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## Cognitive and hippocampal effects of adult male mice exposure to environmentally relevant doses of phthalates

Suzanne Ducroq, Eric Duplus, Valérie Grange-Messent, Francesca Trivelloni,  
Lucille Penalva-Mousset, Isabelle Petropoulos, Sakina Mhaouty-Kodja

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1 **Title:** Cognitive and hippocampal effects of adult male mice exposure to environmentally relevant  
2 doses of phthalates

3

4 **Authors**

5 Suzanne Ducroq<sup>1</sup>, Eric Duplus<sup>2</sup>, Valérie Grange-Messent<sup>1</sup>, Francesca Trivelloni<sup>1</sup>, Lucille Penalva-  
6 Mousset<sup>2</sup>, Isabelle Petropoulos<sup>2</sup>, Sakina Mhaouty-Kodja<sup>1</sup>

7

8 **Author affiliations**

9 <sup>1</sup>Sorbonne Université, CNRS UMR 8246, INSERM U1130, Neuroscience Paris Seine – Institut de  
10 Biologie Paris Seine, 75005 Paris, France.

11 <sup>2</sup>Sorbonne Université, CNRS UMR 8256, INSERM ERL1164, Biological Adaptation and Ageing -  
12 Institut de Biologie Paris-Seine, 75005 Paris, France.

13

14 **Corresponding author**

15 Sakina Mhaouty-Kodja

16 Sorbonne Université, CNRS UMR 8246, INSERM U1130

17 7 quai St Bernard, Bât A 3ème étage 75005, Paris, France.

18 Tel: +331 44 27 91 38

19 email: sakina.mhaouty-kodja@sorbonne-universite.fr

20

21 **Abstract**

22 We recently showed that chronic exposure of adult male mice to environmental doses of DEHP alone  
23 or in a phthalate mixture altered blood brain barrier integrity and induced an inflammatory profile in  
24 the hippocampus. Here, we investigate whether such exposure alters hippocampus-dependent  
25 behavior and underlying cellular mechanisms. Adult C57BL/6J male mice were continuously  
26 exposed orally to the vehicle or DEHP alone (5 or 50 µg/kg/d) or to DEHP (5 µg/kg/d) in a phthalate  
27 mixture. In the Morris water maze, males showed reduced latencies across days to find the platform  
28 in the cue and spatial reference memory tasks, regardless of their treatment group. In the probe test,  
29 DEHP-50 exposed males displayed a higher latency to find the platform quadrant. In the temporal  
30 order memory test, males exposed to DEHP alone or in a phthalate mixture were unable to  
31 discriminate between the most recently and previously seen objects. They also displayed reduced  
32 ability to show a preference for the new object in the novel object recognition test. These behavioral  
33 alterations were associated with a lowered dendritic spine density and protein levels of glutamate  
34 receptors and postsynaptic markers, and increased protein levels of the presynaptic synaptophysin in  
35 the hippocampus. Metabolomic analysis of the hippocampus indicated changes in amino acid levels  
36 including reduced tryptophan and L-kynurenine and elevated NAD<sup>+</sup> levels, respectively, a precursor,  
37 intermediate and endproduct of the kynurenine pathway of tryptophan metabolism. Interestingly, the  
38 protein amounts of the xenobiotic aryl hydrocarbon receptor, a target of this metabolic pathway, were  
39 elevated in the CA1 area. These data indicate that chronic exposure of adult male mice to  
40 environmental doses of DEHP alone or in a phthalate mixture impacted hippocampal function and  
41 structure, associated with modifications in amino acid metabolites with a potential involvement of  
42 the kynurenine pathway of tryptophan metabolism.

43

44 **Keywords**

45 Phthalates; Plasticizers; Nervous system; Behavior; Hippocampus; Learning and memory; Endocrine  
46 Disruptors.

47

48 **Introduction**

49 Plasticizers such as phthalates are widely spread in the environment. Di(2-ethylhexyl)phthalate  
50 (DEHP), the most abundant substance of this family, was identified as an endocrine disruptor for both  
51 human health and environment due to its ability to reduce fetal testosterone production (European  
52 Chemical Agency (ECHA), 2014; European Food Safety Agency (EFSA), 2005; EFSA et al., 2019).  
53 Other phthalates such as benzyl butyl phthalate (BBP), dibutyl phthalate (DBP), diisobutyl phthalate  
54 (DIBP) also detected in the environment were also recognized as substances of very high concern  
55 due to their reprotoxicity and endocrine disrupting activity (ECHA site; EFSA et al., 2019). We have  
56 previously shown that exposure of adult male mice to DEHP at environmental doses reduced the  
57 expression of male sexual behavior possibly through an endocrine mode of action involving down-  
58 regulation of the androgen receptor (AR), without any effects on the estrogen receptor  $\alpha$  (ER $\alpha$ ), in  
59 the neural circuitry underlying this behavior (Dombret et al., 2017). In the male nervous system,  
60 testosterone can act directly through AR activation or be metabolized into neural estradiol, which then  
61 stimulates ERs. These receptors are expressed in several brain areas including the hippocampus and  
62 cortex (Raskin et al., 2009; Simerly et al., 1990). Several studies using pharmacological or genetic  
63 tools that impacted either testosterone levels or AR and ER activation or expression showed that these  
64 signaling pathways modulate spatial memory and temporal processing of objects through molecular  
65 and cellular mechanisms involved in synaptic plasticity in male rodents (Liu et al., 2008; Packard et  
66 al., 1996; Picot et al., 2016). These behaviors could thus be influenced by exposure to endocrine  
67 disrupting compounds such as phthalates, which can impact either gonadal production of testosterone  
68 and/or their neural signaling pathways.

69  
70 The effects of phthalates on learning and memory and related hippocampal processes have been  
71 widely studied in particular for the developmental period of exposure (Barakat et al., 2018; Holahan  
72 et al., 2018; Kougias et al., 2018; Lv et al., 2022; Safarpour et al., 2021; Sun et al., 2014). Whether  
73 adult exposure to these substances also triggers adverse effects was less addressed. Previous studies

74 analyzed the effects of acute or sub-chronic exposure to dibutyl phthalate (DBP) or diisononyl  
75 phthalate (DINP) at doses ranging from 0.2 to 250 mg/kg/d on learning and memory in young adult  
76 mice (Ding et al., 2017; Farzanehfar et al., 2016; Peng, 2015; Song et al., 2021). Exposure for 9 or  
77 14 days to DINP at 150 or 200 mg/kg/d resulted in increased latency to find the platform during the  
78 training and test phases and reduced the time spent in the target quadrant in the retention test  
79 performed two days later in the Morris water maze (Peng, 2015). No effects were observed at lower  
80 DINP doses (0.2, 1.5 or 2 mg/kg/d). Comparable data were reported for an exposure period of 28  
81 days to DINP at 200 mg/kg/d (Song et al., 2021). Using the same task, DBP exposure for 5 weeks  
82 was shown to induce a higher escape latency at the last day of the training phase and reduce time  
83 spent in the target quadrant and swimming speed in the retention task (Ding et al., 2017). These  
84 effects were observed only for the group exposed to DBP at 250 mg/kg/d but not at lower doses (10  
85 and 50 mg/kg/d). Male mice exposed for 14 days to DBP at 6.25 to 200 mg/kg/d exhibited a lower  
86 latency in the passive avoidance test and reduced time spent in the open arms of the elevated plus  
87 maze, but only at the doses of 25 and 200 mg/kg/d (Farzanehfar et al., 2016). It thus appears from  
88 these studies that acute/sub-chronic exposure to phthalates induced effects on learning and memory  
89 at high doses (25 mg/kg/d to 250 mg/kg/d), which are not relevant for the environmental exposure.  
90 We have recently shown that chronic exposure of adult male mice to environmentally relevant doses  
91 of DEHP alone or in a phthalate mixture increased blood brain barrier permeability and affected  
92 endothelial accessory tight junction proteins in the hippocampal CA1 and CA3 areas (Ahmadpour et  
93 al., 2021). This was associated with an inflammatory profile including microglial activation and  
94 induced cyclooxygenase-2 expression. Whether these cellular modifications triggered by low doses  
95 of DEHP alone or in phthalate mixture are accompanied with impaired hippocampus-dependent  
96 behaviors remains to be investigated.

97  
98 The present study was therefore undertaken to investigate the effects of chronic exposure to DEHP  
99 alone or in a phthalate mixture on learning and memory in male mice. Adult C57BL/6J male mice

100 were exposed orally to the vehicle (controls), DEHP alone at 5 or 50 µg/kg/d, or to a phthalate mixture  
101 as previously described (Adam et al., 2021; Ahmadpour et al., 2021). Exposure started eight weeks  
102 before the beginning of behavioral analyses and treatment was maintained throughout the period of  
103 analyses. Males were assessed for spatial memory, temporal order memory and novel object  
104 recognition. As the execution of these behaviors can be altered by the anxiety-state level of animals,  
105 anxiety-related behavior was also assessed. Analyses of dendritic spine density, pre- and post-  
106 synaptic components, synapse ultrastructure and metabolome were conducted in the hippocampus.  
107 General physiological parameters such as body and androgen-sensitive organ weights, and  
108 hippocampal protein amount levels of AR, ER $\alpha$  and the xenobiotic aryl hydrocarbon-receptor (AhR)  
109 were also determined.

110

## 111 **Materials and Methods**

### 112 *Animals and treatments*

113 The study was conducted according to the European legal requirements (Decree 2010/63/UE) and  
114 received the approval of the «Charles Darwin» Ethical committee (project number 01490-01). Male  
115 C57BL/6J mice (Janvier Labs) were housed at 22°C and 12:12 h light-dark cycle (the dark phase was  
116 scheduled from 1:30 P.M. to 1:30 A.M.) and treated as detailed in Supplemental Material and  
117 Methods. Briefly, male mice of eight-week-old were fed with chow containing the vehicle (control  
118 group), DEHP (CAS 117-81-7) at the tolerable daily intake dose of 50 µg/kg/d (EFSA, 2005, 2019),  
119 DEHP at the dose of 5 µg/kg/d in the range of the estimated environmental exposure, or to a phthalate  
120 mixture containing DEHP at 5 µg/kg/d, DBP (CAS 8474-2) at 0.5 µg/kg/d, BBP (CAS 85-68-7) at  
121 0.5 µg/kg/d, DIBP (CAS 84-69-5) at 0.5 µg/kg/d and di-ethylphthalate (DEP; CAS 84-66-2) at 0.25  
122 µg/kg/d. The phthalate doses were weekly adjusted to male body weights. The exposure started eight  
123 weeks before the beginning of analyses and was maintained until sacrifice as described in Figure S1.  
124 Two cohorts of male mice, each including four treated groups, were used in the behavioral tests  
125 (Figure S1). At the end of the tests, animals were euthanized using pentobarbital injection (120  
126 mg/kg). The brains were collected for analyses by Golgi-cox staining, Western blot and transmission  
127 electron microscopy. A third cohort was used for metabolomic analysis. The collected seminal  
128 vesicles and testes from these cohorts were weighed, and the results were expressed as percentage of  
129 body weight.

