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# Disruption of the blood-brain barrier and its close environment following adult exposure to low doses of di(2-ethylhexyl)phthalate alone or in an environmental phthalate mixture in male mice

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1 **Title**

2 Disruption of the blood-brain barrier and its close environment following adult exposure to  
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16

17 **Abstract**

18 We have previously shown that adult male mice exposure to low doses of di(2-  
19 ethylhexyl)phthalate (DEHP) alters neural function and behaviour. Whether such exposure  
20 also affects the integrity and function of the blood-brain barrier (BBB) remained to be  
21 explored. The impact of adult exposure to low doses of DEHP alone or in an environmental  
22 phthalate mixture on the BBB integrity and surrounding parenchyma was studied in male

23 mice. Two-month-old C57BL/6J males were orally exposed for 6 weeks to DEHP alone (0.5,  
24 and 50 µg/kg/day) or to DEHP (5 µg/kg/day) in an environmental phthalate mixture. BBB  
25 permeability, glial activation and neuroinflammation were investigated in the hypothalamic  
26 medial preoptic area (mPOA) and hippocampus involved, respectively on the reproductive  
27 and cognitive functions. Exposure to DEHP alone or in a phthalate mixture increased BBB  
28 permeability and affected the endothelial accessory tight junction protein zona occludens-1  
29 and caveolae protein Cav-1 in the mPOA and the hippocampal CA1 and CA3 areas. This  
30 was associated with an inflammatory profile including astrocyte activation accompanied by  
31 enhanced expression of inducible nitric oxide synthase in the mPOA, and a microglial  
32 activation in the mPOA and the hippocampal CA1 and CA3 areas. The protein levels of the  
33 inflammatory molecule cyclooxygenase-2 were increased in activated microglial cells of the  
34 exposed mPOA. None of the major effects induced by DEHP alone or in a mixture was  
35 detected in the hippocampal dentate gyrus. The data highlight that environmental exposure  
36 to endocrine disruptors such as phthalates, could represent a risk factor for the  
37 cerebrovascular function.

38

## 39 **Keywords**

40 Blood–brain barrier, Endocrine disruptors, Phthalates

41

## 42 1. Introduction

43 Phthalates are chemical compounds found in plasticizers and solvents (Kabir et al., 2015)  
44 and are among the most frequently detected organic pollutants in the environment. Di-2-  
45 ethylhexyl phthalate (DEHP), the most commonly detected phthalate, is widely used to add  
46 flexibility to high-molecular-weight polymers used in the manufacture of polyvinyl chloride  
47 plastic and is therefore found in containers for the storage of food and beverages. DEHP was  
48 also classified by the EU in 2000 as a priority substance “presenting a significant risk to or  
49 via the aquatic environment” in the Water Framework Directive 2000/60/EC, which was  
50 updated in 2008 and 2013 (Directive 2013/39/EU of the European Parliament and of the  
51 Council of 12 August 2013). In addition to DEHP, other phthalates including diethyl phthalate  
52 (DEP), dibutyl phthalate (DBP), butyl benzyl phthalate (BBP), and diisobutyl phthalate (DiBP)  
53 are also detected in the environment worldwide (Gao and Wen, 2016).

54 The majority of the *in vivo* studies addressing the effects of phthalate exposure on the  
55 nervous system have focused on the effects of perinatal exposure. Furthermore, the effects  
56 of exposure to phthalates at low doses during adulthood are still largely under-explored. Our  
57 recent study revealed that chronic exposure during adulthood to DEHP at the tolerable daily  
58 intake dose (TDI) of 50 µg/kg/day (European Food Safety Authority (EFSA) 2005, 2019) or to  
59 a 10-fold lower dose (5 µg/kg/day), close to environmental exposure, disrupts the emission of  
60 courtship vocalizations and therefore the initiation of mating in male mice (Dombret et al.,  
61 2017). This behavioural alteration was not due to modifications of circulating testosterone  
62 levels and/or the integrity of the hypothalamic–pituitary–gonadal axis. It was instead  
63 associated with down-regulation of the androgen receptor (AR) in the hypothalamic medial  
64 preoptic area (mPOA), the main cerebral area involved in the expression of male sexual  
65 behaviour (Dombret et al., 2017). The neural AR plays an important role in the expression of  
66 male sexual behaviour (Raskin et al., 2009) and also in the modulation of hippocampal  
67 functions as shown by the impaired temporal order memory in mice lacking the neural AR  
68 (Picot et al., 2016). The male hippocampus does indeed exhibit high sensitivity to androgens

69 and can thus be targeted by compounds exhibiting anti-androgenic activities. In this context,  
70 a previous study showed that prenatal or perinatal exposure to DEHP at 200 µg/kg/d or 10 to  
71 200 mg/kg/d impaired spatial memory and induced anxiety- and depressive-like behaviour in  
72 adult male mice, and that these effects were associated with AR down-regulation in the  
73 hippocampus (Barakat et al., 2018; Xu et al., 2015).

74 In the brain, including the hypothalamus and hippocampus, the blood-brain barrier (BBB)  
75 present at the level of the cerebral capillary protects cerebral regions from the toxicity of  
76 circulating xenobiotics and pathogens thereby providing cerebral homeostasis (Weiss et al.,  
77 2009). The BBB is formed by endothelial cells (ECs) which are themselves sealed by tight  
78 junctions (TJs) and adherens junctions (Abbott et al., 2010; Weiss et al., 2009), thus limiting  
79 the paracellular diffusion of substances. The BBB also provides a gate-like function for  
80 substance delivery to brain cells by limiting trans-cellular diffusion via selective carrier-  
81 mediated transport systems (Abbott et al., 2010). An extremely low rate of transendothelial  
82 vesicular transport also integrates the transcellular pathway to minimize the uptake of  
83 substances from blood to brain parenchyma (Villaseñor et al., 2019). Among the different  
84 categories of vesicular transport, caveolae appears to be the major element responsible for  
85 transcytosis in cerebral endothelial cells (Villaseñor et al., 2019). At their abluminal side, ECs  
86 are surrounded by basal lamina embedding pericytes, and interact with glial cells (astrocytic  
87 end-feet and microglial cells) and neurons. The whole is called the neurovascular unit (NVU)  
88 (Erdő et al., 2017). The presence of glial cells in the vicinity of the BBB promotes NVU  
89 susceptibility to neuroinflammatory responses. Neuroinflammation, in turn, can contribute to  
90 BBB dysfunction and neurodegenerative process (Vodo et al., 2013).

91 Androgens were also shown to promote cerebral angiogenesis and vasculature formation,  
92 and modulate the cerebrovascular function (see for review, Ahmadpour and Grange-  
93 Messent, 2020). In particular, our previous data indicate that gonadal testosterone supports  
94 the integrity and function of the BBB and prevents gliosis reaction and the up-regulation of  
95 inflammatory proteins in adult male mice (Atallah et al., 2017) and rats (Barreto et al., 2007).

96 In this context, our work aims to document for the first time the impact of oral exposure  
97 during adulthood to low doses of DEHP alone or in a phthalate mixture on the integrity of the  
98 capillary BBB and surrounding parenchyma in male mice. Analyses were processed using  
99 four experimental groups of adult C57BL/6J male mice exposed orally through contaminated  
100 diet in order to mimic the major route of exposure as previously described (Adam et al.,  
101 2021). The first three groups included males exposed for 6 weeks to the vehicle (control),  
102 DEHP at the TDI dose of 50 µg/kg/d, or DEHP at 5 µg/kg/d. The DEHP dose of 5 µg/kg/d is  
103 within the environmental exposure range; this dose induced behavioural alterations in male  
104 mice following adult or pubertal exposure (Capela and Mhaouty-Kodja, 2021; Dombret et al.,  
105 2017). In order to mimic environmental co-exposure to phthalates (Anses, 2015; Martine et  
106 al., 2013), the fourth group of males was exposed for 6 weeks to a phthalate mixture  
107 containing DEHP at 5 µg/kg/d, DBP at 0.5 µg/kg/d, BBP at 0.5 µg/kg/d, DiBP at 0.5 µg/kg/d  
108 and DEP at 0.25 µg/kg/d. Analyses focused on two androgen-sensitive brain areas as  
109 mentioned above the hypothalamic mPOA and the hippocampus. In these brain areas, we  
110 investigated the effects of exposure on BBB permeability using exogenous tracer and  
111 endogenous immunoglobulins G (IgG). Protein levels and distribution of TJ components as  
112 well as glial activation and neuroinflammation were also assessed in the four exposed  
113 groups.

## 114 **2. Material and Methods**

### 115 **2.1. Ethical statement**

116 The experiments have been reported in compliance with the Animal Research: Reporting in  
117 Vivo Experiments (ARRIVE) guidelines. All studies were performed in compliance with the  
118 National Institute of Health guidelines for the care and use of Laboratory Animals (NIH  
119 Guide) and French and European legal requirements (Decree 2010/63/UE). Experiments  
120 were performed accordingly, to minimize animal number and discomfort and were approved  
121 by the “Charles Darwin” Ethical committee (project number 01490-01).

### 122 **2.2. Animals**

123 Males of C57BL/6j strain (Janvier Labs, Le Genest-Saint-Isle, France) bred in our laboratory  
124 were housed in a conventional facility after weaning under controlled photoperiod (12:12h  
125 light dark cycle-lights on at 1 p.m.), maintained at 22°C and relative humidity (60% ± 10%),  
126 and had free access to water and a standard diet (A03–10; Safe-diets, Augy, France). The  
127 mice were housed in nest-enriched polysulfone cages, with polysulfone bottles. Offspring  
128 were mixed at the weaning to avoid potential litter effects with no more than one male per  
129 litter per cage, and were allowed to grow to 8 weeks of age. For these experiments, 9 cohorts  
130 each comprising 9 to 32 animals distributed equally between the 4 treatment groups, were  
131 used. Ultimately, 32 animals from two cohorts were used for BBB permeability assays, 96  
132 animals from five cohorts were used for the capillary-enriched fraction procedure and  
133 Western blot analysis, and then 68 animals from three cohorts were used for the  
134 immunocytochemistry study.

135

### 136 **2.3. Phthalate exposure**

137 Exposure to phthalates (Sigma Aldrich, Saint-Quentin Fallavier, France) was performed for 6  
138 weeks as recently described (Adam et al., 2021). The phthalates were first dissolved in  
139 absolute ethanol (1% of prepared food) and then in water (40% of prepared food) before  
140 incorporated into food as previously described (Adam et al., 2021). Control animals were fed  
141 with chow containing the vehicle i.e. ethanol and water (1% and 40% of prepared food,  
142 respectively). Eight-week-old males were fed ad libitum with chow consisted of their normal  
143 food containing the vehicle (control group), DEHP (CAS 117-81-7) at 50 or 5 µg/kg/d (DEHP-  
144 50 and DEHP-5 groups, respectively), or a phthalate mixture (Mix group) containing DEHP at  
145 5 µg/kg/d, DBP (CAS 84-74-2) at 0.5 µg/kg/d, BBP (CAS 85-68-7) at 0.5 µg/kg/d, DiBP (CAS  
146 84-69-5) at 0.5 µg/kg/d and DEP (CAS 84-66-2) at 0.25 µg/kg/d, reconstituted into pellets as  
147 precisely described previously (Adam et al., 2021). The composition of the phthalate mixture  
148 was based on French and European studies showing an external co-exposure to these  
149 molecules and the presence of their metabolites in urinary samples ( Anses, 2015; Dewalque

150 et al., 2014; Martine et al., 2013). The ratio of DEHP to the other phthalates was determined  
151 on the basis of the estimated daily intake in France and Europe (Dewalque et al., 2014;  
152 Martine et al., 2013).

153 Mice were weighed weekly for the duration of the exposure and phthalate doses were  
154 adjusted to their body weights and calculated for a daily food intake of 5 g per animal  
155 (Dombret et al., 2017), on the basis of previous studies showing this average daily intake for  
156 adult mice of 2 to 19 months old (Cheema et al., Br J Nutrition 2019). Body weight was  
157 comparable between the four treatment groups on the first and last days of exposure  
158 (supplementary Figure S1).

159 The analyses were performed on 5 cohorts each comprising animals distributed equally  
160 between the 4 treatment groups. Briefly, two cohorts were used for BBB permeability assays,  
161 two cohorts were used for immunohistochemistry study and then, one was used for the  
162 capillary-enriched fraction procedure and Western blot analysis.

## 163 **2.4. BBB permeability assay**

164 The BBB permeability assay was performed using an exogenous tracer binding to serum  
165 albumin, the Evans blue dye (Sigma Aldrich, Saint-Quentin Fallavier, France), and  
166 endogenous IgG. The BBB restricts the passage of serum proteins such as albumin and  
167 endogenous IgG from the blood flow into the interstitial tissue (Saunders et al., 2015).

