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► To cite this version:

Noemie Cresto, Margot Courret, Athénaïs Génin, Céline Marie Pauline Martin, Julie Bourret, et al.. Continuous low-level dietary exposure to glyphosate elicits dose and sex-dependent synaptic and microglial adaptations in the rodent brain.. *Environmental Pollution*, 2024, 345, pp.123477. 10.1016/j.envpol.2024.123477 . hal-04444827

HAL Id: hal-04444827

<https://hal.science/hal-04444827v1>

Submitted on 12 Nov 2024

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1 Continuous low-level dietary exposure to glyphosate
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11
12 **Running title:** Neuro-glial cell adjustments to dietary glyphosate.

13 **Keywords:** glyphosate, NOAEL, ADI, perinatal exposure, post-natal exposure,
14 neuronal transmission, neuroglia.

15
16 Number of text pages: 37

17 Number of words: 3842

18 Number of figures: 5

19 Number of Supplemental Figures: 3

20 Number of Supplemental Tables: 1

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32
33
34 Acknowledgment: This work was supported by ANR-Glyflore to LGP, SES, JP, and
35 NM, ANR-Hepatobrain to NM, EnviroDisorders to JP, ANR-CEST-Focus, ANR-
36 EpiCatcher to NM, ANR Microsenso to EA

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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⁺ Homer1⁺ synapses. Microglial Iba1⁺ 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⁺ 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 ¹. 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 ²⁻⁴. Regulatory bodies worldwide have granted
125 glyphosate's approval for use; however, concerns regarding its impact on humans and
126 the environment steadily mount ⁵.

127

128 Glyphosate was originally employed as an inhibitor of 5-enolpyruvylshikimate-
129 3-phosphate synthase in plants ⁶. From this initial framework, glyphosate was shown
130 to impact bacteria, including within the microbiota ⁷, and other physiological functions,
131 such as at the brain level ⁸. Epidemiological studies have suggested a potential
132 association between exposure to environmental contaminants, such as glyphosate,
133 and neurodevelopmental risks ⁸, although controversy exists ⁹. 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 ^{8 10-19}. More
138 recently, a paradigm shift has emerged, involving consumer-relevant modalities for
139 exposure to contaminants ²⁰. 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 ²¹⁻²³.

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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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 μ m-thick slices in an ice-cold solution of artificial cerebrospinal fluid
238 containing sucrose (aCSF sucrose composed in mM of 87 NaCl, 25 NaHCO₃, 75
239 sucrose, 10 D-glucose, 2.5 KCl, 1 NaH₂PO₄, 7 MgCl₂ and oxygenated with carbogen
240 (95% oxygen (O₂) and 5% carbon dioxide (CO₂)) 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 NaHCO₃, 11 D-glucose, 2.5 KCl, 1
244 NaH₂PO₄, 1.3 MgSO₄·7H₂O 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 ²⁴. 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 ²⁵. It consists of applying concentric circles
296 from the center of the cell soma spaced 5 μm 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 μm , 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 μm intervals over a total of 5 μm for 30 optical sections. For image
304 analysis, automated quantification was performed using the Distance Analysis plugin
305 (DiAna)²⁶. 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 μm^3 .

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316 *Statistical analyses.*

317

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.*

345

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) ²⁷. 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 ²⁸. Previous evidence showed that,
397 in mice, glyphosate ingestion triggers neuroinflammatory changes, although evidence
398 refers to high-level exposures ^{29,30}. 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**

441

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 ²³. We should further investigate the functional relevance of the
452 observed neuronal and microglial changes, especially considering their potential role
453 across brain diseases. ³¹⁻³³.

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 ³⁴. In rodents, GBH negatively impacted neuronal transmission and
463 behavioral outputs ^{14,35}. Maternal exposure to GBH at high levels induced autistic-like
464 deficiencies in male offspring ³⁶. Glyphosate or GBH exposure has been reported to

465 cause perturbations in neurodevelopmental processes, leading to long-term
466 neurophysiological changes in animal models³⁷⁻⁴⁰. At doses of 250 or 500 mg/kg GBH,
467 offspring showed behavioral changes¹². Both glyphosate and GBH were shown to
468 cause disruption of neurogenesis accompanied by compensatory responses,
469 modifying synaptic plasticity in the hippocampus⁴⁰. Exposure to GBH leads to
470 glutamate excitotoxicity, oxidative damage, and astrocyte dysfunction in offspring
471 hippocampus¹⁴. TNF-alpha increased brain levels were reported in mice pre-natally
472 exposed to GBH 0.3 mg/Kg³⁰.