130

### 131 *Behavioral tests*

132 Male mice were subjected to the behavioral tests described below and in Figure S1. Tests, which were  
133 conducted during the light phase from 6:00 to 11:00 A.M, were recorded and the videos were analysed  
134 as previously described (Dombret et al., 2020; Picot et al., 2016).

135

### 136 *Morris Water Maze*

137 The tasks were performed in a large circular pool (2.0 m diameter, 0.5 m height) filled with water,  
138 which was maintained at a temperature of  $25^{\circ}\text{C} \pm 1^{\circ}\text{C}$  and made opaque with 400 ml of latex liquid.  
139 After each trial, the animals were dried off and returned to cages placed on heating pads. The test  
140 consisted of two training phases (cue task and spatial reference memory) before the probe test. In the  
141 cue task, mice were trained to locate a cue placed on a visible platform (13 cm diameter) on four trials  
142 per day for three days. Each trial lasted 90 sec, with an inter-trial interval of around 20 to 25 min. The  
143 platform was located in different quadrants of the pool and the mice were released at various points  
144 around the swimming pool. The location of the quadrant and point of release were counterbalanced  
145 every day. The animals were placed on the platform for 30 sec if they did not find it within the 90 sec  
146 trial.

147 In the spatial reference memory task, mice were trained to locate a hidden platform, with visible  
148 spatial landmarks, on four trials per day for five days. Each trial lasted 90 sec, with an inter-trial  
149 interval of around 10 min. The platform was located in one quadrant of the pool and the mice were  
150 released at various points around the swimming pool. The animal was placed on the platform for 30  
151 sec if it did not find it within the 90 sec trial. Ten minutes after the last trial, the platform was removed;  
152 the probe test lasted for 60 seconds. Measures included the latency to find the platform and swimming  
153 velocity.

154

#### 155 *Object recognition tasks*

156 The temporal order task was conducted in a round arena (45 cm diameter) filled with sawdust as  
157 previously described (Picot et al., 2016). During the habituation phase, the animals were put for two  
158 days in the arena in the absence of objects (one 30 min session followed by two daily 10 min sessions)  
159 and then exposed for one day to objects in place in the arena to decrease neophobia. During the  
160 familiarization phase of three sessions, the mice were allowed to explore two objects (a–a, b–b and  
161 c–c) for at least 15 seconds per object, with an interval of 3 min between the sessions. Ten minutes  
162 later, the males were subjected to a 5-min testing phase in the arena, where they were placed in the



163 presence of the first (a) and last (c) object seen. The discrimination index (DI) between objects was  
164 defined as the difference in the time (t) spent exploring the first (tFi) and last objects (tL) divided by  
165 the total time spent exploring the two objects [DI = (tFi - tL)/(tFi + tL)].

166 In the novel object recognition task, the habituation and familiarization phases were performed as  
167 described for temporal order processing. In the testing phase, the animals were exposed to a familiar  
168 object (a) they had seen before and a new object (d). The discrimination index between objects was  
169 defined as the difference in the time (t) spent exploring the novel (tN) and the familiar objects (tFa)  
170 divided by the total time spent exploring the objects [DI = (tN - tFa)/(tN + tFa)].

171

#### 172 *Analysis of anxiety-related behavior*

173 The anxiety state level was measured in the elevated zero maze as previously described (Dombret et  
174 al., 2020; Trouillet et al., 2022). Male mice were first placed in one of the closed arms of the maze.  
175 The test ended after 9 min of free exploration of the maze. The entry in the open arm was taken into  
176 account when all four paws of the mouse were inside this area. The analyses included the number of  
177 entries and the time spent in the open arms.

178

#### 179 *Golgi-cox staining*

180 The brains were fixed for eight days in Golgi-cox solution (NovaUltra Golgi-cox Stain kit, Interchim,  
181 Montluçon, France) as previously described (Jean et al., 2017), and then cut into 30 µm-thick coronal  
182 sections with a vibratome (Leica Microsystems VTC1000S, Wetzlar, Germany). Sections including  
183 the hippocampus, identified using neuroanatomical landmarks of the Mouse Brain Atlas (Paxinos and  
184 Franklin, 2001), were immersed in post-impregnation solution for 10 min, washed in phosphate buffer  
185 saline and mounted with Mowiol. Observations were made using the Zeiss Axiovert 200M  
186 microscope equipped with an AxioCam MRm camera. For each animal, 10 neurons homogeneously  
187 distributed throughout the CA1 (Bregma 2.18, plate 49) or CA2/3 (Bregma 2.30, plate 50) areas were  
188 analyzed. For each neuron, the analyses were performed on a dendritic length of 50 µm localized at

189 30 µm from the start of the dendrite. Thin, stubby and mushroom subtypes of dendritic spines were  
190 determined by applying criteria described by Harris et al. (1992).

191

### 192 *Western blotting*

193 Protein extracts from the CA1 or CA2/3 areas were prepared as previously described for AR and ERα  
194 immunodetection (Capela et al., 2018; Picot et al., 2016) and pre- and post-synaptic proteins (Jean et  
195 al., 2017). Protein samples (20 to 30 µg) were separated on 4-12% polyacrylamide gels (Invitrogen)  
196 and transferred onto nitrocellulose membranes. Blots were incubated overnight at 4°C with  
197 polyclonal anti-AR (1:400, Santa-Cruz Biotechnology), anti-ERα (1:400, Santa-Cruz  
198 Biotechnology), anti-AhR (1:500, Santa-Cruz Biotechnology), anti-spinophilin (1:500, Millipore),  
199 anti-GluN2A (1:500, Millipore) or monoclonal anti-glyceraldehyde 3-phosphate dehydrogenase  
200 (GAPDH; 1:10000, Santa-Cruz Biotechnology), anti-postsynaptic density protein 95 (PSD95; 1:500,  
201 Santa-Cruz Biotechnology), anti-GluN1 (1:500, Millipore), anti-GluN2B (1:500, Millipore) and anti-  
202 synaptophysin (1:500, Abcam). Incubation with secondary peroxidase-conjugated monoclonal or  
203 polyclonal antibodies diluted at 1:5000 (Amersham Bioscience) was performed for 1 h at room  
204 temperature. Signals were revealed with the Pico or Super Signal detection kits (Thermo Scientific)  
205 and quantified using ImageJ software (NIH). The data were normalized with respect to GAPDH  
206 protein amounts.

207

### 208 *Transmission electron microscopy*

209 Three male mice per treatment group were subjected to anesthesia using a lethal dose of pentobarbital  
210 (120 mg/kg, i.p.) before transcardiac perfusion with a 0.9% saline solution followed by a 2.5%  
211 glutaraldehyde solution in 0.1 M cacodylate buffer pH 7.4. Brains were removed and post-fixed in  
212 2.5% glutaraldehyde/0.1 M cacodylate buffer overnight at 4°C. The brains were washed in cacodylate  
213 buffer and cut with a vibratome (Leica VT1000S, Leica Biosystems, Wetzlar, Germany) into four  
214 coronal sections including the hippocampus (100-µm thickness), which were post-fixed in 2%

215 osmium tetroxide diluted in cacodylate buffer (v/v) for 1 h at RT. After dehydration in an alcohol  
216 ascending series, sections were embedded in epoxy resin (Epoxy-Embedding Kit, cat. N° 45359,  
217 Sigma Aldrich, Switzerland). The polymerization step was performed at 60°C for 48 h between two  
218 silicone-coated glass slides. Sections containing the hippocampal CA1 areas, selected using  
219 neuroanatomical landmarks of the Mouse Brain Atlas (Paxinos and Franklin, 2001) indicated above,  
220 were cut in ultrathin sections (70 nm) with an ultramicrotome (Reichert/Leica Ultracut S  
221 ultramicrotome, Leica Microsystems, Bannockburn, IL, USA): four ultrathin sections were collected  
222 per copper mesh grid with four grids per animal. Then, these ultrathin sections were contrasted with  
223 2.5% uranyl salts and Reynold's lead citrate and observed under a transmission electron microscope  
224 (80–120 kV EM 912 Omega ZEISS) equipped with a digital camera (Veleta Olympus).

225

#### 226 *Targeted LC/MS metabolites analyses*

227 The metabolomic analysis was conducted as described previously (de la Calle Arregui et al., 2021).  
228 Hippocampal samples were extracted in a solution containing 50% methanol, 30% acetonitrile (ACN)  
229 and 20% water, and the collected supernatants were stored at -80 °C. The LC/MS analysis was  
230 performed as detailed in Supplemental Materials and Methods. Data were acquired with Thermo  
231 Xcalibur software (Thermo), and raw data were analyzed using MetaboAnalyst 5.0 software. The  
232 peak area of each metabolite was normalized to the total peak areas of all metabolites. The top 40  
233 heatmap corresponds to the 40 most significant metabolites modified between the treatment groups.