### 168 **2.4.1. Evans Blue dye injection**

169 Three awake mice per treatment group were i.p. injected with a 2% Evans Blue solution (4  
170 ml/kg) diluted in normal saline. Three hours later, the mice were deeply anaesthetized with a  
171 lethal dose of pentobarbital (120 mg/kg, i.p.), then transcardially perfused with 0.9% saline  
172 solution followed by 4% paraformaldehyde (PFA) solution diluted in 0.1M phosphate buffer  
173 (PB) pH 7.4. Their brains were carefully removed and post-fixed with the same fixation  
174 solution overnight at 4°C. Afterwards, the brains were cryoprotected with a 20% sucrose  
175 solution for 24 hrs at 4°C before freezing in isopentane (-30°C) and 8 coronal sections (20



176  $\mu\text{m}$  thick) included mPOA or hippocampus were cut using a cryostat and mounted on slides.  
177 In order to visualize blood vessel walls, all sections were co-stained with anti-laminin rabbit  
178 primary antibody (1/100; Table 1) followed by secondary Alexa488-conjugated anti-rabbit IgG  
179 (1:1000, Invitrogen, Villebon sur Yvette, France). To avoid the Evans Blue dye spreading and  
180 to promote optimal visualization of labelled fine structures, sections were quickly immersed in  
181 xylene and mounted with a hydrophobic mounting medium using the method initially  
182 developed by Werner et al., (Werner et al., 1997) and modified by (Atallah et al., 2017). The  
183 Evans blue dye providing a red fluorescence (Steinwall and Klatzo, 1966), fluorescent  
184 signals were observed under a confocal microscope (described below).

#### 185 **2.4.2. Endogenous Immunoglobulin G detection**

186 Mice (n = 5 per treatment group) were deeply anaesthetized with a lethal dose of  
187 pentobarbital (150 mg/kg, i.p.), then were transcardially perfused with 0.9% saline solution  
188 followed by 4% PFA solution diluted in 0.1M PB pH7.4. Their brains were carefully removed  
189 and post-fixed with the same fixation solution overnight at 4° C. Afterwards, the brains were  
190 cryoprotected with a 20% sucrose solution for 24 hrs at 4°C before freezing in isopentane (-  
191 30°C) and 8 coronal sections (20  $\mu\text{m}$  thick) included mPOA or hippocampus were cut using a  
192 cryostat and mounted on slides. The presence of extravasated endogenous mouse IgG from  
193 blood to parenchyma was evaluated using Alexa488-conjugated anti-mouse IgG antibody  
194 (1:1000, Invitrogen, Villebon sur Yvette, France) combined with immuno-labelling of laminin  
195 as described above to detect microvessels. Then fluorescent staining was observed under a  
196 confocal microscope (described below).

### 197 **2.5. Fluorescent immunohistochemistry**

198 The animals (n=5 per treatment group and per immuno-labelling) were deeply anaesthetized  
199 with a lethal dose of pentobarbital (120 mg/kg, i.p.) for the following procedures.

#### 200 **2.5.1. Tissue preparation**

201 The experiments were processed as previously described (Atallah et al., 2017). Briefly, for TJ  
202 proteins (claudin-5, occludin and ZO-1) and the main component of caveolae plasma  
203 membranes, Cav-1 protein, the brains were freshly removed and immediately frozen in  
204 isopentane ( $-30^{\circ}\text{C}$ ). Six to eight serial frozen sections ( $20\text{-}\mu\text{m}$  thickness) included mPOA  
205 and hippocampus were cut and collected on slides, and were then fixed by immersion for 2  
206 min at  $-20^{\circ}\text{C}$  in methanol/acetone (vol/vol) with this followed by the labelling procedure. For  
207 the other proteins (Iba-1, GFAP, S100 $\beta$ , NDRG-2, Cox-2 and iNOS), male mice were deeply  
208 anesthetized using i.p. injection of pentobarbital (120 mg/kg) diluted in with 0.9% saline  
209 solution and then transcardially perfused with 0.9% saline solution followed by 4%  
210 paraformaldehyde solution diluted in 0.1 M PB pH7.4. After post-fixation and cryoprotection  
211 steps as described above, the brains were frozen in isopentane ( $-30^{\circ}\text{C}$ ) and 6 to 8 serial  
212 frozen sections ( $20\ \mu\text{m}$  thickness) included mPOA or hippocampus were cut using a cryostat  
213 and collected on slides.

#### 214 **2.5.2. Immuno-labelling procedure**

215 Non-specific sites were blocked by incubating slide-mounted sections in PBS 1X, 1% bovine  
216 serum albumin (BSA) and 0.2% Triton X-100 for 1 h at room temperature. Then sections  
217 were incubated with one or more primary antibodies (Table 1) overnight at  $4^{\circ}\text{C}$  diluted in the  
218 same phosphate buffer saline (PBS)/bovine serum albumin (BSA)/Triton X-100 solution.  
219 Immune complexes were revealed using secondary Alexa-conjugated anti-mouse, anti-goat  
220 or anti-rabbit IgG (1:1000; Invitrogen, Villebon sur Yvette, France). Fluorescence was  
221 observed with a confocal microscope.

#### 222 **2.6. Confocal microscopy**

223 Simple and multiple fluorescent labelling was visualized with a SP5 upright Leica confocal  
224 laser scanning microscope (Leica Microsystems) equipped with the Acousto-Optical Beam  
225 Splitter (AOBS) and using 63x oil immersion objective. Alexa 488 was excited at 488nm and  
226 observed from 495 to 580 nm; Alexa 555 was excited at 555 nm and observed from 599 to

227 680 nm. The gain and offset for each photomultiplier were adjusted to optimize detection  
228 events. Images (1024x1024 pixels, 16 bits) were acquired sequentially between stacks to  
229 eliminate cross-over fluorescence. The frequency was set up at 400 Hz and the pinhole was  
230 set at 1 Airy. Each optical section (1  $\mu\text{m}$ ) was frame-averaged four times to enhance the  
231 signal/noise ratio. Overlays, projection of the z-stack files and quantification were performed  
232 using the Fiji software (NIH, USA). The presented pictures were the projection of 10–20  
233 successive optical sections into one image, unless otherwise stated in the figure legend.  
234 Quantification of the fluorescent density was performed on three sections sampled at the  
235 level of the mPOA (plate 30 of the Mouse Brain Atlas of Paxinos and Franklin 2001) and on  
236 three sections sampled at the level of the hippocampus (plate 48). The surfaces on which the  
237 fluorescence density quantifications were performed are given in the figure legends.

## 238 **2.7. Western blot protein analysis using cerebral capillary-enriched factions**

### 239 ***2.7.1. Cerebral capillary-enriched fraction procedure***

240 Brains from 12 mice per treatment group were freshly removed and placed quickly on ice.  
241 For each hypothalamic capillary-enriched fraction, two hypothalami were pooled (n = 6 per  
242 treatment group), whereas one hippocampus was used for each hippocampal capillary-  
243 enriched fraction (n = 6 per treatment group). Hypothalamic and hippocampal microvessels  
244 were harvested according to a previously described method (Atallah et al., 2017; Sandoval  
245 and Witt, 2011), in order to obtain microvessel-enriched fractions for each cerebral area.  
246 Briefly, samples were homogenized in a pH 7.4 buffer containing 1% BSA, 2.7 mM KCl, 137  
247 mM NaCl, 1.5 mM  $\text{KH}_2\text{PO}_4$ , 8 mM  $\text{Na}_2\text{HPO}_4$ , 1 mM  $\text{CaCl}_2$ , 0.5 mM  $\text{MgCl}_2$ , 6H<sub>2</sub>O, mM D-  
248 glucose, 1 mM sodium pyruvate, 1 M 4-(2-Hydroxyethyl) piperazine-1-ethanesulfonic acid  
249 (HEPES). Homogenates were centrifuged in an equal volume of 30% Ficoll for 15 min at  
250 4800g at 4°C, supernatants were aspirated and pellets suspended in isolation buffer without  
251 BSA and passed through a 70- $\mu\text{m}$  nylon filter. Filtrates were centrifuged for 10 min at 4 °C at  
252 3000g.

253 **2.7.2. Protein extraction**

254 Protein extraction from pellets of microvascular capillary fractions was performed with a RIPA  
255 buffer containing 50mM Tris—base (pH 7.2), 10 mM EDTA, 10 mM EGTA, 150 mM NaCl,  
256 0.1% sodium dodecyl sulfate, 0.5% deoxycholate acid, 1% Triton X-100, and 1% protease  
257 inhibitor cocktail (Sigma Aldrich) and sonicated 10 times for 30 s. Homogenate samples were  
258 centrifuged at 13000 rpm for 13 min at 4 °C and supernatants containing proteins were  
259 collected. The total protein concentration of each sample was determined using the Bradford  
260 Assay Kit (Thermo Scientific, Courtaboeuf-Villebon sur Yvette, France) according to the  
261 manufacturer's protocol. Protein extracts were stored at -20°C until further processing.

262 **2.7.3. Electrophoresis and immunoblotting**

263 Protein samples were denatured in Laemmli Buffer and heated at 95°C for 5 min.  
264 Electrophoretic migration of 10–20 µg of proteins was carried out on NuPAGE 4–12% Bis–  
265 Tris Gel (Invitrogen, Villebon sur Yvette, France) or Mini-PROTEAN® TGX™ 7.5%  
266 polyacrylamide gels (BIO-RAD, Marnes La Coquette, France). The resolved proteins were  
267 then electrotransferred onto pre-treated polyvinylidene difluoride (PVDF) membranes  
268 (Millipore, Molsheim, France).

269 Membranes with transferred proteins were blocked for 1 h, at RT with a solution of 5% non-  
270 fat milk diluted in PBS 1X with 0.2 % Tween, and then incubated with primary antibodies  
271 (Table 1) diluted in the same blocking solution overnight at 4 °C. Primary antibody binding to  
272 blots was detected by incubation with respectively either secondary HRP–conjugated  
273 (1:5000; Jackson, Cambridgeshire, United Kingdom) or biotin–conjugated (1:2000; Vector,  
274 Burlingame, United States,) anti-rabbit, anti-mouse or anti goat for 2 h, at RT, and then  
275 immune complexes were revealed by the SuperSignal™ West Pico or Femto  
276 Chemiluminescent Substrate kit (Thermo Scientific, Courtaboeuf-Villebon sur Yvette,  
277 France).

278 The signals were quantified by using Fiji software (NIH, USA) and normalized to the value  
279 obtained for the corresponding housekeeper glyceraldehyde- 3-phosphate dehydrogenase  
280 (GAPDH) protein band.

## 281 **2.8. Statistical analysis**

282 The four sample sizes corresponding to the vehicle-treated, DEHP-5 or DEHP-50 and  
283 phthalate mixture were equal. Normal distribution of the four groups was checked using the  
284 Shapiro-Wilk normality test then one-way ANOVA was used to analyze the main effects of  
285 exposure and Tukey tests were used for posthoc analyses to determine group differences.  
286 Differences were considered statistically significant if  $p \leq 0.05$ . In the text, means  $\pm$  S.E.M are  
287 expressed as a percentage of the vehicle group. Two-way ANOVA was used to analyze the  
288 effects of treatment on the body weight of exposed mice. Data are expressed as body weight  
289 means (g)  $\pm$  S.E.M and differences were considered statistically significant if  $p \leq 0.05$ .

290

## 291 **3. Results**

### 292 **3.1. BBB permeability**

293 BBB permeability in the hypothalamic mPOA and hippocampal CA1, CA3 and DG sub-  
294 regions was assessed in mice exposed to the vehicle or DEHP alone or in a phthalate  
295 mixture by using exogenous Evans Blue dye (Figure 1; A-H), which binds to albumin protein,  
296 and endogenous circulating IgG molecules (Figure 1; I-P). Detection of these tracers in the  
297 parenchyma, called extravasation, indicates a BBB leakage. Therefore, to visualize blood  
298 capillary walls, laminin, the main basal lamina protein, was immunodetected.

299 In the hypothalamic mPOA, fluorescent signal analyses showed treatment had had a  
300 significant effect ( $p \leq 0.01$ ) on the extravasation of the Evans Blue dye outside perfused brain  
301 capillaries, with an increased extravasation in DEHP-5-, DEHP-50- and Mix-treated mice  
302 (+110%  $p \leq 0.01$ , +130%  $p \leq 0.01$  and +135%  $p \leq 0.001$ , respectively, above vehicle-treated  
303 mice) determined by posthoc analyses (Figure 1A and B). In the hippocampus, the treatment

304 was found to have had an effect on the extravasation of the Evans Blue dye in the CA1 and  
305 CA3 regions ( $p \leq 0.0001$ ,  $p \leq 0.01$ , respectively). A significant increase above vehicle-treated  
306 mice was evidenced by posthoc analyses for DEHP-5- and DEHP-50-treated mice in the  
307 CA1 parenchyma (+95%  $p \leq 0.01$  and +170%  $p \leq 0.001$  respectively,  $p \leq 0.05$ ), but only for  
308 DEHP-50-treated mice in the CA3 parenchyma (+70%,  $p \leq 0.05$ ) (Figure 1 C-F). In contrast,  
309 there was no treatment effect in the DG capillaries, which greatly restricted Evans blue dye  
310 extravasation to the brain parenchyma (Figure 1G and H).

