objects in the arena, and 24 hours later, one familiar object was replaced by a new one. The discrimination ratio was calculated, revealing a decrease in performance. Males CTR, n=12; ADI, n=12 ; NOAEL, n=9; Females CTR, n=8; ADI, N=11; NOAEL, n=16. One-way ANOVA test (Discrimination ratio Males: p=0.3232; Females: p=0.1502; Males + Females: p=0.041). Asterisks indicate statistical significance (\* p < 0.05), and purple + symbols indicate statistical significance regarding the discrimination ratio value fixed at 0.5 (One sample t-test). (E) The number of synapses was correlated with the discrimination ratio obtained using the novel object recognition test. (F) The number of synapses was correlated with LTP maintenance. (G) The synaptic transmission was correlated with the discrimination ratio obtained using the novel object recognition test for female and male NOAEL. Pink simple linear regression showed non-significant R<sup>2</sup> and P values. Black simple linear regression was calculated by removing the two pink values from the analysis. (H) Correlation coefficients were calculated using the Pearson coefficients and considered statistically significant with \*: R<sup>2</sup>>0.35, P<0.01; \*\*: R<sup>2</sup>>0.4, P<0.001; \*\*\*: R<sup>2</sup>>0.5, P<0.00001. The areas of circles or squares show the absolute value of

833 corresponding correlation coefficients; the colors of circles show both the sign and the absolute  
834 value of the correlation coefficients.  
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837 **Supplemental Figure 1.** Exposure of F0 dams (A) and F1 females (B) and males (C) was  
838 evaluated. The NOAEL dose corresponds to 100 times the ADI level.  
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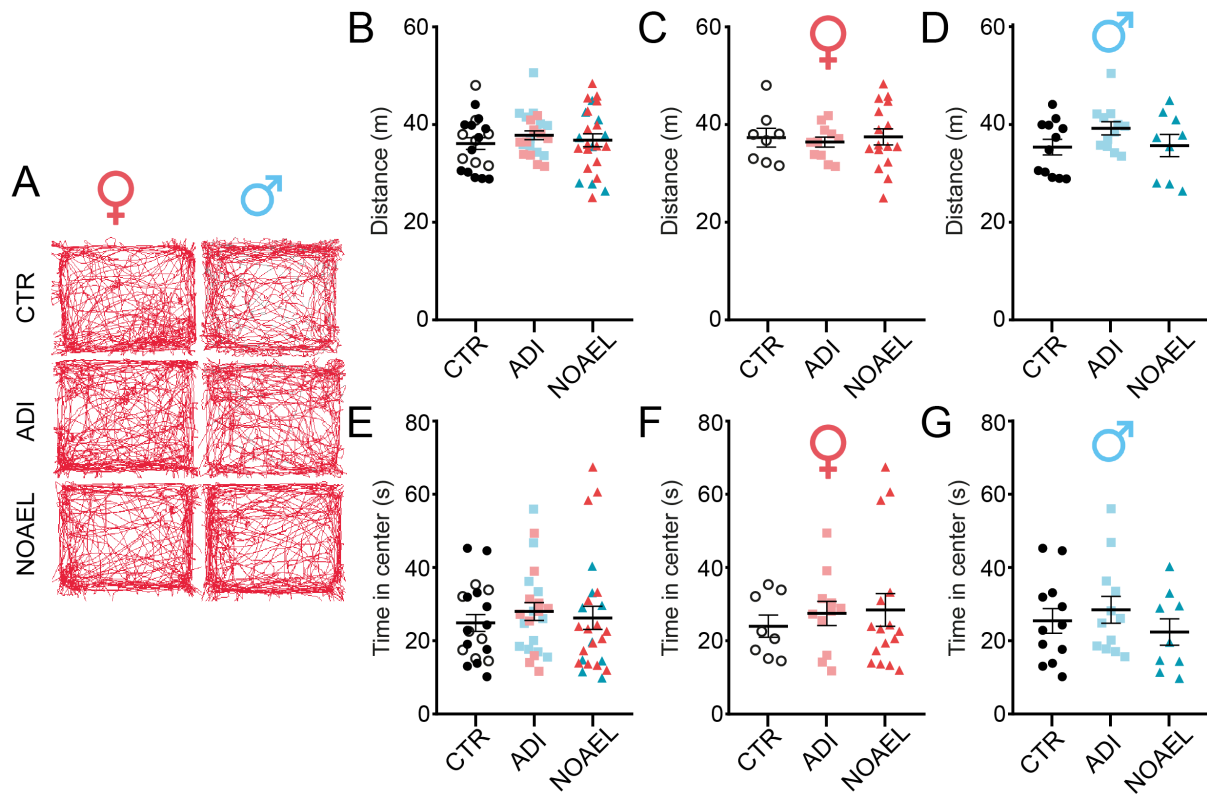
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842 **Supplemental Figure 2. Weight gain, food, and water intake monitoring. (A-B)** Pups' weight gain ( grams), food **(C-D)**, and water intakes **(E-F)** expressed as g or ml/gram of body weight (gBW)/day(d) were monitored from weaning. Males CTR, n=12 ; ADI, n=12 ; NOAEL, n=9 ; Females CTR, n=8 ; ADI, n=11; NOAEL, n=16. Males: Two-way ANOVA test (weight gain:  $p < 0.0001$ ; food intake:  $p = 0.0009$ ; water intake:  $p < 0.0001$ ); Females: Kruskal-Wallis test (weight gain:  $p = 0.7546$ ) and two-way ANOVA test (food intake:  $p = 0.0719$ ; water intake:  $p = 0.2042$ ). Asterisks indicate statistical significance (\*  $p < 0.05$ ; \*\*  $p < 0.01$ ; \*\*\*  $p < 0.001$ ; \*\*\*\*  $p < 0.0001$ ); \*: CTR vs NOAEL; \$: ADI vs NOAEL; #: CTR vs ADI; ns: non-significant. Statistical significance was reported between genotype comparisons under each graphic.