234

#### 235 *Statistics*

236 Data were expressed as means  $\pm$  S.E.M. and analyzed by GraphPad Prism 6 (GraphPad Software).  
237 Normality tests were performed (Kolmogorov-Smirnov and Shapiro-Wilks tests). Two-way analysis  
238 of variance (ANOVA) was used for the main effect of exposure on the latency to reach the platform  
239 and swimming distance in the Cue and Spatial reference memory tasks of the Morris water maze.  
240 Bonferroni's post hoc tests were used to determine group differences. Kruskal-Wallis test was used

241 to analyze the effect of exposure on the latency to reach the platform and swimming distance in the  
242 Probe test of the Morris water maze. Dunn's post hoc tests were used to determine group differences.  
243 One way ANOVA and Fisher's LSD post hoc test were used to analyze metabolomic results.  
244 Metabolic fold changes between two groups were quantified by using Student t-test. One way  
245 ANOVA was used to analyze the effect of exposure on the remaining data. Bonferroni's post hoc  
246 tests were used to determine group differences. P values < 0.05 were considered to be significant.  
247

248 **Results**

249 **Behavioral effects of adult exposure to DEHP alone or in a phthalate mixture**

250 *Spatial learning and memory assessed in the Morris water maze*

251 In the cue task with a visible platform (Figure 1A-B), two-way ANOVA shows an effect of time ( $F_{(2, 88)} = 112.8, p < 0.0001$ ) but not of treatment ( $F_{(3, 44)} = 0.33, p = 0.803$ ). Male mice exhibited improved  
252 performance in finding the platform across days, regardless of their treatment group. Comparable  
253 data were found in the spatial reference memory task with hidden platform and visible spatial  
254 landmarks (Figure 1C-D). There was an effect of time ( $F_{(4, 176)} = 14.95, p < 0.0001$ ) but not of treatment  
255 ( $F_{(3, 44)} = 1.60, p = 0.203$ ), with males again being able to exhibit increased performance over the  
256 training period without any significant difference between the treated groups. In these two tasks, the  
257 distance travelled did not differ between the treatment groups (Figure S2A-B). Ten minutes after the  
258 end of training, males were assessed for their ability to find the platform location (Figure 1E-F). There  
259 was an effect of treatment ( $p = 0.002$ ) on the latency to find the platform quadrant. Post hoc analyses  
260 show a significantly higher latency for the DEHP-50 group (+371% versus the control group).  
261 Although not significant, a tendency of increase was also observed for the latency of DEHP-5 and  
262 Mix groups. The distance travelled was also comparable between the treatment groups (Figure S2C).

264

265 *Temporal order memory and novelty detection tasks*

266 In the temporal order task for visual objects (Figure 2A), the total time spent exploring the objects  
267 did not differ between the treated groups ( $55.84 \pm 4.72$  sec for the Veh group,  $54.32 \pm 4.64$  sec for  
268 the DEHP-5 group,  $53.74 \pm 4.83$  sec for the DEHP-50 group,  $59.68 \pm 4.69$  for the Mix group). Mice  
269 from the vehicle group were more interested ( $p < 0.0001$ ) in exploring the first object seen than the  
270 last one (Figure 2B). By contrast, males from DEHP-5, DEHP-50 and Mix-groups did not  
271 discriminate between the two presented objects ( $p > 0.05$ ). One way ANOVA revealed a significant  
272 main effect of treatment ( $p = 0.0012$ ), with a lower mean discrimination index for DEHP-5, DEHP-  
273 50 and Mix-groups (-94%, -87% and -75% versus the vehicle group, respectively).

274 In the novelty detection task (Figure 2C), the total time spent exploring the objects was comparable  
275 between the treatment groups ( $55.43 \pm 4.64$  sec for the Veh group,  $58.85 \pm 3.69$  sec for the DEHP-5  
276 group,  $63.88 \pm 5.39$  sec for the DEHP-50 group,  $63.57 \pm 4.12$  for the Mix group). Analysis of mean  
277 discrimination index showed that males from the vehicle and Mix groups spent more time exploring  
278 the new object than the familiar one ( $p < 0.001$  for the vehicle group and  $p < 0.01$  for the Mix-group)  
279 as shown in Figure 2D. One-way ANOVA indicated an effect of treatment ( $p = 0.0057$ ), with a lower  
280 discrimination index for DEHP-5, DEHP-50 and Mix-groups (-68%, -67% and -59% versus the  
281 vehicle group, respectively).

282

### 283 *Anxiety-related behavior*

284 In the elevated O-maze, an effect of treatment was observed on the number of entries ( $p = 0.0358$ )  
285 and time spent ( $p < 0.05$ ) into the open arms (Figure 2E-F). Reduced number of entries for the DEHP-  
286 5 and Mix groups (-24% and -25% versus the Veh group, respectively) and time spent in the open  
287 arms for the Mix group (-25% versus the Veh group) were observed, but a tendency to decrease was  
288 also seen for the other groups.

289

### 290 **Dendritic spine density and protein amounts for pre- and post-synaptic markers in the** 291 **hippocampus of adult male mice exposed to DEHP alone or in a phthalate mixture**

292 Behavioral processing and reorganization of circuitries during learning and memory relies on several  
293 cellular processes involved in neuroplasticity. The formation and maturation of spines emanating  
294 from neuronal dendrites and known to constitute excitatory postsynapses are considered to be  
295 essential for neuroplasticity. We therefore assessed whether the behavioral alterations induced by  
296 exposure of adult male mice to DEHP alone or in phthalate mixture were associated with  
297 modifications in the density and morphology of dendritic spines in the hippocampus. For this purpose,  
298 brains from males exposed to the vehicle or DEHP alone or in a phthalate mixture were processed in  
299 Golgi-Cox staining and dendritic spines were analyzed and classified in stubby, thin and mushroom

300 types according to their level of maturation as previously described (Jean et al., 2017). Figure 3A-B  
301 shows that there was an effect of treatment on the number of stubby ( $p = 0.0008$ ), thin ( $p = 0.0014$ )  
302 and mushroom spines ( $p = 0.0003$ ) in the CA1 area. The density was reduced in almost all treatment  
303 groups compared to vehicle-treated males (-35% to -43% for stubby spines, -64% to -78% for thin  
304 spines and -64% to -69% for mushroom spines). This resulted in an average reduction in the total  
305 number of dendritic spines in males exposed to DEHP alone or in phthalate mixture (-46% to -55%)  
306 compared to the vehicle group. These effects were not restricted to the CA1 area since reduced spine  
307 dendritic density was also apparent in the CA2/3 region as shown in Figure S3A-B.

308 Quantification by Western blotting of proteins extracted from the CA1 area showed modifications in  
309 the protein levels of postsynaptic markers. Figure 3C-F shows a significant effect of treatment on the  
310 protein amounts of PSD-95 ( $p = 0.0007$ ), spinophilin ( $p = 0.035$ ), GluN1 ( $p = 0.0006$ ) and GluN2A  
311 receptors ( $p = 0.0014$ ). No significant treatment effect was found on the protein amount of GluN2B  
312 ( $p = 0.25$ ), although a tendency towards a decrease was also observed (Figure 3G). Posthoc analyses  
313 show that the protein levels were reduced for PSD-95 in the DEHP-50 and Mix groups (-53% and -  
314 55%, respectively), for spinophilin in the DEHP-50 group (-50%), for GluN1 in the DEHP-50 and  
315 Mix groups (-85% and -95%, respectively), and for GluN2A in the DEHP-5, DEHP-50 and Mix  
316 groups (-61%, -62% and -45%, respectively) in comparison to the Veh group.

317 The protein levels for the presynaptic vesicle membrane protein synaptophysin were also quantified  
318 in the CA1 area (Figure S4A). There was an effect of treatment ( $p = 0.04$ ), with a higher amount in  
319 the Mix group (+63% versus the vehicle group). We also conducted a qualitative analysis of  
320 ultrastructural morphology of synapses corresponding to dendritic spines in the hippocampal CA1  
321 area. Qualitative random electron microscopy observations were thus performed with 33 to 42 images  
322 for each experimental group including at least one synapse associated with dendrite. Representative  
323 images in Figure S4B show that the synapses observed in the CA1 region of the hippocampus were  
324 clearly identifiable for the vehicle group with, on the one hand, a clean, homogeneous, compact and  
325 electron-dense postsynaptic membrane and, on the other hand, the presence of vesicles with low dense

326 electron density content in the presynaptic element. This ultrastructural profile corresponds to that of  
327 functional synapses. The observation of synapses in the CA1 area of DEHP-5 treated mice showed a  
328 similar ultrastructure (Figure S4B). For mice treated with DEHP-50 alone or in a phthalate mixture,  
329 vesicles were not identifiable, so making the distinction between the pre- and postsynaptic element  
330 hazardous (Figure S4B). The ultrastructure of the mitochondria and myelinated axons attests to the  
331 absence of artefacts linked to the chemical fixation of the brains or to the procedure for preparing  
332 samples for electron microscopy (Figure S4B).

333

### 334 **Metabolomic analysis of the hippocampus of male mice exposed to DEHP alone or in a** 335 **phthalate mixture**

336 We have previously shown that adult exposure to DEHP triggered modifications in the levels of  
337 proteins involved in cellular metabolism in the medial preoptic area in male mice (Dombret et al.,  
338 2017). As metabolic activity is mandatory for the cellular processes involved in neural function and  
339 behavior, we conducted a metabolomic analysis by LC/MS on the hippocampus of adult mice  
340 exposed to the vehicle, DEHP alone or in a phthalate mixture. The heatmap of the top 40 modified  
341 metabolites is shown in Figure 4. Among them, ANOVA analysis shows that several metabolites  
342 were significantly modified by exposure to DEHP alone or in a phthalate mixture (FDR p-adjusted  
343 value < 0.05). Most of them are amino acids, which were down-regulated, and may have an important  
344 role in neurotransmission. This included tyrosine (FDR p-adjusted value = 0.022), valine (FDR p-  
345 adjusted value = 0.022), proline (FDR p-adjusted value = 0.044) and tryptophan (FDR p-adjusted  
346 value = 0.022) which are involved either in catecholamine, serotonin or glutamate metabolism  
347 (Dalangin et al., 2020; Fernstrom & Fernstrom, 2007; Höglund et al., 2019; Jones et al., 2021;  
348 Schwarcz & Stone, 2017). Tryptophan is particularly interesting as it is also involved in the synthesis  
349 of neuroactive compounds via the kynurenine pathway. Tryptophan was down-regulated with a fold  
350 change of 0.563 in the DEHP-50 group ( $p = 0.007$ ) and 0.61 for the Mix group ( $p = 0.009$ ), while  
351 NAD, the endproduct of this metabolic pathway (O'Mahony et al., 2015; Ramos-Chávez et al., 2018),



352 was overproduced with a fold change of 1.325 ( $p = 0.003$ ) for the DEHP-50 group (Table S1). L-  
353 kynurenine, an intermediate of this pathway, was also downregulated in the Mix group ( $FC = 0.275$ ,  
354  $p = 0.043$ ; Table S1), suggesting that tryptophan was actively metabolized via the kynurenine  
355 pathway following phthalate exposure.