311 Immunolabelling of endogenous circulating IgG showed that endogenous IgG were observed  
312 and were restricted to capillary wall or lumen in vehicle-treated mice (Figure 1K and M). In  
313 contrast, the effect of treatment on IgG immunoreactivity in the mPOA ( $p \leq 0.01$ ), with higher  
314 levels in DEHP-5, DEHP-50- and mixture-treated mice (+160%,  $p \leq 0.01$ ; +35% and +15%,  $p$   
315  $\leq 0.05$ ; respectively) in the parenchyma of phthalate-treated mice compared to the vehicle  
316 group (Figure 1I and J) was shown by posthoc analyses. Similarly, a treatment effect ( $p \leq$   
317  $0.0001$ ) was found in the CA1 area with significantly higher levels of endogenous IgG in the  
318 DEHP-5 (+140 %,  $p < 0.05$ ), DEHP-50 (+270 %,  $p < 0.01$ ) and Mix-groups (+900 %,  $p <$   
319  $0.0001$ ) versus the vehicle group (Figure 1K and L) was shown by posthoc analyses. There  
320 was also a treatment effect in the CA3 area ( $p \leq 0.0001$ ) as illustrated in Figure 1M and N;  
321 posthoc analyses showed increased levels for mice exposed to DEHP alone or in a mixture  
322 (+ 86%, +89% and +100%,  $p \leq 0.0001$ , for DEHP-5, DEHP-50 and mixture-group vs. vehicle,  
323 respectively). In accordance with the result obtained for Evans blue dye, no extravasation of  
324 circulating IgG from blood compartment to hippocampal DG parenchyma was detected  
325 (Figure 1O and P).

### 326 **3.2. Protein levels and distribution of TJ components**

327 BBB leakage induced by DEHP alone or in a mixture could be due to an alteration of  
328 capillary inter-endothelial TJs leading to paracellular transport of components. TJs comprise  
329 a complex of transmembrane proteins claudin-5 and occludin, which are associated with  
330 peripheral scaffolding proteins. Therefore, we investigated the distribution and amounts of

331 these proteins using immunofluorescence and Western blot analysis on microvessel-  
332 enriched fractions. No significant effect of treatment was found on the protein amounts of  
333 claudin-5 and occludin in the capillaries of hypothalamus (Figure 2A and B) and  
334 hippocampus (Figure 2D and E). In addition, confocal microscopy analysis of the capillaries  
335 of hypothalamic mPOA and hippocampal CA1, CA3 and DG sub-regions, showed no  
336 significant treatment effect on the distribution of immuno-labelling of claudin-5  
337 (supplementary figure S2) and occludin (supplementary Figure S3).

338 Those endothelial transmembrane components of TJs interact with the actin cytoskeleton via  
339 cytoplasmic TJs accessory proteins with the ZO-1 protein having predominance. Data from  
340 Western blot performed on microvessel-enriched fractions obtained from the hypothalamus  
341 (Figure 2C), highlighted a treatment effect on ZO-1 protein amount ( $p \leq 0.0001$ ), with less  
342 ZO-1 for both DEHP-5- and DEHP-50-treated mice (-75%,  $p \leq 0.0001$ ) and also Mix-treated  
343 mice (-65%,  $p \leq 0.0001$ ) versus the vehicle group following posthoc analyses. A treatment  
344 effect was also found on the amount of ZO-1 in hippocampal capillaries (Figure 2F). Posthoc  
345 analyses showed that the amount of ZO-1 was significantly increased for DEHP-50- treated  
346 mice (+50%,  $p \leq 0.05$ ), whereas no significant difference was detected for DEHP-5- and  
347 mixture-treated groups compared to vehicle (Figure 2F). As for claudin-5 and occludin,  
348 confocal microscopy analysis of the capillaries of hypothalamic mPOA and hippocampal  
349 CA1, CA3 and DG sub-regions, showed no treatment effect on the immuno-labelling of ZO-1  
350 protein (supplementary Figure S4).

### 351 **3.3. Cav-1 protein level in capillaries walls**

352 Caveolae are non-clathrin-coated vesicles with plasma membranes enriched in caveolin  
353 (Cav) proteins, involved in the receptor-independent transcellular transport of molecules from  
354 blood to parenchyma. We analyzed the level of caveolin-1 isoform protein (Cav-1), the main  
355 component of the caveolae plasma membranes. Fluorescence analysis revealed an effect of  
356 treatment ( $p \leq 0.01$ ) on Cav-1 immunoreactivity in capillary walls and posthoc analyses  
357 showed a significant high decrease for DEHP-50- and Mix-treated mice in the mPOA (DEHP-

358 50 group: -50 %;  $p < 0.05$  and mixture-group: -75%;  $p < 0.01$  vs. vehicle; Figure 3A and B)  
359 and in the CA-1 (DEHP-50 group: -60 %;  $p < 0.05$  and mixture-group: -70%;  $p < 0.01$  vs.  
360 vehicle; Figure 3D and E) and CA-3 areas (-70 %;  $p < 0.01$  vs. vehicle in DEHP-50 and  
361 mixture-group; Figure 3F and G). Again, no significant treatment effect was found in  
362 hippocampal DG (Figure 3H and I). These results were confirmed by Western blot analysis  
363 performed on microvessel-enriched fractions showing a treatment effect ( $p \leq 0.001$ ), with a  
364 decrease by -63% and -56% ( $p < 0.01$  in DEHP-50 and mixture-group, respectively) in  
365 hypothalamic vessels (Figure 3C) and -60% ( $p < 0.01$ ) and -50% ( $p < 0.05$ ) in hippocampal  
366 vessels of DEHP-50 and mixture-group respectively in comparison to the vehicle group  
367 (Figure 3J) determined by posthoc analyses.

### 368 **3.4. Glial activation and neuroinflammation**

369 The neuroinflammation process occurs following several brain insults and is often associated  
370 with a dysfunction of the neurovascular unit. Neuroinflammatory responses are underlined on  
371 the one hand by a glial activation involving an increase of glial specific protein levels,  
372 NDRG2, S100 $\beta$  and GFAP for astrocyte, and Iba-1 for microglia, and on the other hand by  
373 an increase of inflammatory molecules such as iNOS and COX-2. The distribution of these  
374 proteins was assessed using immunofluorescence, and protein levels in glial cells closely  
375 associated with capillaries were assessed using Western blot analysis performed on  
376 microvessel-enriched fractions from the whole hypothalamus and hippocampus.

377 In the mPOA, analyses of immunofluorescence results showed an effect of treatment on  
378 NDRG2 and S100 $\beta$  labellings ( $p \leq 0.0001$ ;  $p \leq 0.05$ , respectively). A significant increase of  
379 NDRG2 labelling in astrocytes for mixture-treated mice (+125%,  $p < 0.0001$  vs. vehicle,  
380 Figure 4A and B) and a significant increase of S100 $\beta$  labelling in astrocytes for DEHP-50-  
381 and mixture-treated mice (+92% and +82% vs. vehicle, respectively,  $p < 0.05$ , Figure 4A and  
382 C) were determined by posthoc analyses. All NDRG2-positive astrocytes are immunoreactive  
383 for S100 $\beta$  but few S100 $\beta$ -positive astrocytes express NDRG2. This astrocytic activation was  
384 confirmed by a significant enhancement of GFAP immunofluorescence density in the



385 parenchyma and in the vicinity of capillaries for DEHP-50-treated mice (+ 100%;  $p < 0.05$  vs.  
386 vehicle, Figure 4D and E). By contrast, Western blot analysis performed on microvessel-  
387 enriched fractions showed an effect of treatment ( $p \leq 0.0001$ ), with a significant decrease of  
388 GFAP amount in the astrocytic end-feet which remained in contact with the isolated vessels  
389 for DEHP-5-, DEHP-50- and mixture-treated mice compared to vehicle (-40%,  $p < 0.01$ , -  
390 55%,  $p < 0.0001$  and -37%,  $p < 0.01$ , respectively vs. vehicle, Figure 4G), determined by  
391 posthoc analyses.

392 Immunofluorescent analysis showed an effect of treatment ( $p \leq 0.01$ ), with a stronger signal  
393 for iNOS colocalized with GFAP surrounding capillaries not only for DEHP-5-, DEHP-50-  
394 treated mice (+280 %;  $p < 0.01$  vs. vehicle) but also for mixture-treated mice compared to  
395 vehicle (+360 %;  $p < 0.001$  vs. vehicle) (Figure 4D and F). Conversely, no significant effect  
396 was highlighted for iNOS protein level using Western blot analysis performed on  
397 hypothalamic microvessel-enriched fractions (Figure 4H). Immunohistochemical labelling for  
398 the specific marker of microglial cells, Iba-1, was also changed by the treatment ( $p \leq 0.01$ )  
399 and posthoc analyses showed a significant increase in the mPOA parenchyma for DEHP-50-  
400 treated mice (+125%;  $p < 0.01$  vs. vehicle, Figure 6A and B), whereas hypothalamus  
401 capillary-enriched fraction showed no difference in the amount of Iba-1 protein among the  
402 experimental groups (Figure 6C). COX-2 protein was immuno-detected in Iba-1-  
403 immunopositive microglial cells in the mPOA (Figure 6A) especially for DEHP-50-treated  
404 group compared to vehicle (+130 %;  $p < 0.01$ , Figure 6D). However, no significant difference  
405 in the COX-2 protein amount was measured in the capillaries of hypothalamus of phthalate-  
406 treated mice compared to those of vehicle-treated mice (Figure 6E).

407 In the hippocampus, analysis of the environment of the capillaries of CA1, CA3 and DG sub-  
408 regions showed no significant treatment effect for immuno-labelling intensity of NDRG2 and  
409 S100 $\beta$  for all phthalate-treated groups compared to the vehicle (Figure 5A-F). Observations  
410 using confocal microscopy confirmed these results (supplementary figure S5). Quantification  
411 of GFAP immunoreactivity displayed no significant treatment effect between control and

412 phthalate-treated mice not only in the CA1 (Figure 5G and H) but also in the CA3 (Figure 5J  
413 and K) and in the DG region (Figure 5M and N). Western blot analysis performed on  
414 hippocampal microvessel-enriched fractions did not show any modification of GFAP protein  
415 content in the hippocampus, regardless of the experimental group (Figure 5P).  
416 Immunofluorescent analysis showed that iNOS labelling was not co-localized with GFAP  
417 labelling in the CA1 region (Figure 5G), but was significantly impacted by the treatment ( $p \leq$   
418 0.01) and enhanced for DEHP-5-, DEHP-50 and mixture-treated mice compared to the  
419 vehicle (+180 %;  $p < 0.01$ , +125 %;  $p < 0.05$ , and +115 %;  $p < 0.05$ , respectively vs. vehicle,  
420 Figure 5I) determined by posthoc analyses. In the hippocampal CA3 region, the level of  
421 iNOS immunoreactivity, not detected in astrocyte (Figure 5J), was equivalent between  
422 animals treated with the vehicle and DEHP alone or in a mixture (Figure 5L). In the  
423 hippocampal DG, iNOS immunoreactivity was affected by the treatment ( $p \leq 0.001$ ) and  
424 significantly increased only for DEHP-5-treated mice (+270%;  $p < 0.001$ , vs. vehicle, Figure  
425 5O). Finally, no effect on iNOS protein amount was measured (Figure 5Q) in the  
426 hippocampal microvessels.

427 Iba-1 immunoreactivity was affected by treatment ( $p \leq 0.05$ ) and increased in the CA1  
428 (+115%, + 125% and + 118%;  $p < 0.05$  vs. vehicle in DEHP-5, DEHP-50 and mixture-group  
429 respectively; Figure 6F and G), and in CA3 areas (DEHP-5 group: + 200%;  $p < 0.0001$ ;  
430 DEHP-50 group: + 90 %;  $p < 0.05$ ; mixture-group: + 80%;  $p < 0.05$  vs. vehicle; Figure 6I and  
431 J). No treatment effect was measured in the DG (Figure L and M). In the hippocampal  
432 capillaries-enriched fractions, no significant treatment effect was observed on the protein  
433 amounts of Iba-1 (Figure 6O). Confocal microscopy and Western blot analysis showed no  
434 significant effect on COX-2 immunolabelling (Figure 6H, K and N) and the amount of protein  
435 in microvessel-enriched fractions of the hippocampus (Figure 6P).

#### 436 **4. Discussion**

437 This study aimed to characterize the impact of a subchronic oral exposure to environmental  
438 doses of DEHP alone or in a phthalate mixture on the neurovascular unit in the  
439 hypothalamus and hippocampus of adult male mice. To our knowledge of the available  
440 literature, this study is the first to assess the effects of adult exposure to low doses of  
441 phthalates on the blood-brain barrier.

442 The data obtained show that exposure to DEHP alone or in a phthalate mixture significantly  
443 increased the BBB permeability in the hypothalamic mPOA and the hippocampal CA1 and  
444 CA3 regions as evidenced by the use of two different techniques assessing extravasation of  
445 exogenous Evans blue tracer and circulating endogenous IgG. It is generally accepted that  
446 increased permeability of the BBB reflects the failure of its functional integrity (Erdő et al.,  
447 2017), which can be manifested by an alteration at the molecular and / or morphological level  
448 of the constituents of these TJs (Obermeier et al., 2013). Our results show that exposure to  
449 DEHP alone or in a phthalate mixture did not affect the expression of the major TJs  
450 transmembrane proteins, claudin-5 and occludin, indicating that the increased BBB  
451 permissiveness was not due to changes in these protein amounts in the two brain structures  
452 we assessed. Our results show that the increased BBB permeability induced by exposure to  
453 DEHP alone at 5 or 50 µg/kg/d or DEHP 5 µg/kg/d in a phthalate mixture was associated with  
454 a dramatically decreased amount of the accessory junctional protein ZO-1 in hypothalamic  
455 microvessel-enriched fractions. An opposite effect was observed in hippocampal  
456 microvessel-enriched fractions in the group exposed to DEHP at 50 µg/kg/d. The underlying  
457 cause of these changes could, at least in part, be the observed BBB integrity failure induced  
458 by exposure to DEHP alone or in a mixture, since interaction between claudin, occludin and  
459 ZO-1 is pivotal for facilitating tight junction assembly, and regulating the effectiveness of TJs  
460 complex function (Abbott et al., 2010). However, it is important to note that no difference was  
461 detected in ZO-1 immunoreactivity when assessed by immunohistochemistry in the mPOA,  
462 or CA1 and CA3 regions. A possible explanation could be that in the brain sections submitted  
463 to immunohistochemical analyses, the quantifications were carried out from 2-dimensional

464 images and therefore the absence of the third dimension may impede the complete  
465 evaluation of the components of TJ proteins.