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^{21,23}. 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
478²³. Sex-dependent modifications in social preference were found to result from NOAEL
479 dietary exposure²¹. Assessing memory using novel object recognition, we previously
480 found that male mice are not affected by NOAEL glyphosate exposure⁴¹. 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 deficits¹².

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 ⁴², and high-exposure intoxications can occur ^{43,44}. Glyphosate was
493 reported to cross the placental and blood-brain barrier, events that could favor
494 neurodevelopmental modifications ^{45–47}. 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 ^{48 49}.

497

498 As working hypotheses, exposure to glyphosate could trigger peripherally-
499 mediated (e.g., immune responses ⁴⁵ or modified microbiota within a gut-brain axis
500 ^{50,51}) or direct damage to the brain borders and cerebrovascular permeability ²².
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 ²³. Our results revealed cellular adjustments in the brain, although we did not delve
504 into specific mechanisms ^{5,8}. 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 ^{52–55}. Their function is fundamental for synaptic
510 maturation and establishing functional circuits ⁵⁶. 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 ²⁸. 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 ¹³ and
535 modify brain monoaminergic neurotransmitter levels in rodents ¹⁹. 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 ⁵⁷. 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 ^{22 38}.

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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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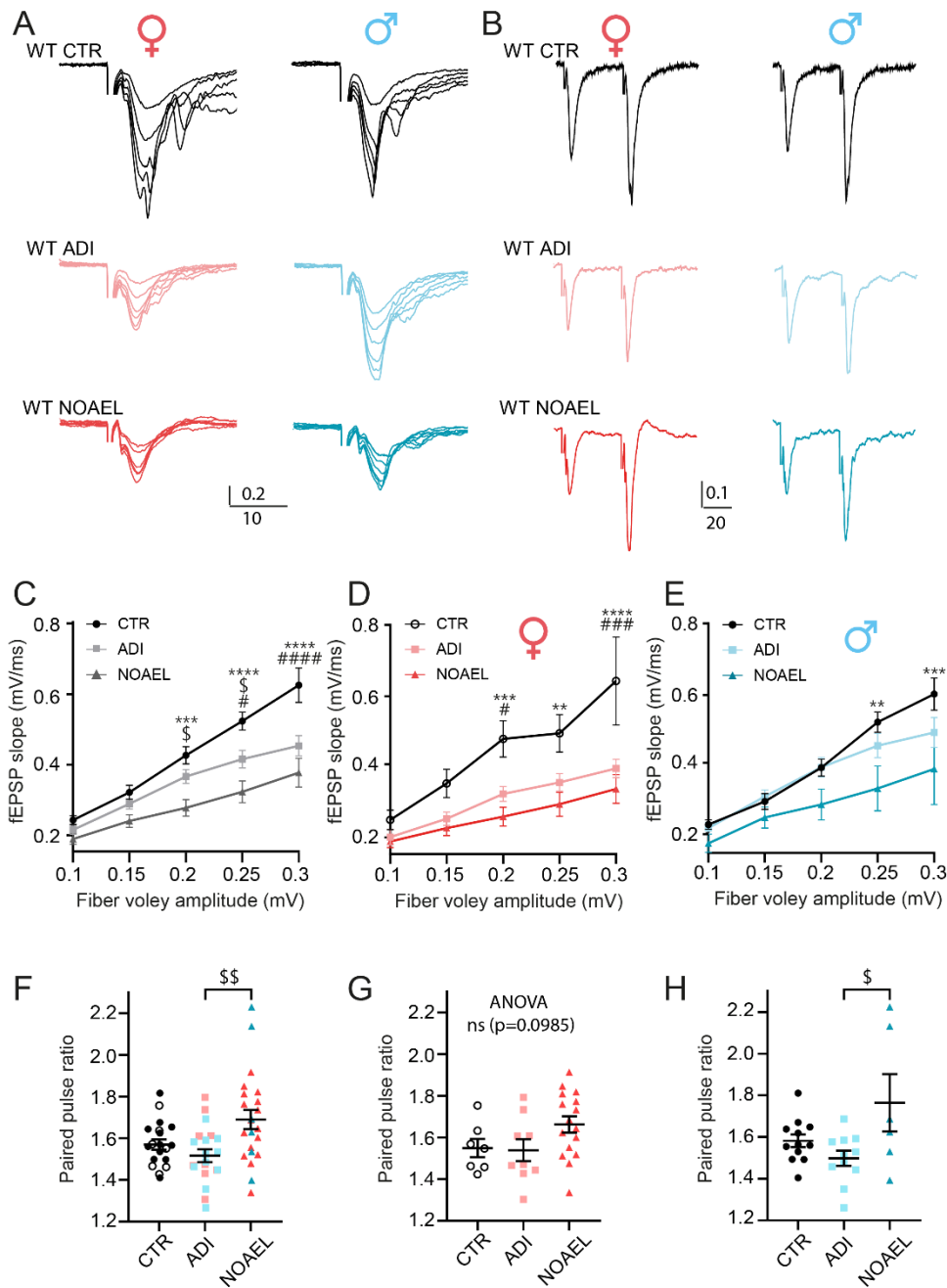
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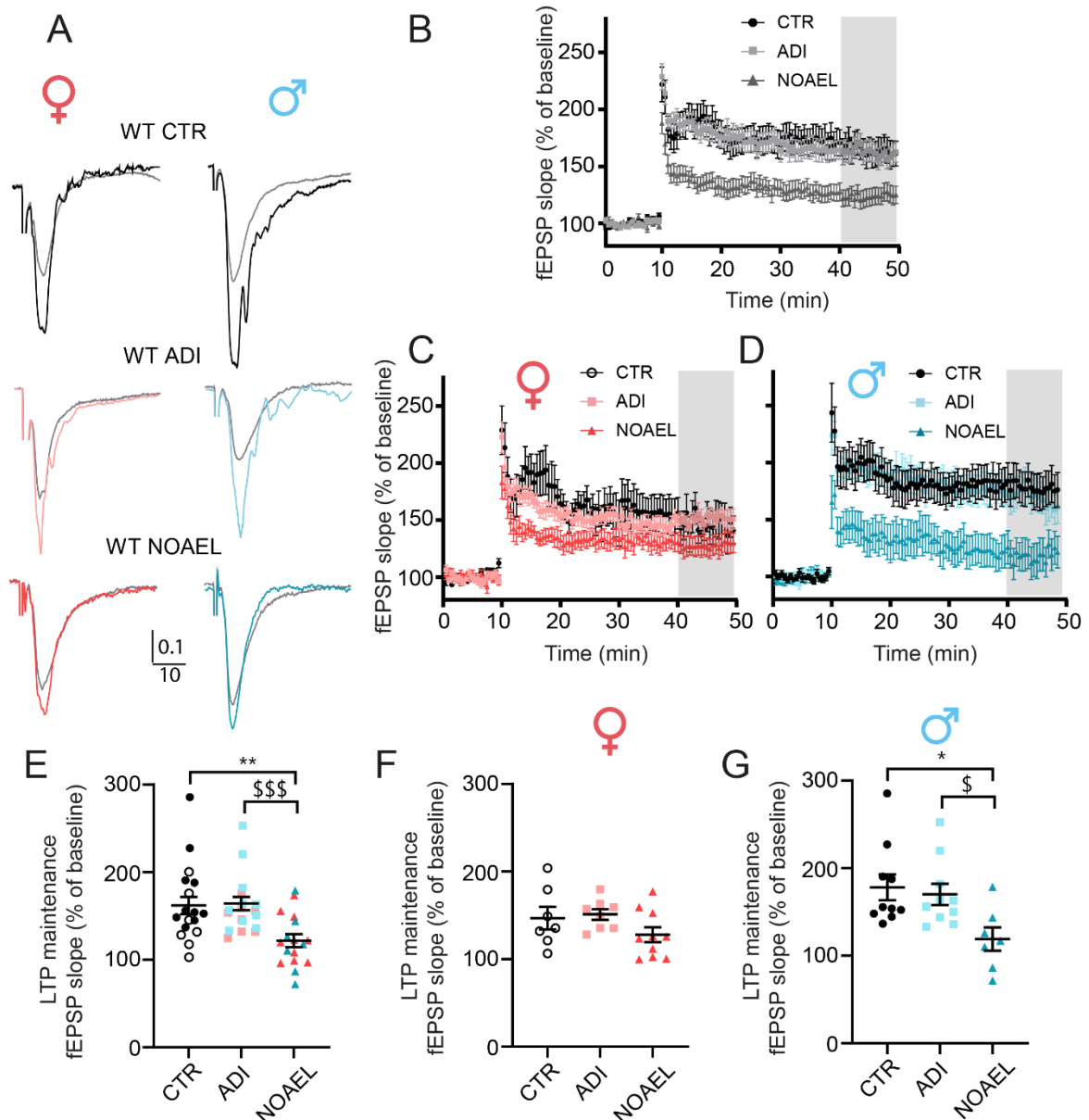