356

357 **Protein amounts of AR, ER $\alpha$  and AhR in the hippocampus of adult male mice exposed to DEHP**  
358 **alone or in a phthalate mixture**

359 Our previous data showed that adult DEHP exposure did not impact the gonadotropic axis or  
360 circulating levels of testosterone, but rather impacted *AR* expression levels (Dombret et al., 2017).  
361 Accordingly, analyses of body weight and relative weights of androgen-sensitive tissues such as the  
362 testis and seminal vesicles did not show any effect of the treatment with DEHP alone or in a phthalate  
363 mixture (Figure 5A-C). We thus quantified the protein amounts of the AR and ER $\alpha$  by Western  
364 blotting in the CA1 area. Data illustrated in Figure 5D-E show an effect of treatment on AR protein  
365 amounts ( $p = 0.041$ ), but not on ER $\alpha$  ( $p = 0.425$ ), with significantly reduced protein levels in the  
366 DEHP-50 group. An effect of treatment ( $p = 0.014$ ) was also found on the protein amount of the  
367 xenobiotic receptor AhR (Figure 5F), a target of the kynurenine pathway of tryptophan metabolism  
368 (Ramos-Chávez et al., 2018) and a negative regulator of AR expression (Arabnezhad et al., 2020;  
369 Ghotbaddini et al., 2018). Significantly higher protein levels were observed for the DEHP-5 group  
370 (+78%,  $p < 0.01$ ) compared to the vehicle group. Although not significant, a tendency to increase was  
371 also seen for the other treated groups.

372

373 **Discussion**

374 The present study shows that chronic exposure of adult male mice to environmental doses of  
375 DEHP alone or in a phthalate mixture impaired short-term memory in spatial, temporal order and  
376 novelty tasks. These behavioral alterations were associated with reduced dendritic spine density and  
377 protein levels of postsynaptic markers in the hippocampus. Metabolomic modifications including  
378 changes in the levels of metabolites involved in tryptophan metabolism via the kynurenine pathway  
379 were also observed together with higher protein levels of the AhR, a xenobiotic receptor target of this  
380 metabolic pathway.

381

382 Adult male mice chronically exposed to DEHP at 50 µg/kg/d exhibited altered spatial memory  
383 in the Morris Water Maze. A tendency to increase for the latency to find the platform quadrant was  
384 also observed for DEHP-5 and Mix-treated groups, despite a comparable training phase between the  
385 control and the three treated groups. Males exposed to DEHP alone at 5 or 50 µg/kg/d or in a phthalate  
386 mixture displayed also altered temporal processing of visual objects as they were unable to  
387 discriminate between the first and last objects seen. This could be related to either reduced temporal  
388 order processing and/or diminished capacity to discriminate between new and familiar objects since  
389 these male groups exhibited a reduced performance in the novelty task. The higher anxiety state level  
390 of male mice exposed to DEHP alone or in a phthalate mixture compared to the vehicle group could  
391 only partially account for the observed alterations since a session to decrease neophobia towards  
392 objects was included in the two object recognition tasks.

393 Further analyses of the hippocampus, which plays a key role in the processing of spatial and temporal  
394 order memory, showed a lower density of dendritic spines in the CA1 region of males exposed to  
395 DEHP alone or in a phthalate mixture compared to the vehicle group. The effect of exposure on these  
396 excitatory postsynapses was observed for all spine types ranging from the transient (thin) to the most  
397 mature and stable spine (mushroom). In agreement with this reduced dendritic spine density, analysis  
398 of postsynaptic proteins in the CA1 area showed lower levels for PSD-95 and spinophilin as well as

399 for glutamate receptors (GluN1, GluN2A) in males exposed to DEHP alone or in a phthalate mixture.  
400 Interestingly, a low dendritic spine density was also observed in the CA2/3 area, suggesting that these  
401 structural alterations were not restricted to the CA1 area. As male mice were exposed to DEHP alone  
402 or in a phthalate mixture during eight weeks before the beginning of behavioral tests, with exposure  
403 maintained during the whole period of analyses until sacrifice, we suggest that such exposure  
404 prevented structural modifications necessary for neuroplasticity processes related to dendritic spines  
405 to occur in the CA areas, thus resulting in a low neuronal connectivity and behavioral processing.  
406 Qualitative ultrastructural analysis of synapses corresponding to dendritic spines in the hippocampal  
407 CA1 area showed normal morphology, but the presynaptic vesicles appeared hardly identifiable in  
408 DEHP-50 and Mix-exposed male mice. In addition, higher protein levels for the presynaptic  
409 synaptophysin were observed in the CA1 area of males exposed to DEHP alone or in a phthalate  
410 mixture. While the exact role of synaptophysin in neurotransmission is still not clear, it was recently  
411 suggested that the synaptophysin/gyrin family might negatively regulate excitocytosis (Raja et al.,  
412 2019). It thus remains to determine whether adult exposure to DEHP alone or in a phthalate mixture  
413 at low doses affects also neurotransmission processes.

414  
415 The metabolomic approach highlighted changes in several metabolites involved in  
416 neurotransmitter synthesis or aminoacid metabolism following exposure to DEHP alone or in a  
417 phthalate mixture. The reduced tryptophan and L-kynurenine levels associated with the elevated  
418 levels of NAD are of particular interest, Tryptophan and L-kynurenine levels depend not only on  
419 tryptophan abundance but also on the activity of the enzymes involved in this pathway. This could  
420 explain the discrepancy observed between the concentrations of the metabolites and the endproduct,  
421 NAD (Covarrubias et al., 2021). Hence, the reduced tryptophan and L-kynurenine suggests that  
422 tryptophan concentration is limited and that both tryptophan and L-kynurenine are fed into NAD+  
423 synthesis.

424 Altogether, these observations suggest that exposure to DEHP alone or in a phthalate mixture  
425 triggered modifications in the kynurenine pathway of tryptophan metabolism. First, exposure to high  
426 doses of phthalates was previously reported to disturb the kynurenine pathway of tryptophan  
427 metabolism in both male rats (Fukuwatari et al., 2002a; 2002b; 2004) and men (Nassan et al., 2019).  
428 Phthalates share, indeed, structural similarities with tryptophan metabolites, which allow binding and  
429 inhibition of  $\alpha$ -amino- $\beta$ -carboxymuconate- $\epsilon$ -semialdehyde decarboxylase, an enzyme that prevents  
430 the accumulation of kynurenine pathway metabolites (Singh et al., 2018). Second, disruption of the  
431 kynurenine pathway is suggested to be involved, through its neuroactive metabolites, in glutamatergic  
432 toxicity and inflammatory processes via a mechanism involving activation of the xenobiotic receptor  
433 AhR (Ramos-Chávez et al., 2018). The relevance of environmental doses of phthalates to induce such  
434 metabolic modifications is supported by a recent study showing that urinary concentrations of several  
435 phthalate metabolites were positively associated with kynurenine-derived neuroactive metabolites in  
436 women (Nassan et al., 2020). Furthermore, we have recently shown that exposure of adult male mice  
437 to environmental doses of DEHP alone or in a phthalate mixture, under similar experimental  
438 conditions, induced microglial activation and increased BBB permeability in the hippocampus  
439 (Ahmadpour et al., 2021). Based on all these observations, we suggest that chronic exposure to low  
440 doses of phthalates may primarily induce several metabolic changes including but not limited to the  
441 dysregulation of tryptophan metabolism, which may then trigger microglial activation and BBB  
442 impairment, leading to reduced neuronal connectivity and neurotransmission and thus to behavioral  
443 deficits.

444

445 The present data indicate that exposure to low environmental doses of phthalates impacted the  
446 hippocampus and related cognitive function. This contrasts with previous studies showing effects of  
447 phthalates at high but not at low doses (Ding et al., 2017; Peng, 2015; Song et al., 2021). The  
448 discrepancy with our data is probably due to the short duration of exposure used in these studies  
449 compared to the chronic continuous exposure used here. The currently described cognitive effects

450 following exposure of male mice to environmental doses of DEHP alone or in a phthalate mixture  
451 can be relevant for humans. A recent human study indicates that exposure to phthalates is associated  
452 with poor performance in immediate and delayed recall tests in the U.S. elderly (Weng et al., 2022).  
453 The effects induced by exposure to DEHP alone or in a phthalate mixture are not restricted to the  
454 hippocampus and its related cognitive function. Indeed, reduced sexual behavior in male mice  
455 exposed to DEHP alone or in a phthalate mixture, under similar experimental conditions, was also  
456 associated with alterations in metabolic activity and structural plasticity in the preoptic area (Ducroq  
457 et al., under revision). The underlying mode of action in this hypothalamic region seemed to involve  
458 reduced AR protein amounts, associated with AhR up-regulation, a known negative regulator of AR  
459 expression (Arabnezhad et al., 2020; Ghotbaddini et al., 2018) and receptor target of the kynurenine  
460 pathway of tryptophan metabolism (Ramos-Chávez et al., 2018). By comparison, the data obtained  
461 in the hippocampus show reduced AR protein levels but only for the DEHP-50 group, while AhR  
462 protein levels were significantly higher in the DEHP-5 group with a tendency to increase in DEHP-  
463 50 and Mix-groups. This suggests that AR down-regulation in the hippocampus possibly participated  
464 to the behavioral effects observed in male mice exposed to DEHP alone or in a phthalate mixture, but  
465 other mechanisms were probably also involved. In support of this hypothesis the fact that male mice  
466 deleted for the neural AR exhibited impaired temporal order memory but displayed normal novel  
467 object recognition (Picot et al., 2016), while male mice exposed to DEHP alone or in a phthalate  
468 mixture exhibited deficits for both behaviors.

469

## 470 **Conclusion**

471 Chronic exposure of adult male mice to DEHP alone at doses equivalent to or below the TDI  
472 dose or in a phthalate mixture disrupted spatial and temporal order memory. The comparable effects  
473 of DEHP alone and in a phthalate mixture found for the majority of data suggest that DEHP drives  
474 the effect in the mixture. Possible underlying mechanisms of these behavioral deficits could be the  
475 metabolomic alteration observed in the hippocampus and including changes in the kynurenine

476 pathway of tryptophan metabolism. This could participate to induce microglial activation and BBB  
477 impairment (Ahmadpour et al., 2021), and alter structural neuroplasticity (present study) in the  
478 hippocampus. It is possible that exposure to phthalates altered other brain regions involved in  
479 cognitive processes such as the cortex or related to other neural functions. We have recently described  
480 reduced expression of sexual behavior and altered cellular and molecular processes in the  
481 hypothalamus of male mice exposed in similar experimental conditions to equivalent doses of DEHP  
482 and phthalate mixture (Dombret et al., 2017; Ahmadpour et al., 2021; Ahmadpour et al., 2022). This  
483 highlights the vulnerability of the adult nervous system to continuous exposure to phthalates at doses  
484 equivalent and below the established reference doses for other endpoints (EFSA, 2005; EFSA et al.,  
485 2019; US EPA, 1987).