466 In addition to this possible impairment in the organization of endothelial TJs, our results also  
467 highlight potential modifications in caveolae-mediated transcellular transport. Indeed, adult  
468 exposure to DEHP at 50 µg/kg /d or to DEHP 5 µg/kg /d in the phthalate mixture led to a  
469 significant decrease in Cav-1 immunoreactivity in the mPOA and CA1 and CA3 regions, and  
470 in the amount of protein of the microvessel-enriched fractions of the hypothalamus and  
471 hippocampus.

472 In the DG, none of the modifications induced by phthalate exposure on BBB permeability and  
473 amounts of proteins involved in endothelial TJs and Cav-1 were observed, indicating that  
474 BBB integrity was preserved in this hippocampal vascular network. These results suggest  
475 that, within the same brain structure, the BBB may exhibit differential sensitivity to exposure  
476 to DEHP alone or in a mixture. This difference in sensitivity within the hippocampus suggests  
477 that maintaining BBB would involve different signalling pathways, with the DG containing  
478 neural circuits different from those of the CA1 and CA3 subregions and being the site of adult  
479 neurogenesis.

480 The modifications induced by exposure to DEHP alone or in a phthalate mixture in the BBB  
481 integrity were associated with astrocyte and microglial activation in the hypothalamic mPOA.  
482 Astrocyte activation was characterized by the increased immunoreactivity for both GFAP,  
483 NDRG2 and S100β in the parenchyma of the mPOA. Firstly, this is in accordance with our  
484 recent study reporting that chronic adult exposure to DEHP at 50 µg/kg/d induced an up-  
485 regulation of GFAP in the parenchyma of the mPOA in male mice (Dombret et al., 2017).  
486 Secondly, the data also indicate that adult exposure of male mice to DEHP alone or in a  
487 phthalate mixture was capable of affecting different steps of cellular activation and states.  
488 NDRG2 is induced in the early phase of astrocyte activation preceding GFAP expression (Lin  
489 et al., 2015), while S100β is defined as a state in which astrocytes lose their neural stem cell  
490 potential and acquire a more mature developmental stage (Raponi et al., 2007). Western blot

491 analysis performed with hypothalamic microvessel-enriched fractions showed a decreased  
492 GFAP amount in astrocyte end-feet associated with microvessels. This difference in the data  
493 protein obtained between the two techniques suggests a reorganization of the intermediate  
494 filament cytoskeleton of astrocytes involved in the BBB. Reactive astrocytes are known to  
495 express the pro-inflammatory molecule iNOS in response to various stimuli to produce an  
496 excessive amount of nitric oxide (NO), a gaseous signalling molecule involved in most cases  
497 with a neurodegenerative and neuroinflammatory status (Saha and Pahan, 2006). A Western  
498 blot performed on microvessel-enriched fractions from the whole hypothalamus showed no  
499 changes in the iNOS protein levels, but higher iNOS labelling was colocalized with GFAP  
500 labelling astrocyte end-feet associated with capillaries in the mPOA. This suggests that the  
501 inflammatory reaction mediated by astrocytes was initiated at, or limited to, the level of the  
502 BBB. The astroglial activation in the parenchyma of the mPOA induced by the exposure to  
503 DEHP at 50 µg/kg/d was accompanied by an activation of microglial cells, which also  
504 exhibited a higher immunoreactivity of the inflammatory molecule COX-2. The association  
505 between COX-2 expression and microglial activation could lead to NVU impairment through  
506 neuronal damage as has been previously reported (Vijitruth et al., 2006). Further studies will  
507 compare the impact of phthalate exposure on neuronal integrity.

508 In the hippocampus, no changes in astrocyte activation markers were induced by adult  
509 exposure to DEHP alone or in a phthalate mixture, suggesting an absence of astrocyte  
510 activation in this region despite the increased BBB permeability. In contrast, microglial  
511 activation was observed in the CA1 and CA3 sub-regions following exposure to DEHP alone  
512 or in a phthalate mixture, whereas no changes in COX-2 immunoreactivity were  
513 demonstrated. A recent investigation has shown that prenatal exposure to DEHP at 200  
514 µg/kg/d increased the level of COX-2 in pyramidal neurons of the CA-2/3 region in adult male  
515 mice (Barakat et al., 2018). This suggests that differences in induced COX-2 expression by  
516 phthalates may depend on the period of exposure. Alternatively, a longer exposure period in  
517 adult males may be a more efficient way of inducing astrocyte activation and COX2

518 expression. Nevertheless, exposure to DEHP alone or in a mixture-induced iNOS activation  
519 was noted in the CA1 and DG sub-regions but not in the CA3 area, while BBB leakage was  
520 detected in both the CA1 and CA3 areas. The iNOS labelling was thus not colocalized with  
521 the astrocytic marker GFAP, suggesting that astrocytes of the BBB may not be the only cells  
522 capable of producing NO under these conditions. Indeed, different brain cell types including  
523 neurons, macrophages and microglia, can respond to different stimuli with iNOS production  
524 and take part in brain inflammation (Heneka and Feinstein, 2001; Sierra et al., 2014). It is  
525 thus possible that an increased amount of iNOS protein in the hippocampus may arise from  
526 an additional direct impact of DEHP alone or in a mixture on the parenchyma. Phthalates are  
527 able to cross the BBB thus eliciting direct effects through neuronal damage,  
528 neuroinflammation, oxidative stress and alteration of monoaminergic, cholinergic and amino-  
529 acids transmission as has been reported for adult or developmental exposure to DEHP or  
530 DBP at high doses ranging from 30 to 750 mg/kg/d (Kassab et al., 2019; You et al., 2018).  
531 Thus, the question remains as to whether the BBB dysfunction is a cause or a consequence  
532 of a neuroinflammatory state.

533 Altogether, these data show that the BBB dysfunction and generally associated astroglial  
534 activation and neuroinflammation were differently induced by adult exposure to DEHP alone  
535 or in a phthalate mixture (Table 2). Differences were seen between the hypothalamic mPOA  
536 and hippocampus, in particular for the astrocyte activation. Differences in sensitivity to  
537 exposure were also suggested by the obtained data for each brain area. In the mPOA, while  
538 BBB permeability and iNOS induction were affected since 5 µg/kg/d of DEHP alone or in a  
539 mixture, the other parameters were rather altered at 50 µg/kg/d of DEHP and /or the mixture,  
540 suggesting that the other phthalates add to the effects of DEHP-5. In the hippocampus, the  
541 DG appeared less sensitive showing only an induced iNOS in response to DEHP treatment  
542 while the CA1 and CA3 areas were more reactive, with effects induced since 5 µg/kg/d of  
543 DEHP alone for BBB permeability, microglial activation and neuroinflammation.

544 The down-regulation of the androgen receptor (AR) induced by exposure to DEHP in the  
545 hypothalamic mPOA has been previously shown (Dombret et al., 2017). Thus, the alterations  
546 triggered by adult exposure to DEHP alone or in a phthalate mixture in the neurovascular unit  
547 of the mPOA may be also linked, at least in part, to the down-regulation of the AR.  
548 Furthermore, in another study, we have shown that testosterone depletion in adult male  
549 mice, leading to a down-regulation of the neural AR, triggered i) BBB permeability for Evans  
550 Blue and endogenous IgG with a disorganization of TJ structure including a reduced protein  
551 amount of ZO-1, ii) increased activation of astrocytes and microglia and iii) up-regulation of  
552 inflammatory molecules such as iNOs and COX2 (Atallah et al., 2017). Finally, the AR  
553 mediated-transcriptional activity is, at least in part, positively modulated by the caveolae  
554 transmembrane Cav-1 protein (Bennett et al., 2010; Lu et al., 2001). The tight regulation by  
555 androgens of several proteins involved in these processes is detailed in a recent review  
556 (Ahmadpour and Grange-Messent, 2020).

557 The hippocampus is also an androgen-sensitive area, as evidenced by a high AR expression  
558 in male mice and altered related functions in a mouse model lacking the neural AR  
559 (Mhaouty-Kodja, 2018; Picot et al., 2016; Raskin et al., 2009). It is thus possible that the  
560 alterations reported here in the neurovascular unit of this structure may also depend, at least  
561 in part, on an impact on the AR signalling pathway. This can explain the comparable data  
562 obtained on BBB permeability, endothelial TJs and trans-endothelial vesicular transport in  
563 the mPOA and CA1 and CA3 regions following exposure to DEHP alone or in a phthalate  
564 mixture (Table 2). The CA1 and CA3 regions contain much more AR-immunoreactive  
565 neurons than the DG (Raskin et al., 2009), which may explain the reduced sensitivity of this  
566 latter area to DEHP exposure.

567 The differences observed in astrocyte activation and COX-2 expression between the mPOA  
568 and the hippocampal CA1 and CA3 regions, and also within the same brain area for the  
569 different components of the neurovascular unit could be underlined by different expression  
570 patterns of AR but also of estrogen receptors (ERs) in neurons, endothelial cells, astrocytes

571 and microglia, which also express these receptors (Hajszan et al., 2007; Kerr et al., 1995;  
572 Picot et al., 2016). Indeed, in the male rodent nervous system, testosterone exerts its effects  
573 through direct activation of the AR, but can also be metabolized into estradiol, which then  
574 stimulates ER $\alpha$  and ER $\beta$ .

575 The effects induced by DEHP exposure i.e. BBB permeability, astroglial activation and  
576 neuroinflammation in adult male mice may participate, in particular in the mPOA, to the  
577 altered sexual behaviour observed under similar experimental conditions (Dombret et al.,  
578 2017). Current studies explore the cognitive impact of male mice exposure to low doses of  
579 phthalate but only one recent study addressed the effects of adult DEHP exposure on spatial  
580 learning and memory at high doses of 100 and 300 mg/kg/d in male rats (Ran et al., 2019).  
581 Besides behavioural alterations, the BBB leakage and inflammatory signs induced by oral  
582 exposure to low doses of phthalates reported in the present study may participate in other  
583 neural alterations. Although they appear moderate, these modifications could have  
584 deleterious long-term consequences in the case of chronic environmental exposure. Indeed,  
585 BBB impairment and neuroinflammation are suspected to be involved in neurodegenerative  
586 diseases and aging processes (Erdő et al., 2017).

587 Our analyses addressing the impact of DEHP exposure on behavioural responses (Dombret  
588 et al., 2017) and BBB integrity (present study) in male mice show that DEHP-induced effects  
589 can be observed at low doses equivalent or below the tolerable daily intake dose of 50  
590  $\mu\text{g}/\text{kg}/\text{d}$ , which was established and recently updated by the European Food Safety Authority  
591 on the basis of reduced fetal testosterone production. This indicates that the nervous system  
592 including the neurovascular unit is highly sensitive to phthalates and should be also  
593 considered as a relevant endpoint in risk assessment for these molecules. In this context, it  
594 is important to stress that the human cerebrovascular function also seems sensitive to  
595 androgen, although these effects are less defined than for estrogens (see for review,  
596 (Robison et al., 2019)).

## 597 **5. Conclusion**



598 The present study shows that phthalates, a family of endocrine disruptors, can impact the  
599 cerebrovascular function at doses close to the environmental exposure in adult male mice.  
600 Subchronic exposure to low doses of DEHP alone or in a phthalate mixture increases BBB  
601 permeability of two brain regions leading to a dysfunctionality of this selective interface,  
602 which is needed to ensure a tight regulation between the circulatory system, the immune  
603 system and the brain parenchyma. In addition, we also show similarities and differences in  
604 the vulnerability of the NVU of the two brain regions, one controlling male sexual behaviour,  
605 and the second one involved in cognitive function. Based on our previous studies, we  
606 suggest that phthalates may operate through a mode of action involving at least a partial  
607 disruption of neural *AR* expression in the mPOA, but the ER and/or other signalling pathways  
608 may be also involved in the hippocampus.

609 These data suggest that exposure to endocrine disruptors may be considered as an  
610 environmental risk factor for the cerebrovascular function.

611

## 612 **Author contributions**

613 **Delnia Ahmadpour:** Investigation, Formal analysis, Writing-original draft. **Valérie Grange-**  
614 **Messent:** Conceptualization, Supervision, Writing-review & editing. **Sakina Mhaouty-Kodja:**  
615 Funding acquisition, Review & editing.

616

## 617 **Declaration of Interest Statement**

618 The authors declare they have no actual or potential competing financial interests or  
619 personal relationships that could have appeared to influence the work reported in this paper.