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733 **Figure 1. Basal excitatory synaptic transmission is reduced in the adult brain of ADI and**
 734 **NOAEL glyphosate-exposed mice. (A, C to E)** The input/output curve shows that basal
 735 excitatory synaptic transmission at CA1 Schaffer collateral 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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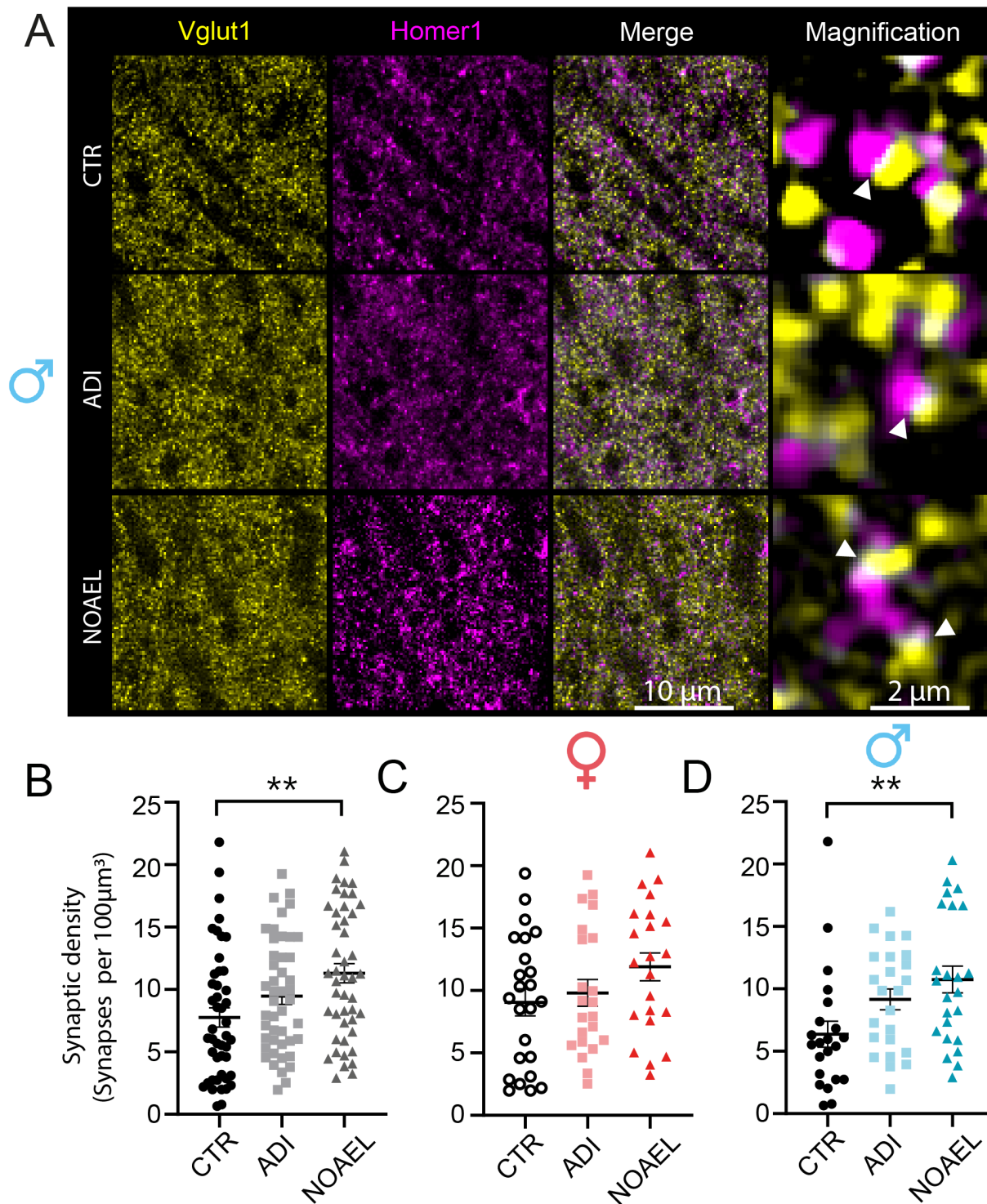
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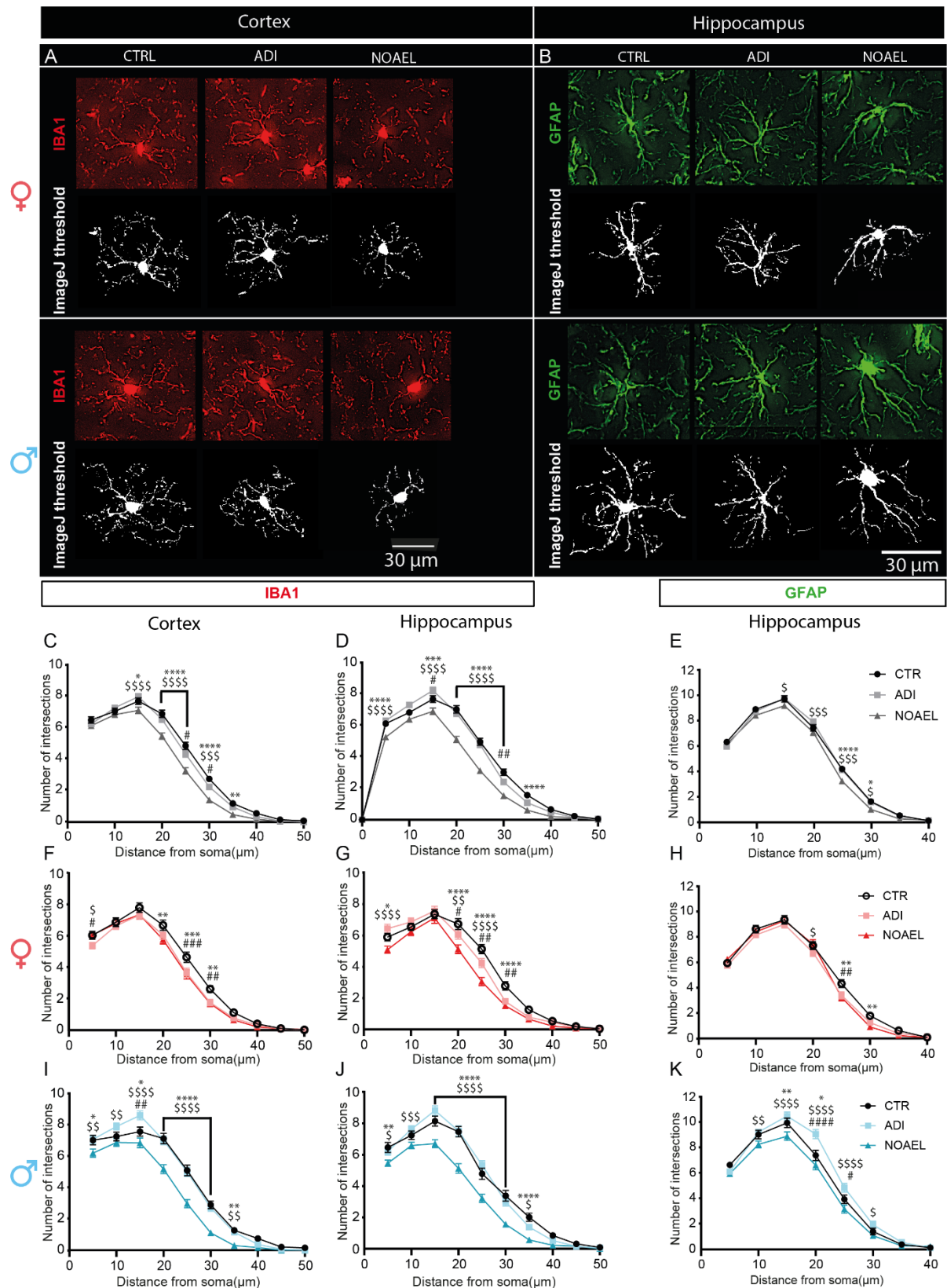