486

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493

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672

673 **Figures**

674 **Figure 1. Effects of adult exposure to DEHP alone or in a phthalate mixture on spatial learning**  
675 **and memory assessed in the Morris water maze.** Adult male mice were exposed to the vehicle  
676 (Veh), DEHP at 5 (DEHP-5) or 50 µg/kg/d (DEHP-50) or to a phthalate mixture (Mix). **A-B.** Latency  
677 of to reach the visible platform in the Cue task. Data are as means ± S.E.M. for 11-12 males per  
678 treatment group; there was an effect of training ###p < 0.001. **C-D.** Latency to reach the hidden  
679 platform with spatial cues around the maze during the Spatial reference memory. The effect of  
680 training is indicated (###p < 0.001). **E-F.** Latency to reach the platform quadrant with spatial cues  
681 around the maze during the Probe test. Effect of treatment (##p < 0.01) and post hoc comparisons (\*p  
682 < 0.001) are indicated.

683

684 **Figure 2. Effects of adult exposure to DEHP alone or in a phthalate mixture on temporal order**  
685 **memory, novel object recognition and anxiety-related behavior.** **A-B.** The testing sequence of  
686 object presentation and testing phase of the temporal order task are indicated (A). The discrimination  
687 index is shown as means ± S.E.M. for 11-12 males per treatment group (B). Only the Veh group  
688 displayed more interest in exploring the first object (a) seen, +++p < 0.001. The effect of treatment  
689 (##p < 0.01) and post hoc analyses (\*p < 0.05, \*\*p < 0.01, \*\*\*p < 0.001) versus the Veh group are  
690 indicated. **C-D.** The testing sequence of object presentation and testing phase of the novelty task are  
691 indicated (C). Only the Veh and Mix groups displayed more interest in exploring the new object (d),  
692 +++p < 0.001, ++p < 0.01 (D). The effect of treatment (##p < 0.01) and post hoc analyses (\*p < 0.05)  
693 versus the Veh group are indicated. **E-F.** Total number of entries (E) and time spent in the open arms  
694 (F) of the elevated O-maze. The effect of treatment (#p < 0.05) and post hoc comparisons (\*p < 0.05)  
695 are shown.

696

697 **Figure 3. Effects of adult male mice exposure to DEHP alone or in a phthalate mixture on neural**  
698 **structure in the CA1 area of the hippocampus.** **A.** Representative images of dendritic spines in the



699 CA1 area of the hippocampus of male mice treated with the vehicle (Veh), DEHP at 5 (DEHP-5) or  
700 50 (DEHP-50)  $\mu\text{g}/\text{kg}/\text{d}$ , or to a phthalate mixture (Mix). Thin (t), stubby (s) and mushroom (m)  
701 dendritic spines type are indicated. Scale bar: 10  $\mu\text{m}$  **B**. Quantification of total and subtype dendritic  
702 spine (10 neurons per mouse). Data are presented as means  $\pm$  S.E.M. from 4 males per treatment  
703 group. Treatment effects (### $p < 0.001$ , ## $p < 0.01$ ) and post hoc comparisons (\*\*\* $p < 0.001$ , \*\* $p <$   
704 0.01) are indicated. **C-H**. Quantification of protein amounts for PSD95 (**C**), spinophilin (**D**), GluN1  
705 (**E**), GluN2A (**F**) and GluN2B (**G**) normalized to GAPDH in the CA1 area of the hippocampus. Data  
706 are expressed as means  $\pm$  S.E.M. from 4-6 males per treatment group. Treatment effects (### $p <$   
707 0.001, ## $p < 0.01$ , # $p < 0.05$ ) and post hoc comparisons (\*\* $p < 0.01$ , \* $p < 0.05$ ) are indicated.

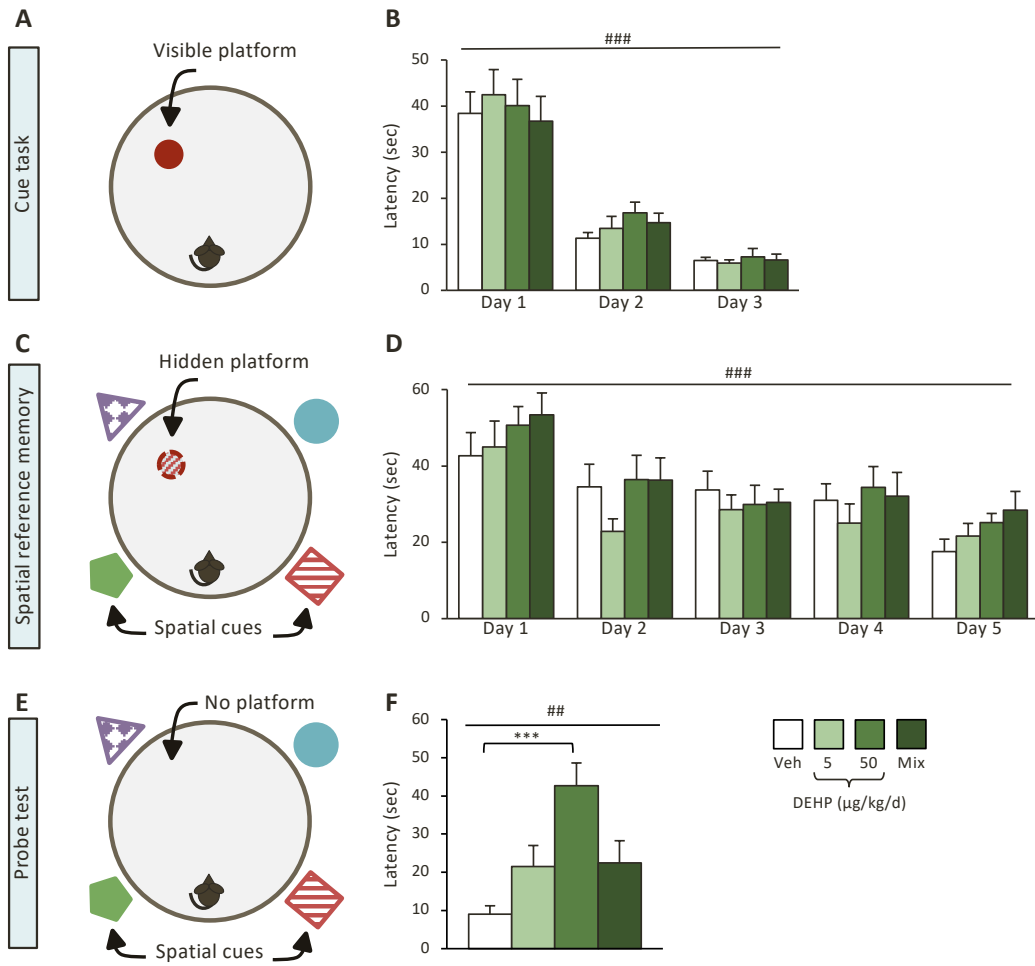
708

709 **Figure 4. Pan-metabolomic analysis in the hippocampus of males mice exposed to DEHP alone**  
710 **or in a mixture of phthalates.** Data are means of 7-8 males per treatment group. Relative levels vary  
711 from high (red) to low (blue). ADP, adenosine diphosphate; CDP, cytosine diphosphate; CMP,  
712 cytosine monophosphate; GDP, guanosine diphosphate; GSSG, oxidized glutathione;  $\text{NAD}^+$ ,  
713 nicotinamide adenine dinucleotide;  $\text{NADP}^+$ , nicotinamide adenine dinucleotide phosphate; UDP,  
714 uridine diphosphate; UMP, uridine monophosphate.

715

716 **Figure 5. Effects of adult male mice exposure to DEHP alone or in a phthalate mixture on**  
717 **physiological parameters and the protein amounts of androgen (AR) and estrogen receptor**  
718 **(ER $\alpha$ ), and aryl hydrocarbon receptor (AhR) in the CA1 area. A.** Growth curve for the total  
719 duration of exposure for male mice exposed to the vehicle (Veh), DEHP at 5 or 50  $\mu\text{g}/\text{kg}/\text{day}$ , or to a  
720 phthalate mixture (Mix). The blue line indicates the duration of the behavioral analyses. Data are  
721 expressed as means  $\pm$  S.E.M. of 23-24 males per treatment group. **B-C**. The weight of testes (**B**) and  
722 seminal vesicles (**C**) are expressed as percentage of body weight (bw). **D-E-F**. Quantification of the  
723 protein amounts for the AR (**D**), ER $\alpha$  (**E**), and AhR (**F**) normalized to GAPDH. Data are expressed

724 as the means  $\pm$  S.E.M. of 4-6 males per treatment group. Treatment effect ( $\#p < 0.05$ ) and post hoc  
725 analyses ( $*p < 0.05$ ,  $**p < 0.01$ ) are indicated.  
726