620

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625

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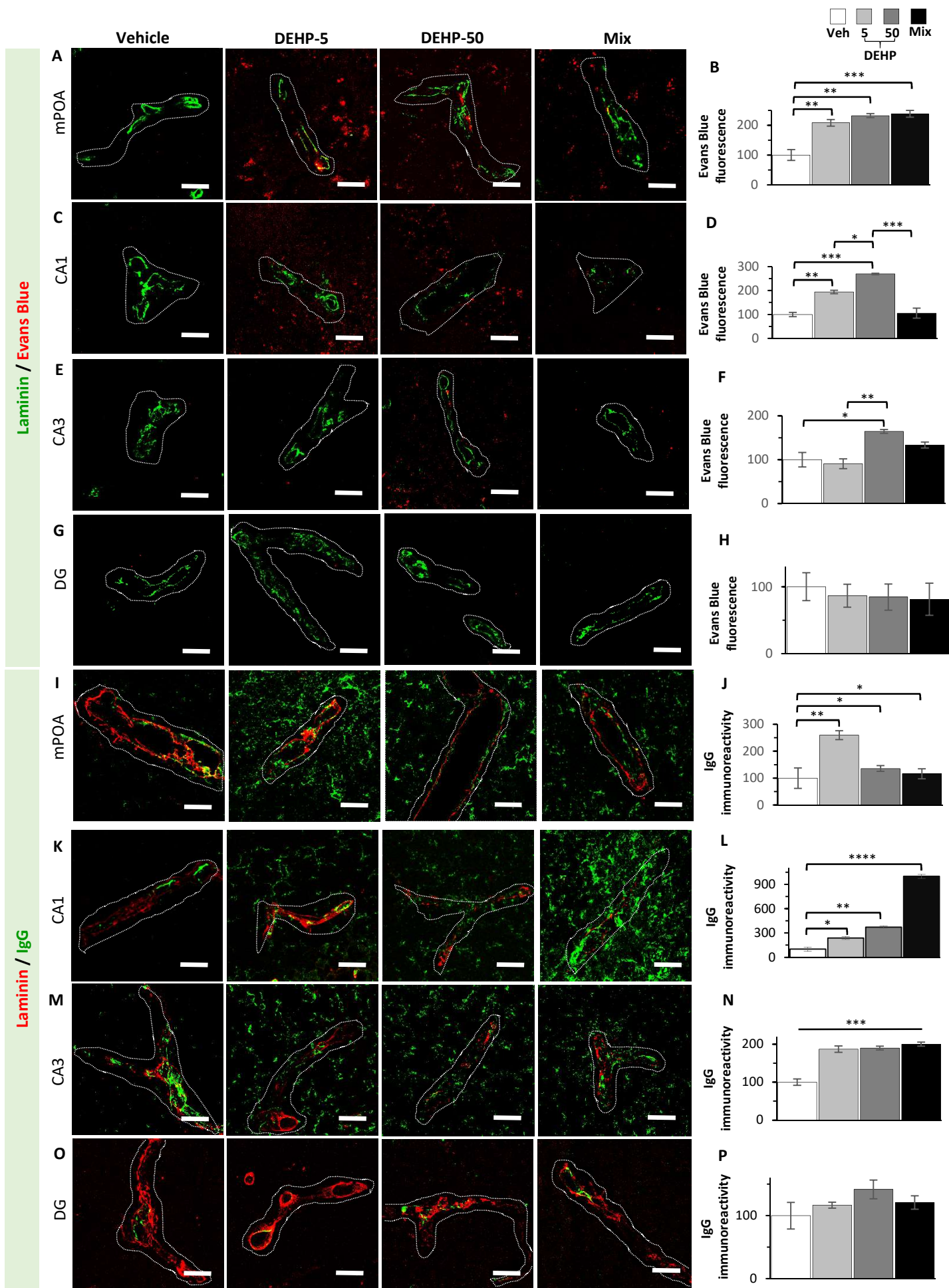
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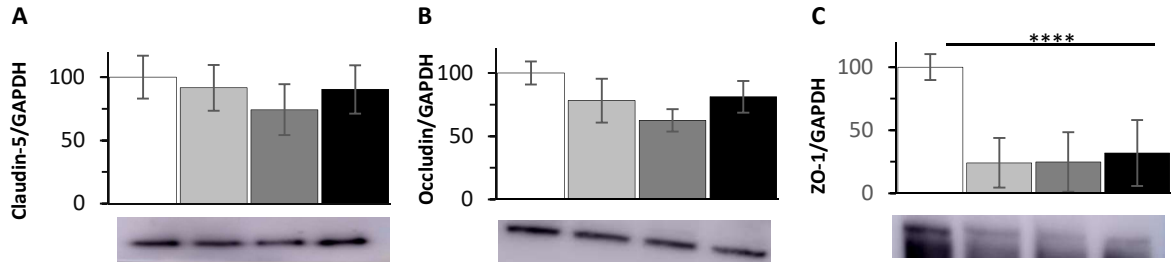
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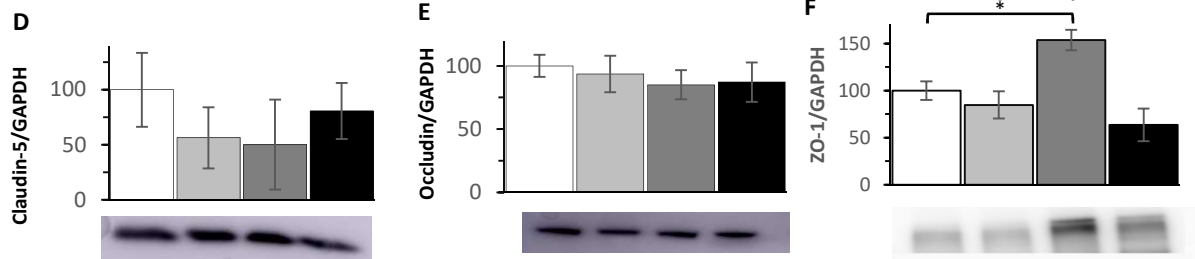


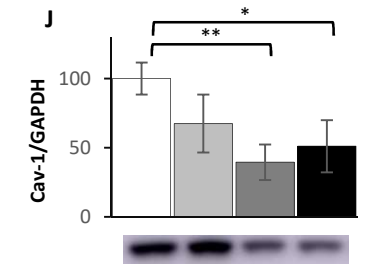
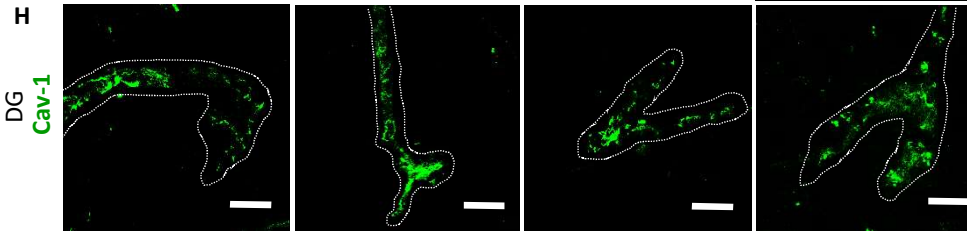
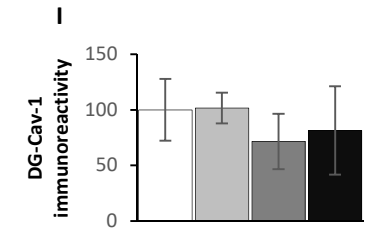
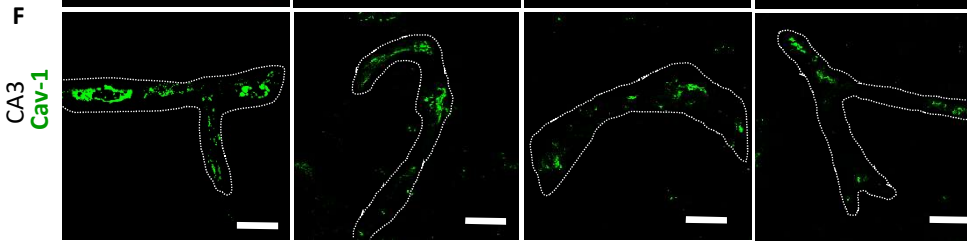
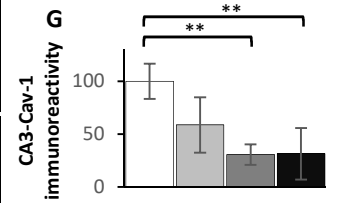
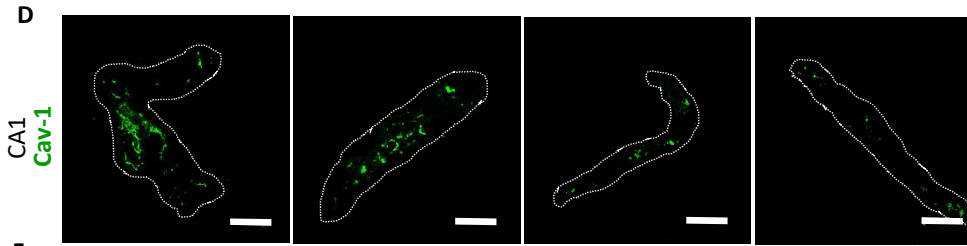
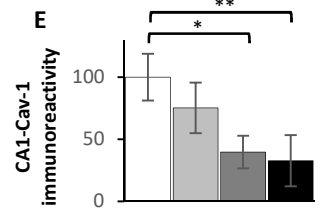
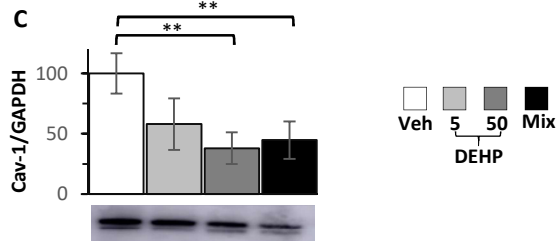
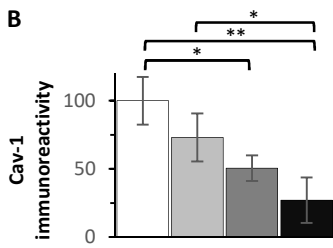
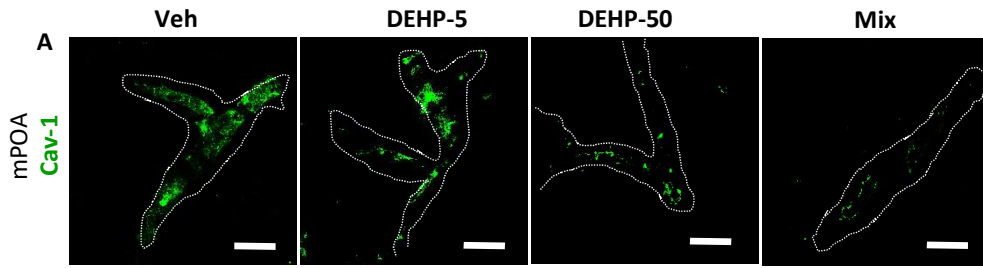
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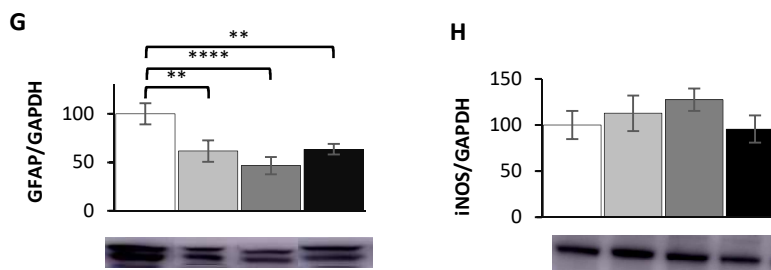
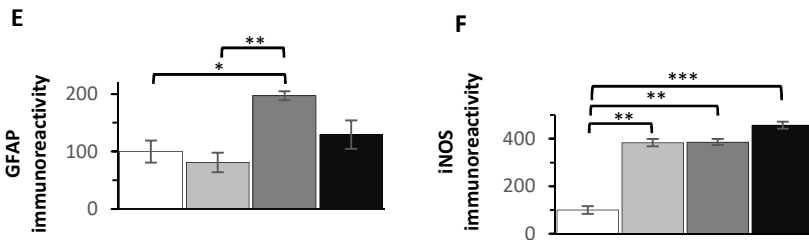
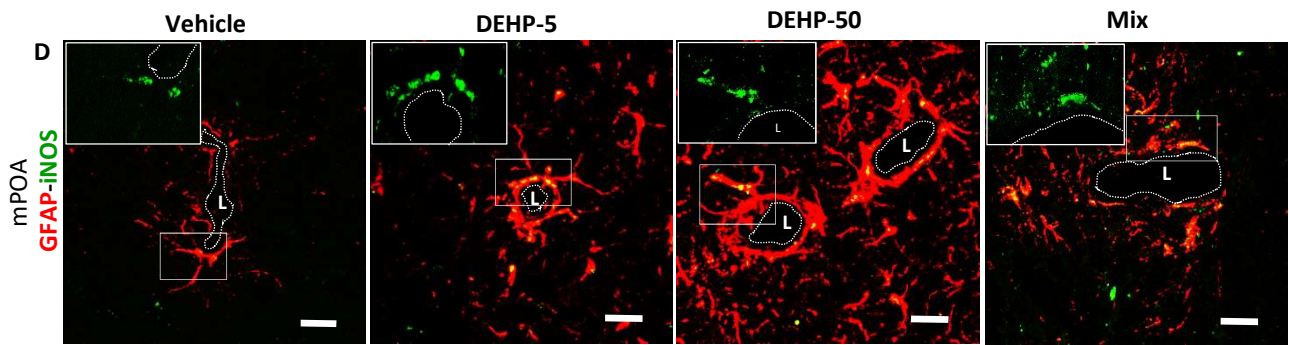
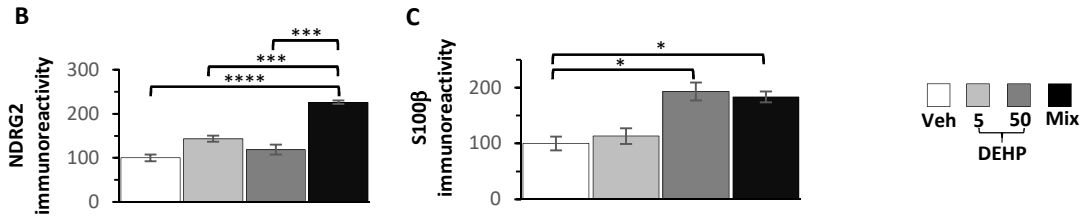
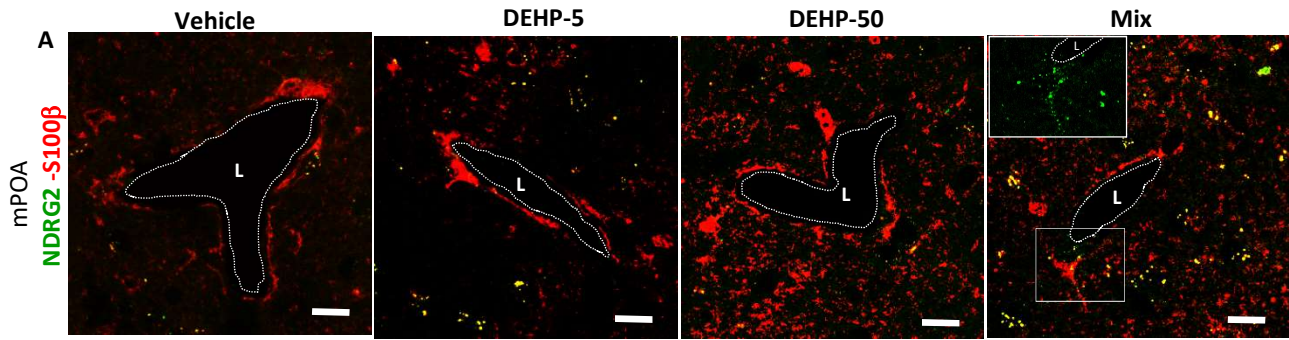
Veh 5 50 Mix  
DEHP