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855 **Supplemental Figure 3. Pre- and post-natal continuous glyphosate exposure and**  
 856 **adulthood behavior screening.** Mice were tested for the OF (A). The total distance traveled  
 857 (B-D) and time spent in the center zone (E-G) were measured over 10 minutes. Males CTR,  
 858 n=12; ADI, n=12 ; NOAEL, n=9 ; Females CTR, n=8 ; ADI, n=11 ; NOAEL, n=16. Males: One  
 859 way ANOVA test (Distance:  $p=0.2085$ ; Time spent in the center zone:  $p=0.5171$ ); Females:  
 860 One way ANOVA test (Distance:  $p=0.8771$ ) and Kruskal-Wallis test (Time spent in the center  
 861 zone:  $p=0.8258$ ); Males and Females: One way ANOVA test (Distance:  $p=0.5969$ ) and  
 862 Kruskal-Wallis test (Time spent in the center zone:  $p=0.524$ ).

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		WT CTR		WT ADI		WT NOAEL		P value
		Mean	SEM of discrepancy	Mean	SEM of discrepancy	Mean	SEM of discrepancy	
NEUROTRANSMISSION Fig. 1	Input/output test M							
	Shapiro-Wilk normality test M	yes		yes		yes		
	Two-way ANOVA (group effect) M							0,2748
	Input/output test F							
	Shapiro-Wilk normality test F	yes		yes		yes		
	Two-way ANOVA (group effect) F							0,1929
	Input/output test M+F							
	Shapiro-Wilk normality test M+F	yes		yes		yes		
	Two-way ANOVA (group effect) M+F							0,0074 ***
	Paired pulse facilitation test M	1,582 (N=12)	0,03019	1,498 (N=11)	0,03605	1,764 (N=6)	0,1372	
	Shapiro-Wilk normality test M	yes		yes		yes		
	Ordinary one-way ANOVA M+F							0,0236 *
Paired pulse facilitation test F	1,549 (N=7)	0,04379	1,539 (N=9)	0,0525	1,662 (N=16)	0,03939		
Shapiro-Wilk normality test F	yes		yes		yes			
Ordinary one-way ANOVA F							0,0985	
Paired pulse facilitation test M+F	1,57 (N=19)		1,516 (N=20)		1,69 (N=22)			
Shapiro-Wilk normality test M+F	yes		yes		yes			
Ordinary one-way ANOVA M+F							0,0033 ***	
NEURONAL PLASTICITY Fig. 2	Long-term potentiation maintenance test M	178,2 (N=10)	14,77	170,0 (N=10)	12,2	119,0 (N=7)	13,45	
	Shapiro-Wilk normality test M	no		yes		yes		
	Kruskal-Wallis test M							0,0109 *
	Long-term potentiation maintenance test F	146,7 (N=7)	12,9	151,0 (N=8)	6,112	127,8 (N=10)	8,495	
	Shapiro-Wilk normality test F	yes		yes		yes		
	Ordinary one-way ANOVA F							0,1657
	Long-term potentiation maintenance test M+F	162,0 (N=17)	9,933	164,3 (N=17)	7,555	121,8 (N=16)	7,424	
	Shapiro-Wilk normality test M+F	no		no		yes		
	Kruskal-Wallis test M+F							0,0003 ****
	SYNAPTIC DENSITY Fig. 3	synaptic density M	4058 (N=6)	618,9	4868 (N=6)	444,4	5709 (N=6)	570,7
Shapiro-Wilk normality test M	yes		no		no			
Kruskal-Wallis test M								0,0964
Synaptic density F	4808 (N=6)	573,6	5214 (N=6)	572,9	6324 (N=6)	594,9		
Shapiro-Wilk normality test F	yes		yes		yes			0,1692
Ordinary one-way ANOVA F								
Synaptic density M+F	4467 (N=6)	419,7	5034 (N=6)	355,8	6003 (N=6)	409,8		
Shapiro-Wilk normality test M+F	no		yes		yes			
Kruskal-Wallis test M+F								0,0243 *
NEUROINFLAMMATION MICROGLIA CORTEX Fig. 4	Sholl analysis M	(N=105)		(N=120)		(N=120)		
	Two-way ANOVA (group effect) M							<0,0001 ****
	Sholl analysis F	(N=120)		(N=117)		(N=120)		
	Two-way ANOVA (group effect) F							<0,0001 ****