**Figure 1**

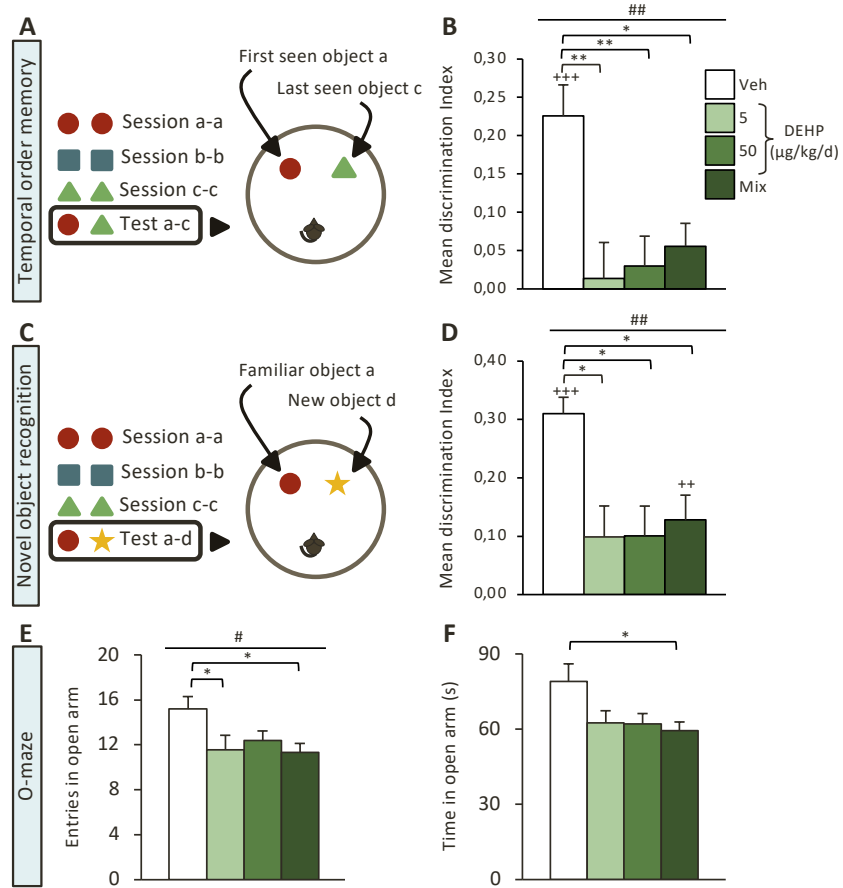
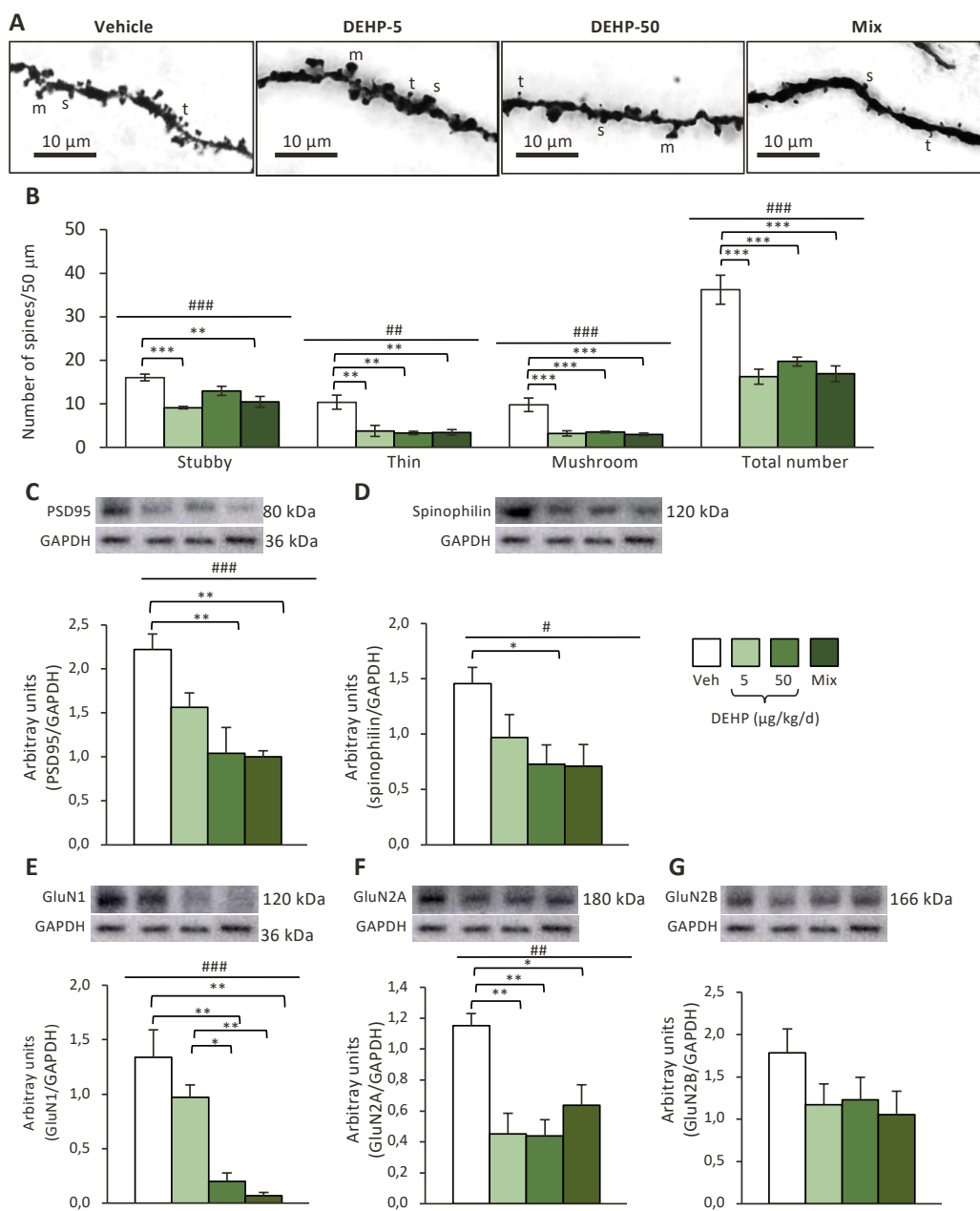