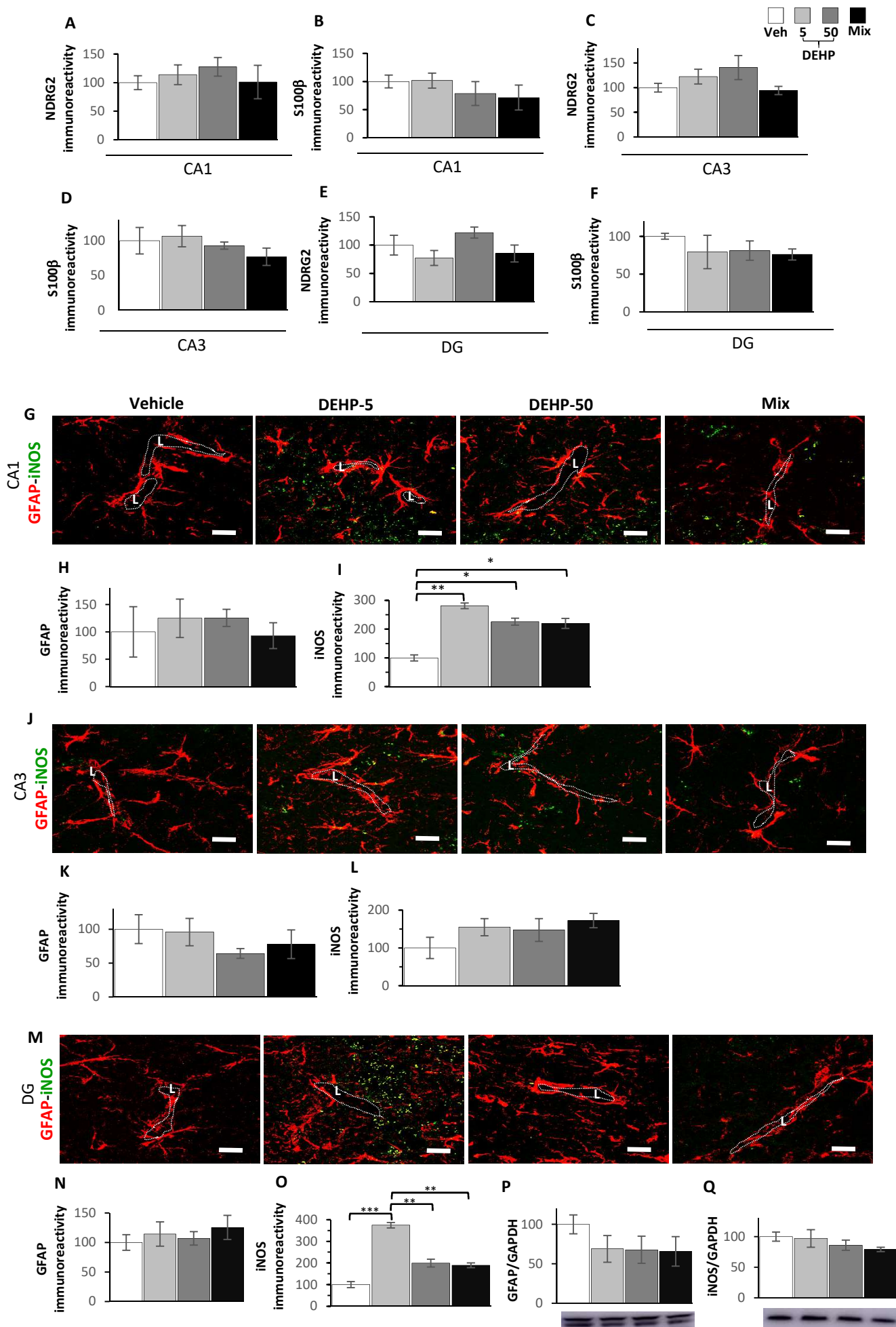
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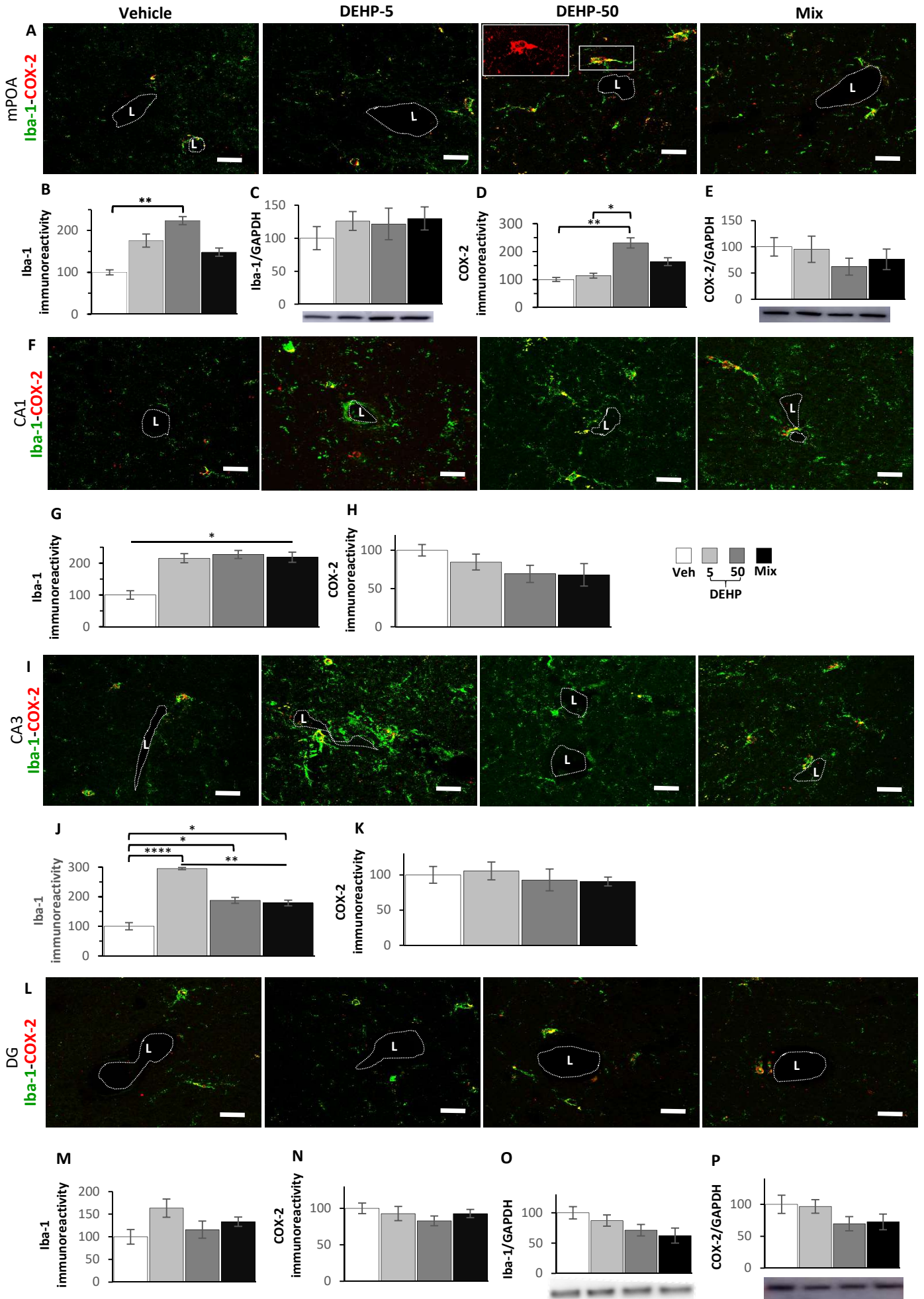












## 1 **Figure legends**

### 2 **Figure 1. Exposure to DEHP alone or in a phthalate mixture increased BBB permeability** 3 **to Evans blue dye and endogenous IgG.**

4 The permeability of BBB was analyzed using Evans blue dye (n=3 per group, A to H) and  
5 immunolabeled endogenous IgG (n=5 per group, I to P) in the hypothalamic medial preoptic  
6 area (mPOA) and hippocampal CA1, CA3 and DG. Images were selected from brain sections  
7 containing the sub-regions of interest in adult male mice orally exposed to vehicle (Veh; first  
8 column), DEHP at 5 µg/kg/d (DEHP-5; second column), DEHP at 50 µg/kg/d (DEHP-50; third  
9 column) and a phthalate mixture (Mix; fourth column). (A-H) Representative images and  
10 corresponding quantitative analysis of the six to eight serial sections for each brain examined  
11 of the mPOA (A, B), CA1 (C, D), CA3 (E, F) and DG (G, H) for Evans blue dye tracer (red),  
12 capillaries were labeled with anti-laminin (green). (I-P) Representative images and  
13 corresponding quantitative analysis of the six to eight serial sections for each brain examined  
14 of the mPOA (I, J), CA1 (K, L), CA3 (M, N) and DG (O, P) of endogenous circulating IgG  
15 (green), capillaries were labeled with anti-laminin (red).

16 \* $p < 0.05$ ; \*\* $p < 0.01$ ; \*\*\* $p < 0.001$ ; \*\*\*\* $p < 0.0001$  compared to vehicle-treated mice compared  
17 to the vehicle group. All data are expressed as mean percentages  $\pm$  S.E.M of vehicle (100%).

18 The pictures presented are the projection of 3 successive optical sections (1 µm) into one  
19 image.

20 Scale bar: 10 µm. The dotted lines delimit the outer contour of the capillaries. The  
21 quantifications of fluorescence density were measured over the entire surface of the images.

22

### 23 **Figure 2. Exposure to DEHP alone or in a phthalate mixture affected endothelial tight** 24 **junction protein ZO-1 levels but not claudin-5 and occludin protein levels.**

25 Western blot analysis (n = 6 per treatment group) of tight junction proteins performed on  
26 microvessel-enriched fractions from hypothalamus (A-C) and hippocampus (D-F) of mice  
27 exposed to the Vehicle (Veh), DEHP at 5 µg/kg/d (DEHP-5), DEHP at 50 µg/kg/d (DEHP-50)  
28 or the phthalate mixture (Mix). All data are expressed as mean percentages ± S.E.M of vehicle  
29 (100%). \**p* < 0.05; \*\**p* < 0.01; \*\*\**p* < 0.001; \*\*\*\**p* < 0.0001 compared to the vehicle group. Data  
30 were normalized to GAPDH level.