748 **Figure 2. Long-term potentiation in the adult brain is reduced in NOAEL glyphosate**
 749 **exposure conditions.** In all mice groups, LTP was measured by field potential recordings
 750 (fEPSPs) at CA1 Schaffer collateral synapses. The light gray box highlights the last 10 min of
 751 the recording 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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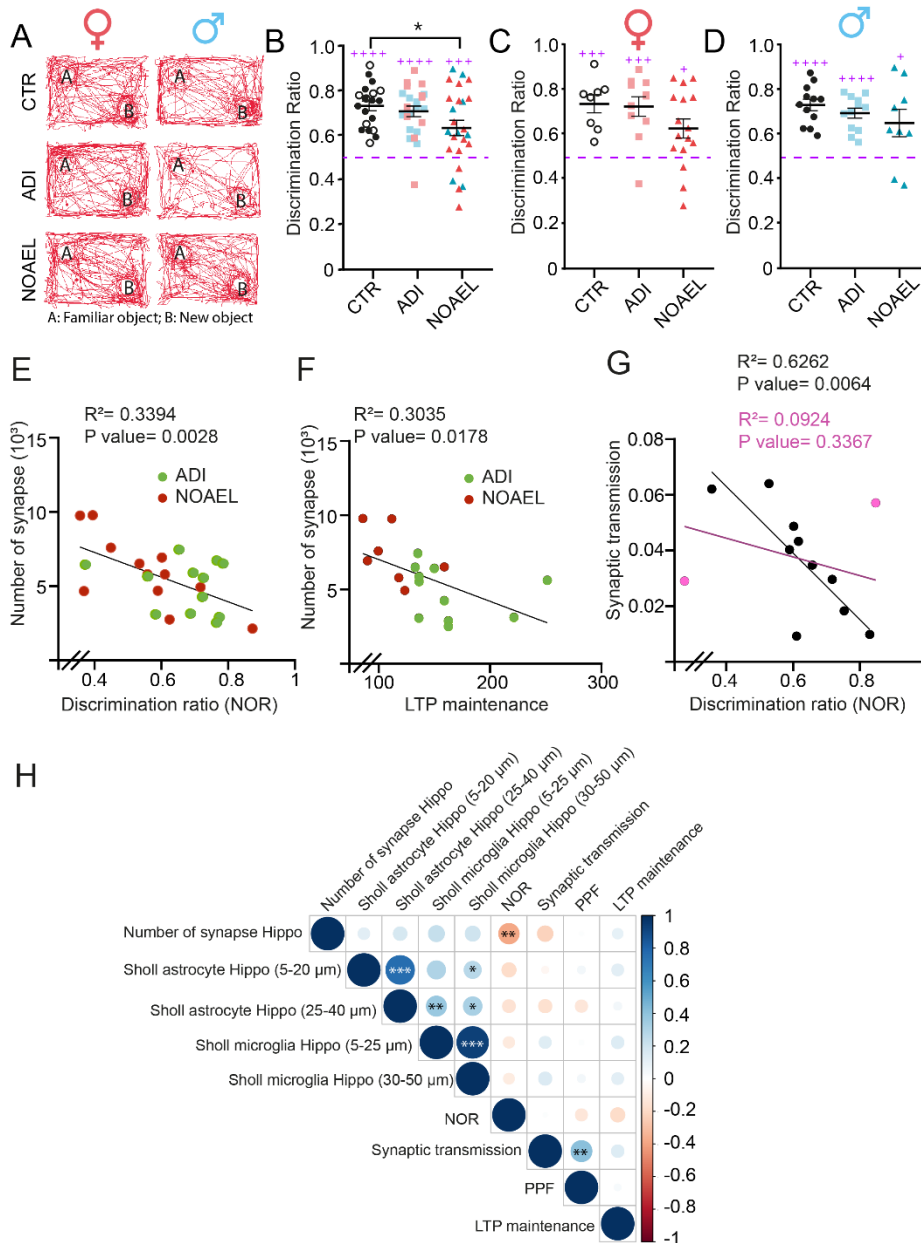
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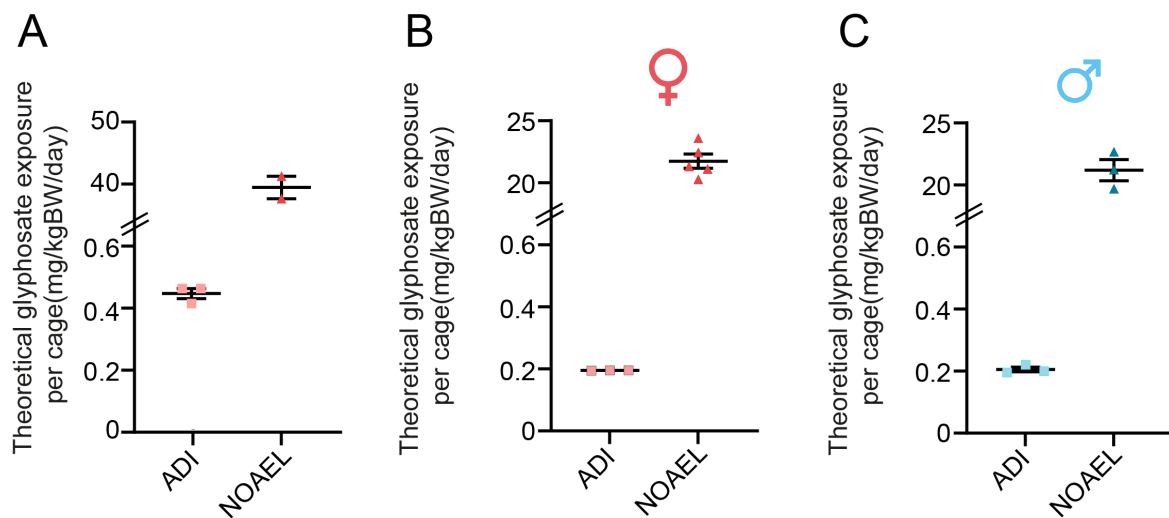