Figure 2



**Figure 3**

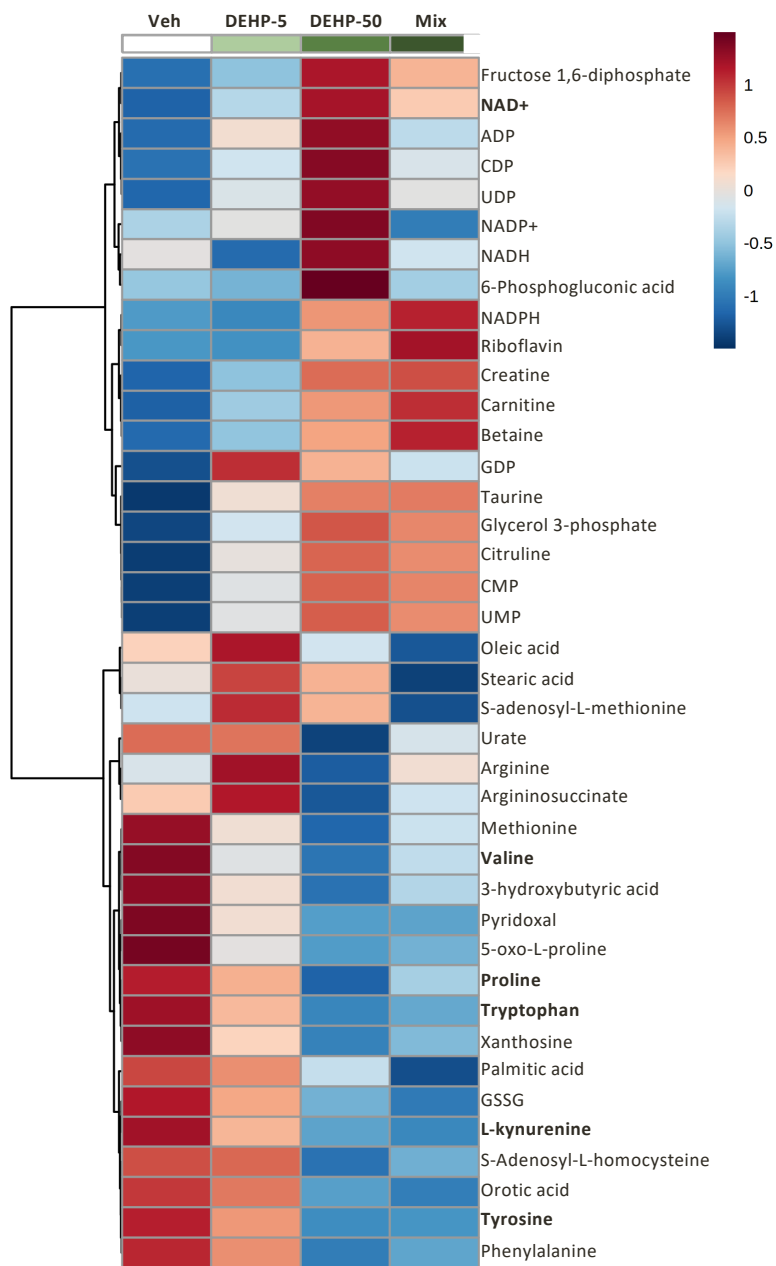


Figure 4

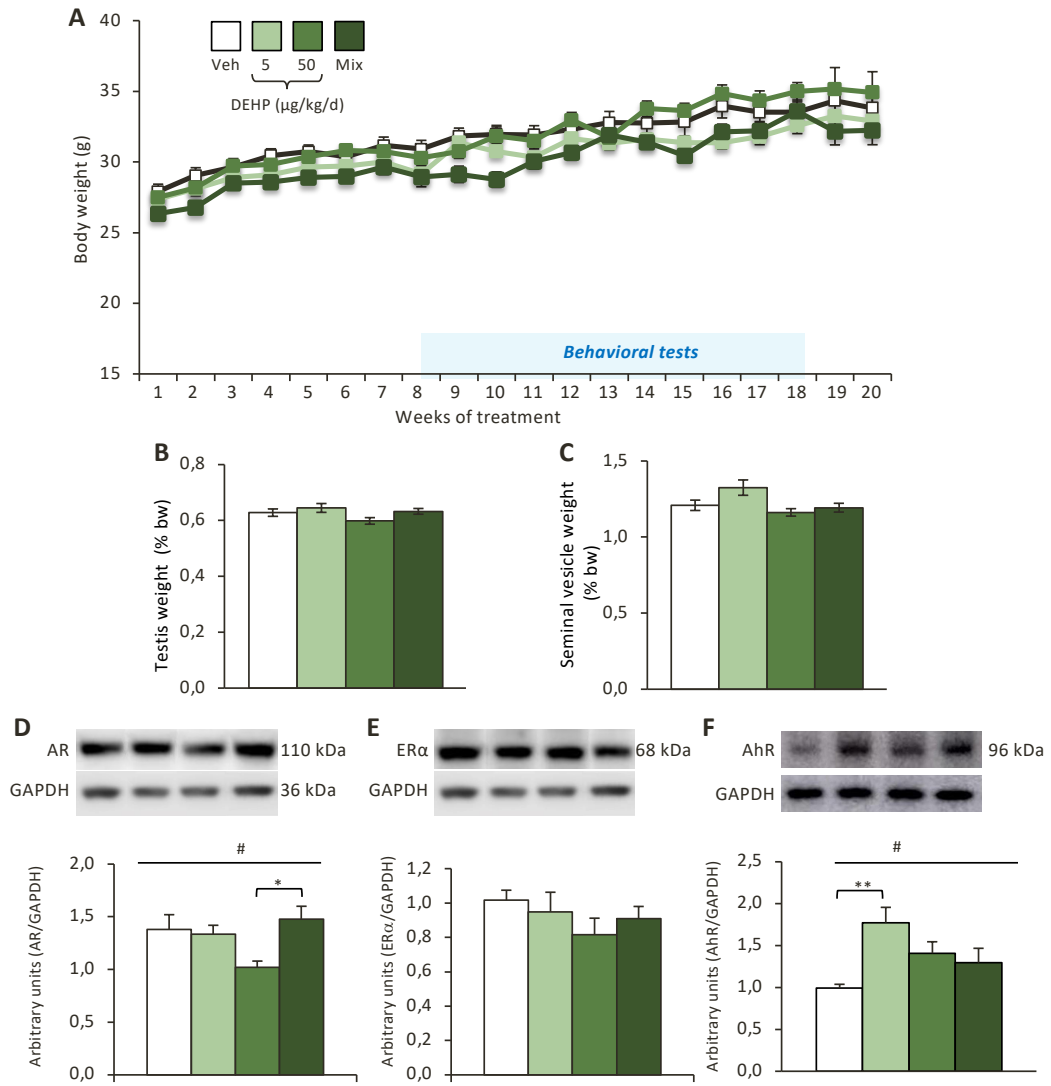


Figure 5

731

732

### 733 **Supplemental Materials and Methods**

#### 734 *Animals and treatments.*

735 Male mice used in this study were produced from the breeding of male and female C57BL/6J mice  
736 (Janvier Labs). Animals were housed in a nest-enriched environment using polysulfone cages and  
737 bottles, at 22°C and 12:12 h light-dark cycle (the dark time began at 1:30 P.M. and ended at 1:30  
738 A.M.), and ad libitum access to food (A03-10; Safe-diets) and water. Male offspring from 11 litters  
739 for cohort 1, 10 litters for cohort 2 and 9 litters for cohort 3 were mixed at weaning. The exposure  
740 started at the adult age of 8 weeks. All the analyses were conducted according to the European legal  
741 requirements (Decree 2010/63/UE) and received the approval of the «Charles Darwin» Ethical  
742 committee (project number 01490-01).

743 Oral exposure was performed through incorporation of phthalates (Sigma-Aldrich) into food as  
744 previously described (Adam et al., 2021; Adam et al., 2022; Ahmadpour et al., 2021; Ahmadpour et  
745 al., 2022; Dombret et al., 2017). Phthalates (Sigma-Aldrich, Saint-Quentin Fallavier, France) were  
746 dissolved in ethanol and water (1% and 40% of prepared food, respectively) and then incorporated  
747 into powdered food (A03-10; Safe-diets), which was then reconstituted into pellets.

748 Male mice of eight-week-old had ad libitum access to chow containing the vehicle (control group),  
749 DEHP (CAS 117-81-7) at the tolerable daily intake dose of 50 µg/kg/d (EFSA, 2005, 2019), DEHP  
750 at the dose of 5 µg/kg/d in the range of the estimated environmental exposure, or to a phthalate  
751 mixture containing DEHP at 5 µg/kg/d, DBP (CAS 8474-2) at 0.5 µg/kg/d, BBP (CAS 85-68-7) at  
752 0.5 µg/kg/d, DiBP (CAS 84-69-5) at 0.5 µg/kg/d and DEP (CAS 84-66-2) at 0.25 µg/kg/d. The  
753 composition of the mixture was based on French and European studies reporting external co-exposure  
754 to these substances (Martine et al., 2013) and presence of their metabolites in urinary samples (Anses  
755 2015; Dewalque et al., 2014). The phthalate doses were weekly adjusted to male body weights, and  
756 calculated for a daily food intake of 5 g per animal (Dombret et al., 2017).

757 The exposure started eight weeks before the beginning of analyses and was maintained until sacrifice.

758 Two cohorts of male mice, each including four treated groups, were used in the behavioral tests



759 (Figure S1). At the end of the tests, male mice were sacrificed and brains and tissues were collected  
760 for analyses by Golgi-cox staining, Western blot and transmission electron microscopy. An additional  
761 cohort was used for metabolomic analysis.

762 The analyses were performed by blind observers using only the numbers attributed at weaning.

763

#### 764 *Targeted LC/MS metabolites analyses*

765 Animals were sacrificed by cervical dislocation and the isolated hippocampus from collected brains  
766 were frozen in liquid nitrogen. Metabolomic analysis was conducted as described previously (de la  
767 Calle Arregui et al., 2021). The extraction solution (50% methanol, 30% acetonitrile (ACN) and 20%  
768 water) adjusted to the weight of the tissue (1 ml per 50 mg), was added to hippocampal samples,  
769 which were then vortexed for 5 min at 4 °C and centrifuged at 16,000 g for 15 min at 4 °C. The  
770 collected supernatants were stored at -80 °C. The LC/MS step was performed using QExactive Plus  
771 Orbitrap mass spectrometer equipped with an Ion Max source and a HESI II probe coupled to a  
772 Dionex UltiMate 3000 UPLC system (Thermo). A standard calibration mixture was used for external  
773 mass calibration every seven days. The samples (5 µl) were injected onto a ZIC-pHILIC column (150  
774 mm × 2.1 mm; i.d. 5 µm) with a guard column (20 mm × 2.1 mm; i.d. 5 µm) (Millipore). Buffer A  
775 (20 mM ammonium carbonate and 0.1% ammonium hydroxide (pH 9.2)) and buffer B (CAN) were  
776 used. The chromatographic gradient was run at a flow rate of 0.200 µl min<sup>-1</sup> as follows: 0-20 min,  
777 linear gradient from 80% to 20% of buffer B; 20–20.5 min, linear gradient from 20% to 80% of buffer  
778 B; 20.5–28 min, 80% buffer B. The mass spectrometer was operated in full scan, polarity switching  
779 mode with the spray voltage set to 2.5 kV and the heated capillary held at 320 °C. The sheath gas  
780 flow was set to 20 units, the auxiliary gas flow to 5 units and the sweep gas flow to 0 units. Metabolites  
781 were detected across a mass range of 75–1,000 *m/z* at a resolution of 35,000 (at 200 *m/z*) with the  
782 automatic gain control target at 10<sup>6</sup> and the maximum injection time at 250 ms. Lock masses were  
783 used to ensure mass accuracy below 5 ppm. Data were acquired with Thermo Xcalibur software  
784 (Thermo). The peak areas of metabolites were determined using Thermo TraceFinder software

785 (Thermo), identified by the exact mass of each singly charged ion and by the known retention time  
786 on the HPLC column.

787 The raw data were analyzed with MetaboAnalyst 5.0 software. The peak area of each metabolite was  
788 normalized for each sample to the total peak areas of all metabolites. The top 40 heatmap served to  
789 evaluate the 40 most significant metabolites changing between the treatment groups. Metabolites  
790 presenting equivalent abundance patterns were positioned close together.

791

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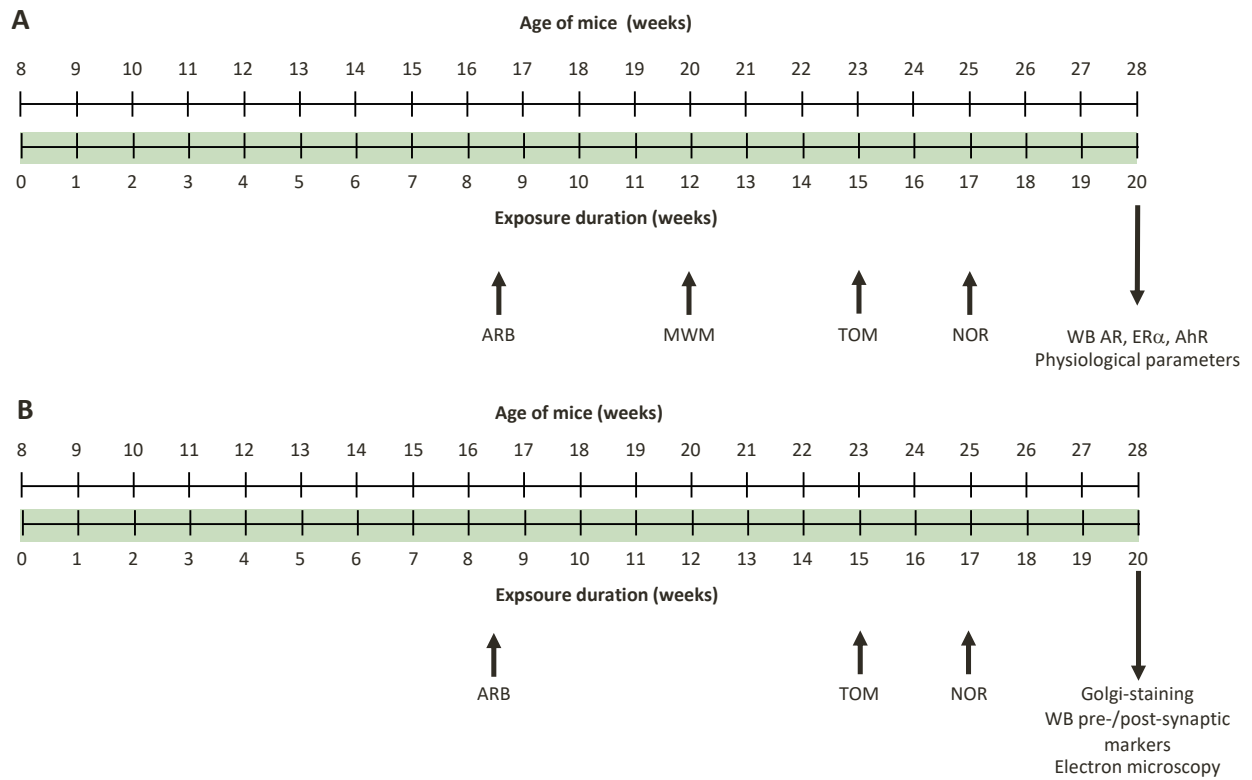
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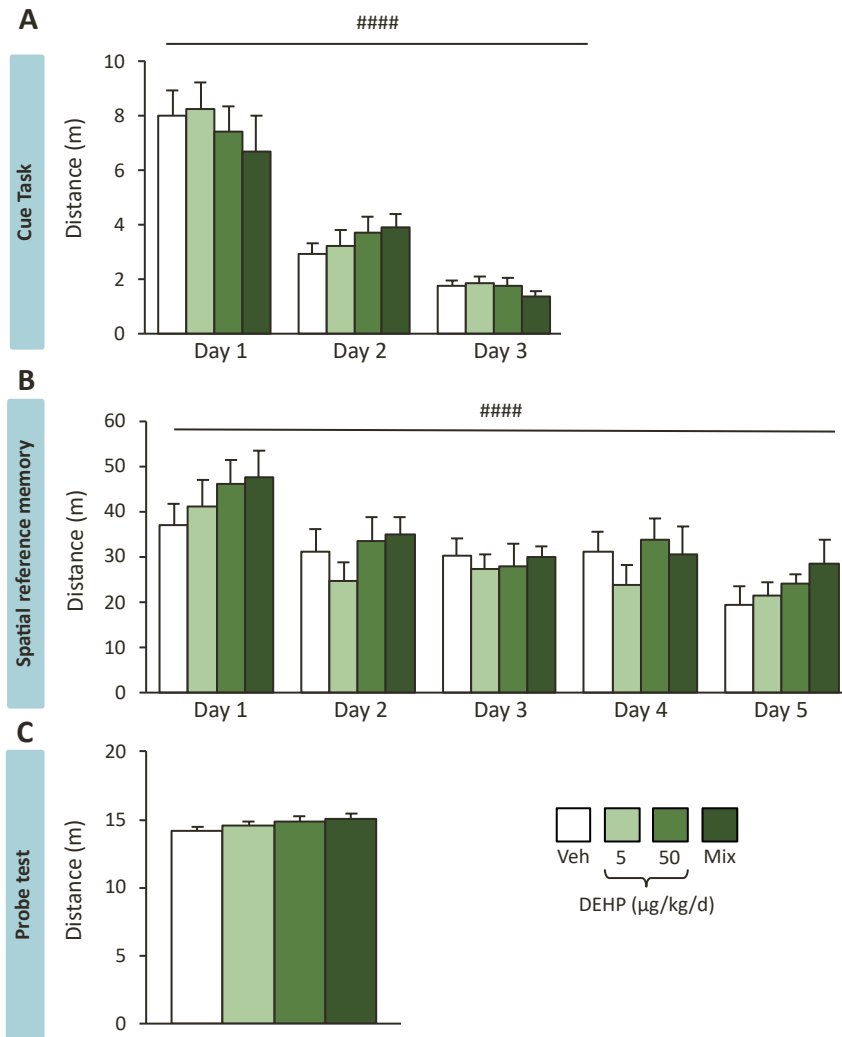