31

32 **Figure 3. Exposure to DEHP alone or in a phthalate mixture decreased protein levels of**  
33 **the caveolae-associated membrane protein Cav-1.**

34 Images were selected from brain sections containing the sub-regions of interest in adult male  
35 mice orally exposed to vehicle (Veh; first column), DEHP at 5 µg/kg/d (DEHP-5; second  
36 column), DEHP at 50 µg/kg/d (DEHP-50; third column) and a phthalates mixture (Mix; fourth  
37 column). (A, B) Representative images of immunodetection of Cav-1 in the mPOA (A) and the  
38 corresponding quantitative analysis of the Cav-1 immunoreactivity density (B) of the six to eight  
39 serial sections for each brain examined (n = 5 per treatment group). Values represent mean  
40 percentages ± S.E.M of vehicle (100%). \**p* < 0.05; \*\**p* < 0.01 compared to the vehicle group.  
41 (C) Western blot analysis of Cav-1 performed on microvessel-enriched fractions. \**p* < 0.05; \*\**p*  
42 < 0.01 compared to the vehicle group (n = 6 per treatment group). Data were normalized to  
43 GAPDH levels.

44 (D-I) Representative images of immunodetection of Cav-1 in the hippocampus (D: CA1; F:  
45 CA3; H: DG) and the corresponding quantitative analysis of the Cav-1 immunoreactivity  
46 density (E: CA1; G: CA3; I: DG) of the six to eight serial sections for each brain examined (n=5  
47 per group). \**p* < 0.05; \*\**p* < 0.01 compared to the vehicle group. (J) Western blot analysis of  
48 Cav-1 performed on microvessel-enriched fractions from hippocampus. \**p* < 0.05; \*\**p* < 0.01  
49 compared to the vehicle group (n = 6 per treatment group). Data were normalized to GAPDH  
50 level.



51 Scale bar: 10  $\mu$ m. The dotted lines delimit the outer contour of the capillaries. The  
52 quantifications of fluorescence density were measured over the entire surface of the images.  
53 All values represent mean percentages  $\pm$  S.E.M of vehicle (100%).

54

55 **Figure 4. Exposure to DEHP alone or in a phthalate mixture induced astrocyte activation**  
56 **and iNOS expression in the medial preoptic area (mPOA).**

57 (A-C) Representative images (A) and corresponding quantitative analysis (B, C) of the six to  
58 eight serial sections for each brain examined of the co-immunolabeling of NDRG2 (green,  
59 insert) and S100 $\beta$  (red) in the mPOA of mice exposed to the vehicle (Veh), DEHP at 5  $\mu$ g/kg/d  
60 (DEHP-5), DEHP at 50  $\mu$ g/kg/d, or phthalate mixture (Mix) (n = 5 per treatment group). (D-F)  
61 Representative images (D) and corresponding quantitative analysis (E, F) of the six to eight  
62 serial sections for each brain examined of the co-immunolabeling of iNOS (green, inserts) and  
63 GFAP (red) in the mPOA (n=5 per group). \* $p$  < 0.05; \*\*\* $p$  < 0.001; \*\*\*\* $p$  < 0.0001 compared to  
64 the vehicle group.

65 (G, H) Western blot analysis (n = 6 per treatment group) of GFAP (G) and iNOS (H) performed  
66 on microvessel-enriched fractions from the hypothalamus. \* $p$  < 0.05; \*\* $p$  < 0.01; \*\*\* $p$  < 0.001;  
67 \*\*\*\* $p$  < 0.0001 compared to the vehicle group (n=6 per group). Data were normalized to  
68 GAPDH level.

69 Scale bar: 10  $\mu$ m. L: Lumen delimited by the dotted lines. The quantifications of fluorescence  
70 density were measured over the entire surface of the images. All values represent mean  
71 percentages  $\pm$  S.E.M of vehicle (100%).

72

73 **Figure 5. Exposure to DEHP alone or in a phthalate mixture had no effect on astrocyte**  
74 **activation in the hippocampus but induced iNOS expression in the CA1 and DG.**

75 (A-F) Quantitative analysis of the immunoreactivity of NDRG2 and S100 $\beta$  in the hippocampus  
76 (A, B: CA1; C D: CA3; E, F: DG) of the six to eight serial sections for brain collected from mice  
77 exposed to the vehicle (Veh), DEHP at 5  $\mu$ g/kg/d (DEHP-5), DEHP at 50  $\mu$ g/kg/d, or phthalate  
78 mixture (Mix) (n = 5 per treatment group). No significant difference was measured compared  
79 to vehicle-treated controls.

80 (G-O) Representative images of co-immunodetection of GFAP (red) and iNOS (green) in the  
81 hippocampus (G: CA1; J: CA3; M: DG) and their corresponding quantitative analysis of the  
82 immunoreactivity density (H, I: CA1; K, L: CA3; N, O: DG) of the six to eight serial sections for  
83 each brain examined (n=5 per group). \* $p$  < 0.05; \*\* $p$  < 0.01; \*\*\* $p$  < 0.001 compared to vehicle-  
84 treated controls.

85 (P, Q) Western blot analysis (n=6 per group) of GFAP (P) and iNOS (Q) performed on  
86 microvessel-enriched fractions from hippocampus. Data were normalized to GAPDH level. No  
87 significant difference was measured compared to vehicle-treated controls.

88 Scale bar: 10  $\mu$ m. L: Lumen delimited by the dotted lines. The quantifications of fluorescence  
89 density were measured over the entire surface of the images. All values represent mean  
90 percentages  $\pm$  S.E.M of vehicle (100%).

91

92 **Figure 6. Exposure to DEHP alone or in a phthalate mixture induced microglia activation**  
93 **in the testosterone-sensitive cerebral regions, the medial preoptic area (mPOA) and the**  
94 **hippocampus, and increased COX-2 expression in the mPOA.**

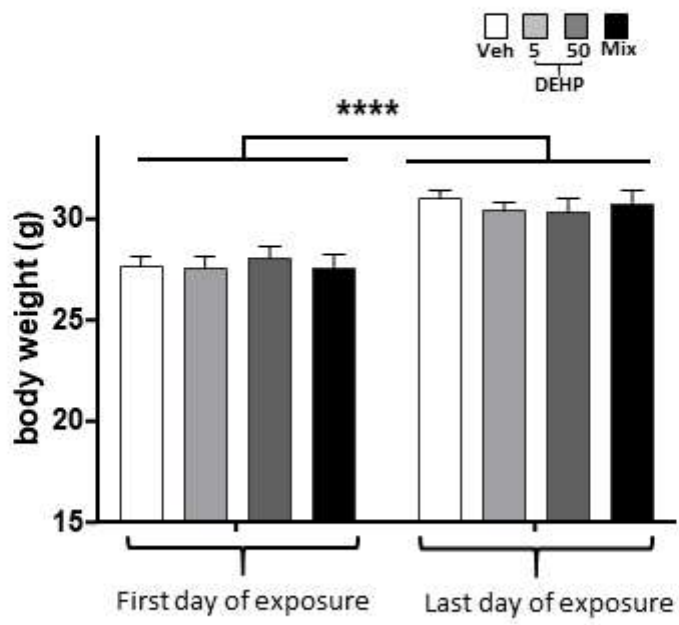
95 (A, B, D) Representative images (A) and corresponding quantitative analysis (B, D) of the six  
96 to eight serial sections for each brain examined of the co-immunolabeling of Iba-1 (green) and  
97 COX-2 (red, insert) in the mPOA of mice exposed to the vehicle (Veh), DEHP at 5  $\mu$ g/kg/d  
98 (DEHP-5), DEHP at 50  $\mu$ g/kg/d, or phthalate mixture (Mix) (n = 5 per treatment group). \* $p$  <  
99 0.05; \*\* $p$  < 0.01 compared to the vehicle group. (C, E) Western blot analysis (n = 6 per  
100 treatment group) of Iba-1 (C) and COX-2 (E) performed on microvessel-enriched fractions from

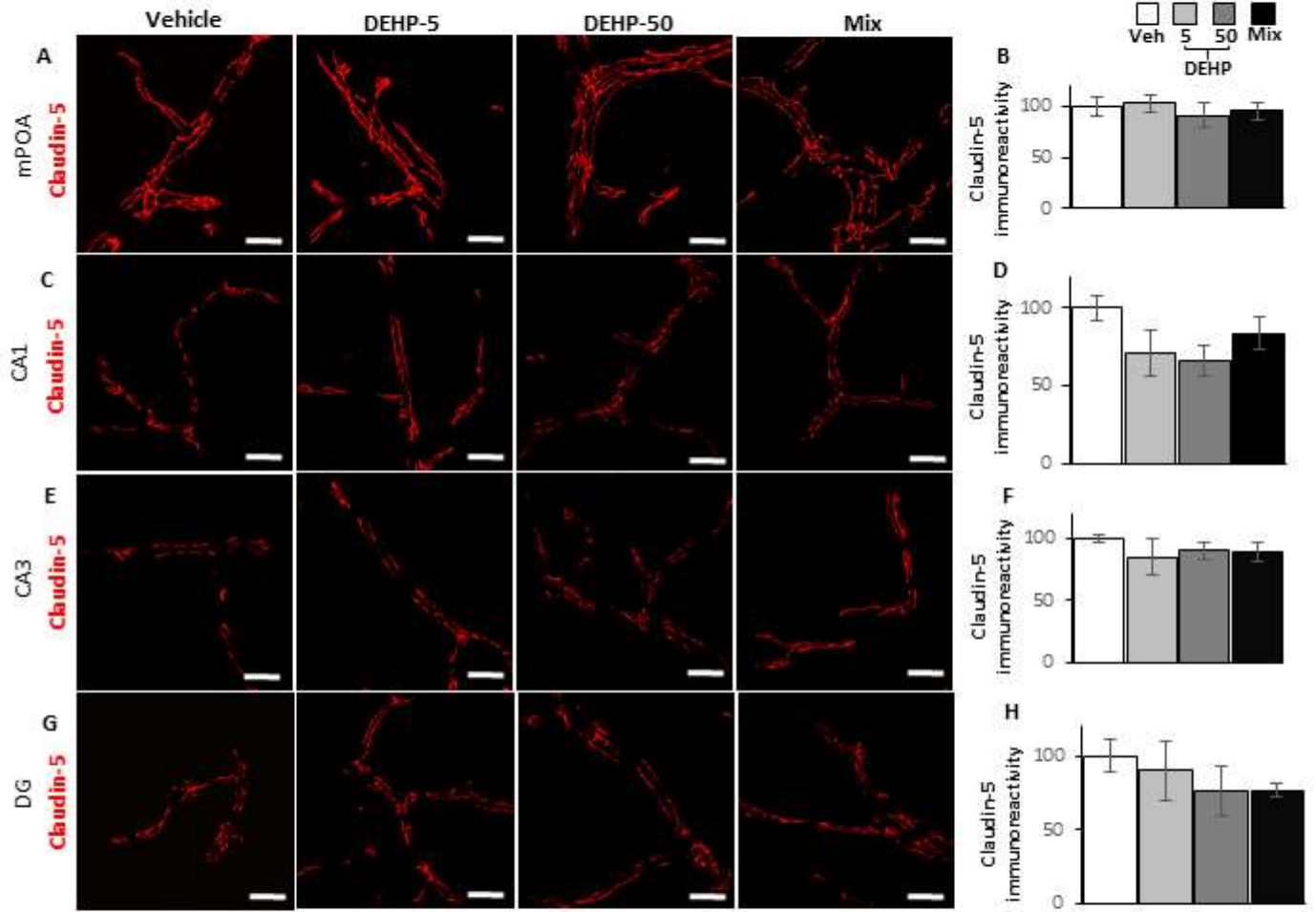
101 hypothalamus. \* $p < 0.05$ ; \*\* $p < 0.01$  compared to the vehicle group. Data were normalized to  
102 GAPDH level.