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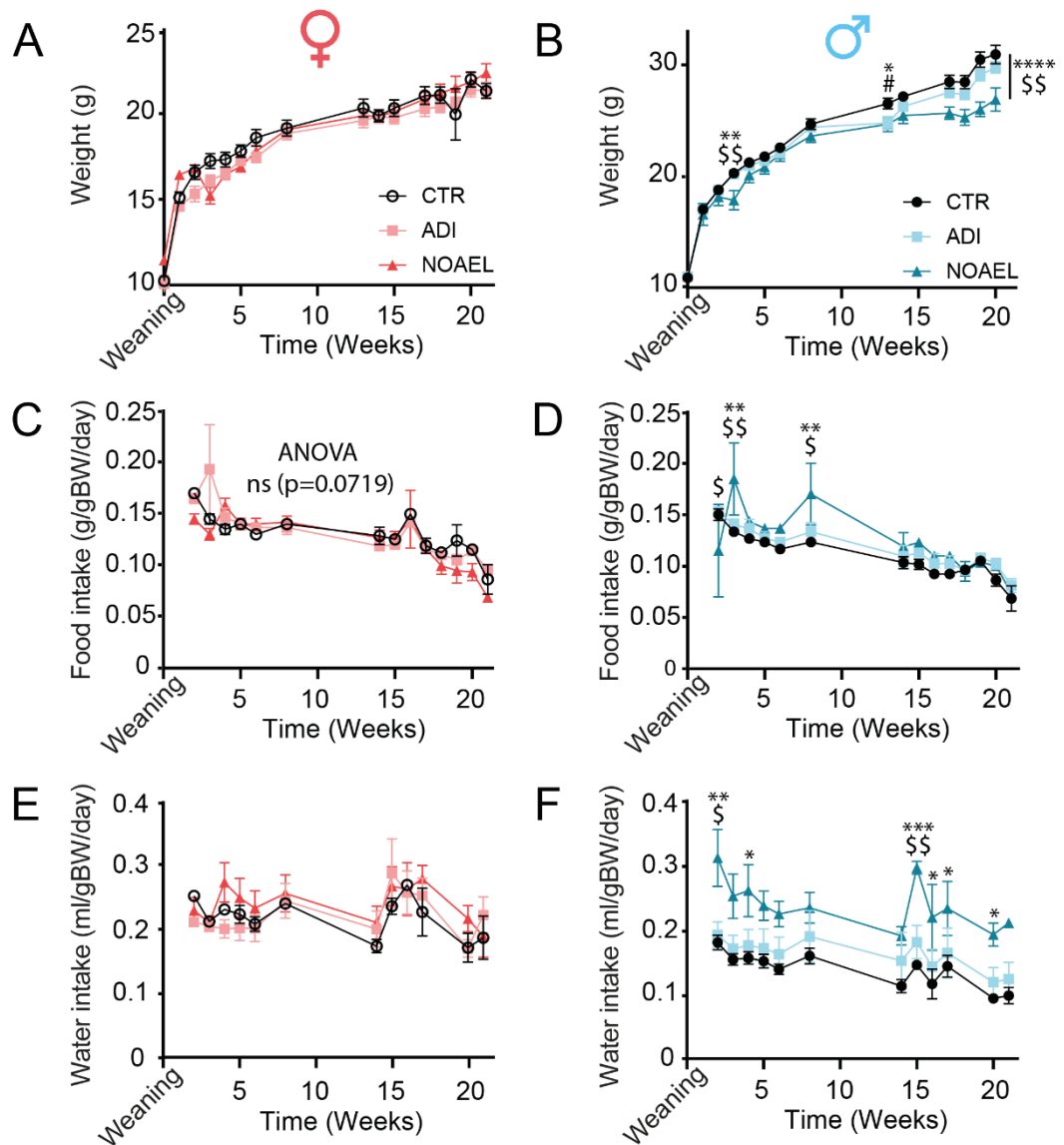
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Figure 5. Mouse-specific cross-correlation of neurophysiological and histological data set. (A-D) 24 hours after the open field test, mice were exposed to two similar objects in the arena, and 24 hours later, one familiar object was replaced by a new 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 R² 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 *: R²>0.35, P<0.01; **: R²>0.4, P<0.001; ***: R²>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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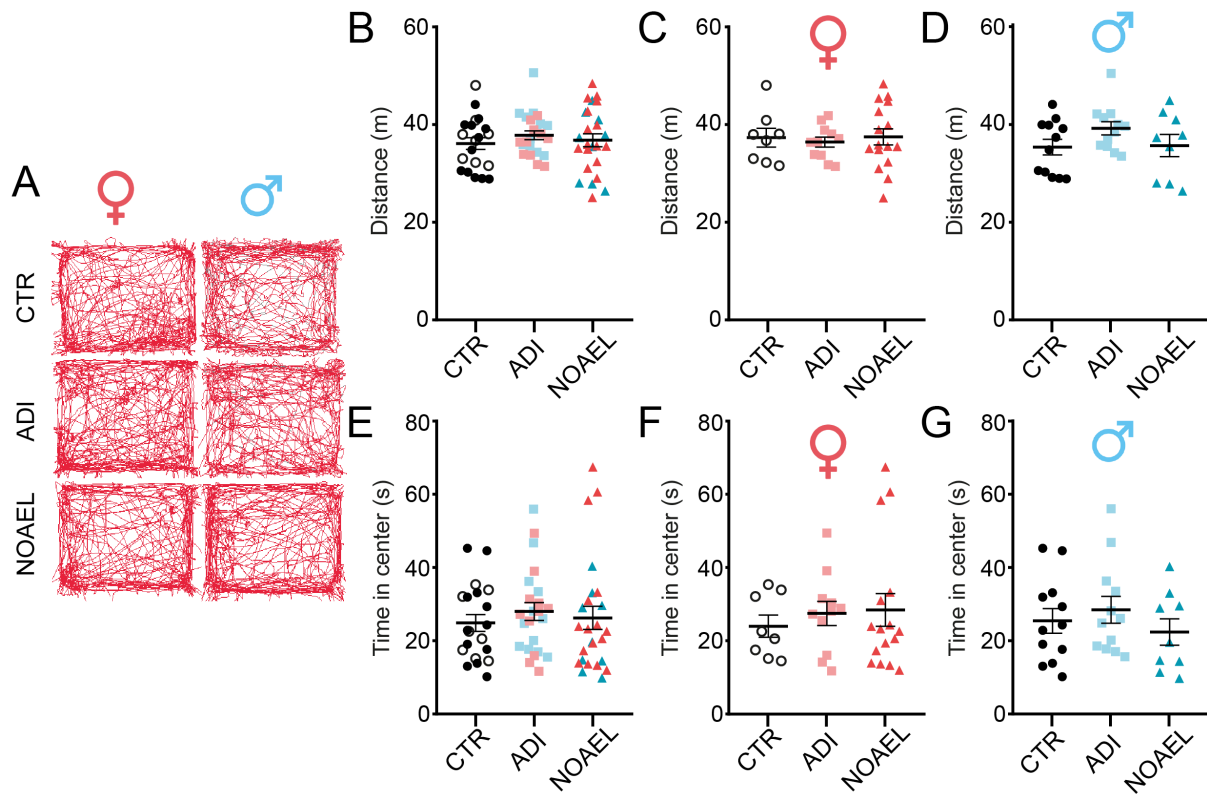
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842 **Supplemental Figure 2. Weight gain, food, and water intake monitoring. (A-B)** Pups' weight gain (grams), food **(C-D)**, and water intakes **(E-F)** expressed as g or ml/gram of body weight (gBW)/day(d) were monitored from weaning. Males CTR, 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 <i>Fig. 1</i>	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 <i>Fig. 2</i>	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 <i>Fig. 3</i>	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 <i>Fig. 4</i>	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 <i>Fig. 4</i>	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 <i>Fig. 4</i>	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 <i>Fig. 5 and supplemental figure 3</i>	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 <i>Supplemental figure 2</i>	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.