837

838 **Figure S1. A-B.** Male mice from the first (A) and second cohorts (B) were exposed since 8 weeks of  
 839 age. The duration of exposure is indicated in green, with males exposed for 8 weeks before the first  
 840 analyses and the exposure maintained until sacrifice. AR: androgen receptor; ER: estrogen receptor;  
 841 ARB: Anxiety-related behavior; MWM: Morris Water Maze; TOM: Temporal Order Memory; NOR:  
 842 Novel Object Recognition; WB: Western blotting.

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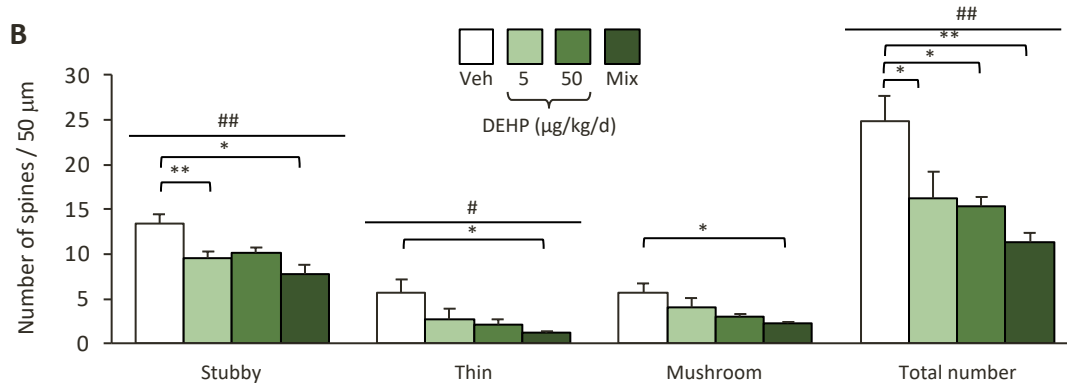
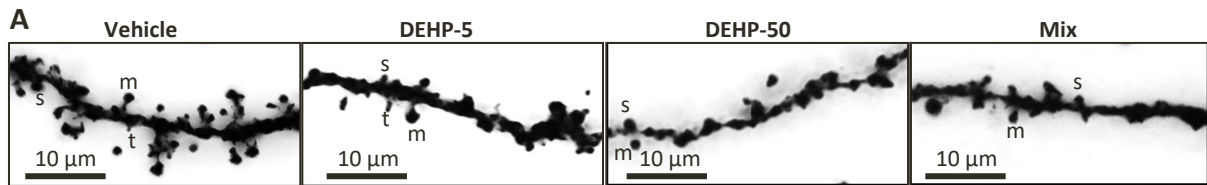


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847 **Figure S2. Effects of adult exposure to DEHP alone or in a phthalate mixture on spatial learning**  
 848 **and memory in the Morris water maze.** Male mice were exposed to the vehicle (Veh), DEHP at 5  
 849 or 50  $\mu\text{g}/\text{kg}/\text{d}$ , or to a phthalate mixture (Mix). **A-C.** Distance to reach the platform in the Cue (**A**)  
 850 and spatial reference memory tasks (**B**), and at the probe test (**C**). Distance (meters) is shown as  
 851 means  $\pm$  S.E.M. for 11-12 males per treatment group; ####  $p < 0.001$ : effect of time.

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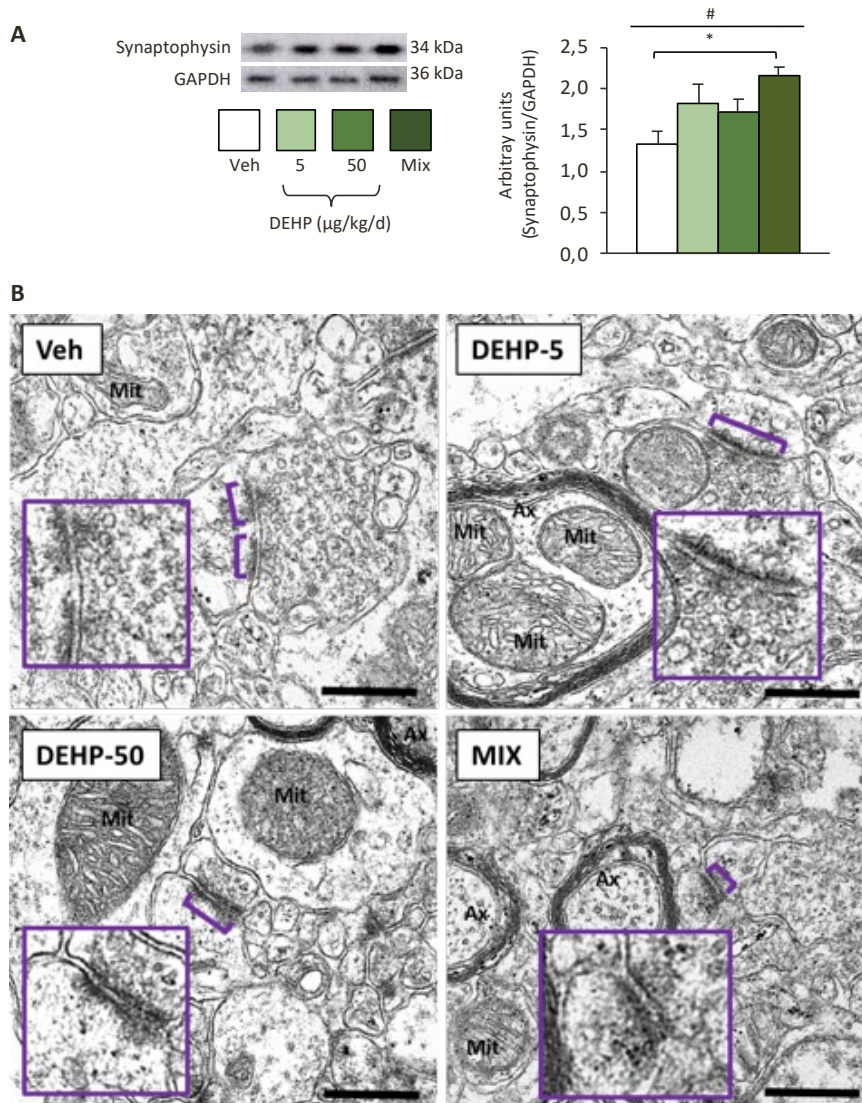


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854

855 **Figure S3. Effects of adult male mice exposure to DEHP alone or in a phthalate mixture on**  
 856 **dendritic spine density in the hippocampal CA2/3 area. A.** Representative images of dendritic  
 857 spines in the CA2/3 area of the hippocampus of males mice treated with the vehicle (Veh), DEHP at  
 858 5 (DEHP-5) or 50 (DEHP-50)  $\mu\text{g}/\text{kg}/\text{d}$ , or to a phthalate mixture (Mix). Thin (t), stubby (s) and  
 859 mushroom (m) dendritic spines type are indicated. **B.** Quantification of total and subtype dendritic  
 860 spines (10 neurons per mouse). Data are presented as means  $\pm$  S.E.M. from 4 males per treatment  
 861 group. Treatment effects (## $p < 0.01$ , # $p < 0.05$ ) and post hoc comparisons (\*\* $p < 0.01$ , \* $p < 0.05$ )  
 862 are indicated.

863



864

865

866 **Figure S4. Effects of adult male mice exposure to DEHP alone or in a phthalate mixture on**

867 **synaptophysin levels and ultrastructural morphology in the CA1 area of the hippocampus. A.**

868 Quantification of protein amount of synaptophysin normalized to GAPDH in the CA1 area of male

869 mice exposed to vehicle (Veh), DEHP at 5 (DEHP-5) or 50 (DEHP-50)  $\mu\text{g}/\text{kg}/\text{d}$ , or to a phthalate

870 mixture (Mix). Data are expressed as means  $\pm$  S.E.M. from 4 males per treatment group. Treatment

871 effects ( $\#p < 0.05$ ) and post hoc comparisons ( $*p < 0.05$ ) are indicated. **B.** Representative electron

872 micrographs of three independent experiments per treatment showing synapses in the CA1 area. For

873 the Veh, synapses are clearly identifiable with the presynaptic element containing vesicles, and the

874 compact and electron-dense postsynaptic membrane (purple bracket), delimiting the synaptic cleft.

875 For DEHP-5, the ultrastructure of the synapses appears similar to that of the vehicle group. For

876 DEHP-50 and Mix, the presynaptic vesicles are not observable and it is difficult to distinguish the  
877 presynaptic element from the post-synaptic element. Scale bar: 500 nm. Mit: mitochondria; Ax:  
878 myelinated axon. Purple squares: inserts showing at higher magnification the corresponding synapses  
879 indicated by the purple brackets.

880

881