NEUROINFLAMMATION MICROGLIA HIPPOCAMPUS Fig. 4	Sholl analysis M+F	(N=225)		(N=117)		(N=120)		
	Two-way ANOVA (group effect) M+F							<0,0001 ****
	Sholl analysis M	(N=90)		(N=119)		(N=115)		
	Two-way ANOVA (group effect) M							<0,0001 ****
NEUROINFLAMMATION ASTROCYTE HIPPOCAMPUS Fig. 4	Sholl analysis F	(N=120)		(N=113)		(N=120)		
	Two-way ANOVA (group effect) F							<0,0001 ****
	Sholl analysis M+F	(N=168)		(N=203)		(N=184)		
	Two-way ANOVA (group effect) M+F							<0,0001 ****
BEHAVIOR Fig. 5 and supplemental figure 3	Sholl analysis M	(N=75)		(N=90)		(N=90)		
	Two-way ANOVA (group effect) M							<0,0001 ****
	Sholl analysis F	(N=93)		(N=113)		(N=94)		
	Two-way ANOVA (group effect) F							0,0022 **
	Sholl analysis M+F	(N=168)		(N=203)		(N=184)		
	Two-way ANOVA (group effect) M+F							<0,0001 ****
	Open field distance (cm) M	3537 (N=12)	160,7	3921 (N=12)	134,4	2639 (N=9)	227,9	
	Shapiro-Wilk normality test M	yes		yes		yes		
	Ordinary one-way ANOVA M							0,2085
	Open field distance (cm) F	3154 (N=8)	191,9	3139 (N=11)	104	2492 (N=16)	165,6	
	Shapiro-Wilk normality test F	yes		yes		yes		
	Ordinary one-way ANOVA F							0,8771
Open field distance (cm) M+F	3613 (N=20)	121,9	3784 (N=23)	89,37	3681 (N=25)	132,2		
Shapiro-Wilk normality test M+F	yes		yes		yes			
Ordinary one-way ANOVA M+F							0,5969	
Open field time in center (s) M	25,47 (N=12)	3,373	28,49 (N=12)	3,672	22,44 (N=9)	3,607		
Shapiro-Wilk normality test M	yes		yes		yes			
Ordinary one-way ANOVA M							0,5171	
Open field time in center F	23,99 (N=8)	3,039	27,48 (N=11)	3,311	28,41 (N=16)	4,462		
Shapiro-Wilk normality test F	yes		yes		yes			
Kruskal-Wallis test F								0,8258
Open field time in center M+F	24,88 (N=20)	2,311	28,01 (N=23)	2,432	26,26 (N=25)	3,141		
Shapiro-Wilk normality test M+F	yes		yes		no			
Kruskal-Wallis test M+F								0,524
Discrimination ratio M	0,7286 (N=12)	0,02573	0,692 (N=12)	0,02257	0,6479 (N=9)	0,06177		
Shapiro-Wilk normality test M	yes		yes		yes			
Ordinary one-way ANOVA M							0,3232	
Discrimination ratio F	0,7332 (N=8)	0,04032	0,7214 (N=11)	0,04381	0,6225 (N=16)	0,04318		
Shapiro-Wilk normality test F	yes		yes		yes			
Ordinary one-way ANOVA F							0,1502	
Discrimination ratio M+F	0,7305 (N=20)	0,02167	0,7061 (N=23)	0,02366	0,6316 (N=25)	0,03478		
Shapiro-Wilk normality test M+F	yes		yes		yes			
Ordinary one-way ANOVA M+F							0,041 *	
PHYSIO PARAMETERS Supplemental figure 2	Weight gain (%) M							
	Shapiro-Wilk normality test M	yes		yes		yes		
	Two-way ANOVA (group effect) M							0,0182 *
	Weight gain (%) F							
	Shapiro-Wilk normality test F	no		no		yes		
	Kruskal-Wallis test F							0,7546
	Food intake (g/kg bw/d) M							
	Shapiro-Wilk normality test M	yes		yes		yes		
	Two-way ANOVA (group effect) M							0,1705
	Food intake (g/kg bw/d) F							
	Shapiro-Wilk normality test F	yes		yes		yes		
	Two-way ANOVA (group effect) F							0,1229
Water intake (g/kg bw/d) M								
Shapiro-Wilk normality test M	yes		yes		yes			
Two-way ANOVA (group effect) M							0,9994	
Water intake (g/kg bw/d) F								
Shapiro-Wilk normality test F	yes		yes		yes			
Two-way ANOVA (group effect) F							0,9957	

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Supplemental Table 1. Summary of statistical analyses.