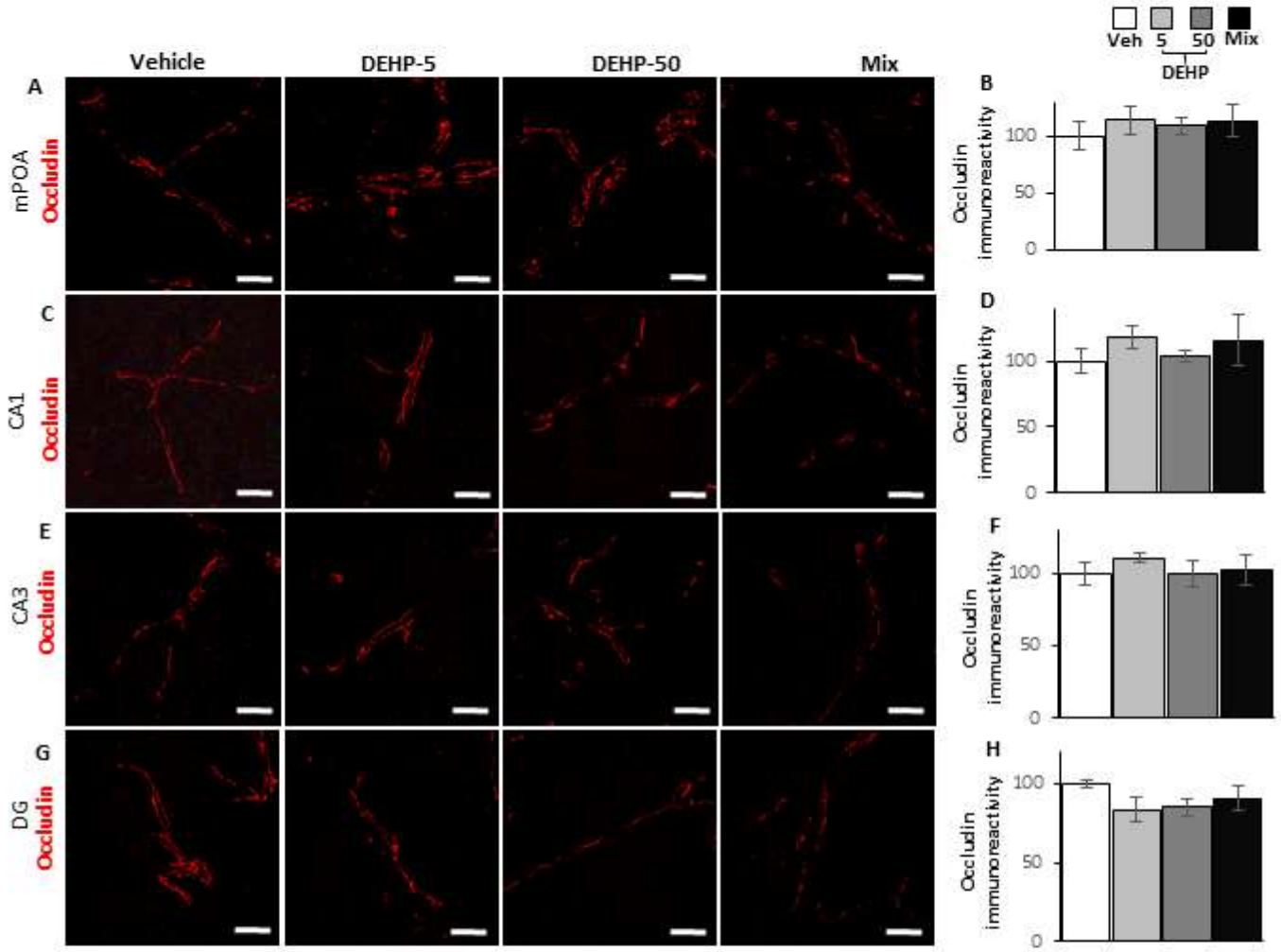
103 (F-N) Representative images of co-immunodetection of Iba-1 (green) and COX-2 (red) in the  
104 hippocampus (F: CA1; I: CA3; L: DG) and their corresponding quantitative analysis of the  
105 immunoreactivity density (G, H: CA1; J, K: CA3; M, N: DG) of the six to eight serial sections  
106 for each brain examined ( $n = 5$  per treatment group). \* $p < 0.05$ ; \*\* $p < 0.01$ ; \*\*\* $p < 0.001$ ; \*\*\*\* $p$   
107  $< 0.0001$  compared to vehicle-treated controls. (O, P) Western blot analysis ( $n = 6$  per  
108 treatment group) of Iba-1 (O) and COX-2 (P) performed on microvessel-enriched fractions from  
109 hippocampus. Data were normalized to GAPDH level. No significant difference was measured  
110 compared to vehicle-treated controls.

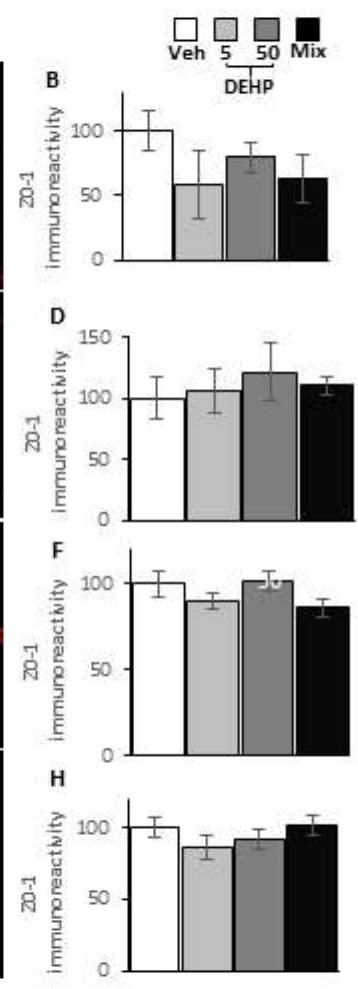
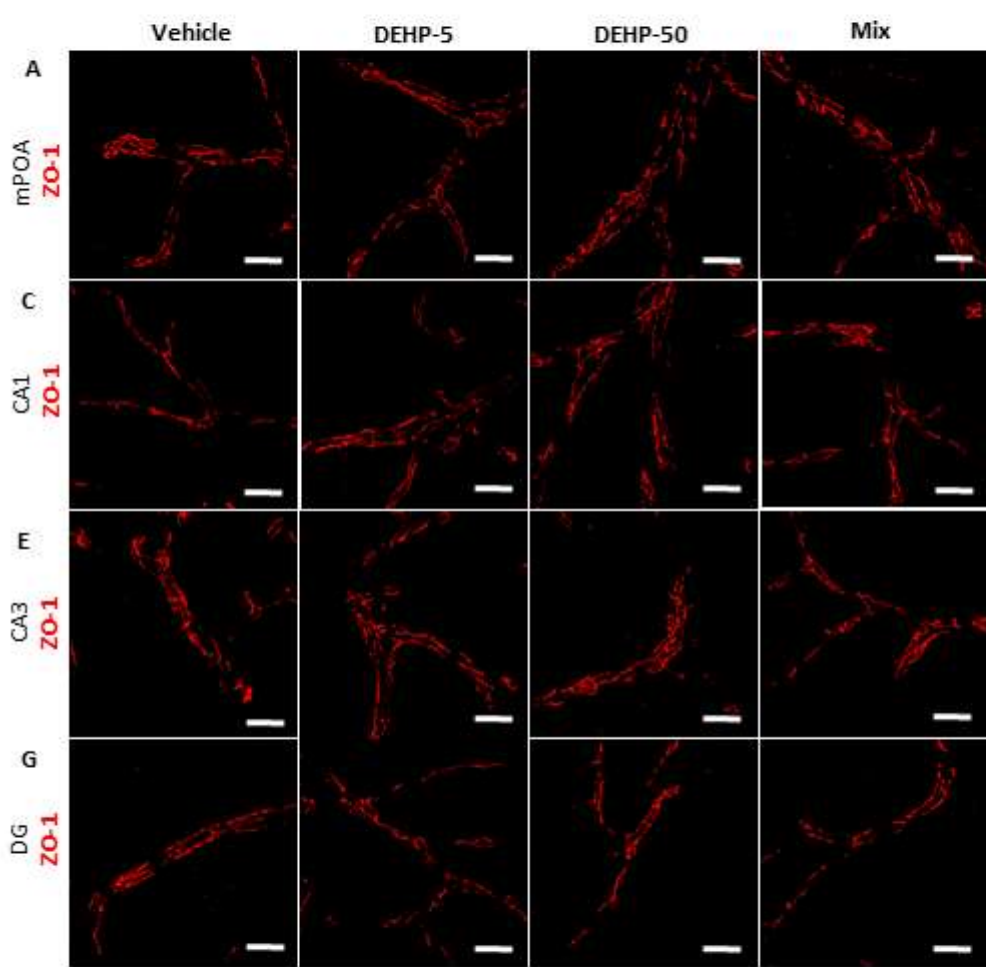
111 Scale bar: 10  $\mu\text{m}$ . L: Lumen delimited by the dotted lines. The quantifications of fluorescence  
112 density were measured over the entire surface of the images. All values represent mean  
113 percentages  $\pm$  S.E.M of the vehicle (100%).

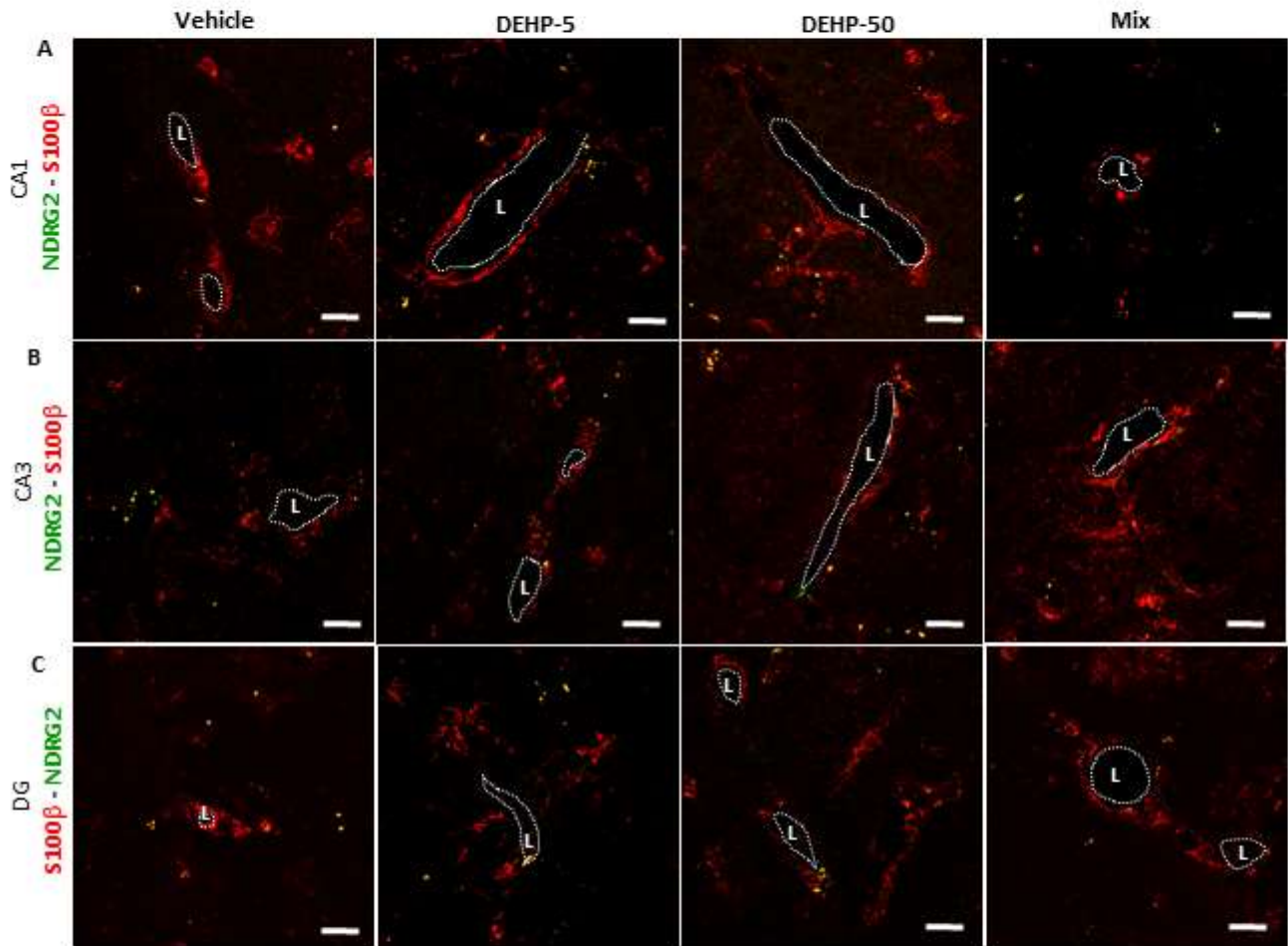
114













**Figure S1. Exposure to DEHP alone or in a phthalate mixture did not affect the body weight of treated mice.**

The body weight of male mice exposed to the vehicle (Veh), DEHP at 5 µg/kg/d (DEHP-5), DEHP at 50 µg/kg/d (DEHP-50), or a phthalate mixture (Mix), is reported for the first and last days of exposure. The values represent body weight means (g) ± S.E.M (n = 12 per treatment group). There was an effect of time ( $F_{(1, 88)} = 48.8$ ,  $p < 0,0001$ ) but not of treatment ( $F_{(3, 88)} = 0,1063$ ,  $p = 0.9562$ ).

**Figure S2. Exposure to DEHP alone or in a phthalate mixture did not affect claudin-5 immunoreactivity.**

(A, B) Representative images of immunodetection of claudin-5 in the mPOA of mice exposed to the vehicle (Veh), DEHP at 5 µg/kg/d (DEHP-5), DEHP at 50 µg/kg/d (DEHP-50), or phthalate mixture (Mix) (A) and their corresponding quantitative analysis of the immunoreactivity density (B) of the six to eight serial sections for each brain examined (n = 5 per treatment group). No significant difference was measured compared to vehicle-treated controls. (C-H) Representative images of immunodetection of claudin-5 (C: CA1; E: CA3; G: DG) and the corresponding quantitative analysis of the immunoreactivity density (D: CA1; F: CA3; H: DG) of the six to eight serial sections for each brain examined (n=5 per group). No significant difference was measured compared to vehicle-treated controls.

Scale bar: 10 µm. The quantifications of fluorescence density were measured over the entire surface of the images and the values represent mean percentages ± S.E.M of vehicle (100%).

**Figure S3. Exposure to DEHP alone or in a phthalate mixture did not affect occludin immunoreactivity.**

(A, B) Representative images of immunodetection of occludin in the mPOA of mice exposed to the vehicle (Veh), DEHP at 5 µg/kg/d (DEHP-5), DEHP at 50 µg/kg/d (DEHP-50), or phthalate mixture (Mix) (A) and their corresponding quantitative analysis of the immunoreactivity density (B) of the six to eight serial sections for each brain examined (n=5 per group). No significant difference was measured compared to vehicle-treated controls. (C-H) Representative images of immunodetection of occludin in the hippocampus (C: CA1; E: CA3; G: DG) and the corresponding quantitative analysis of the immunoreactivity density (D: CA1; F: CA3; H: DG) of the six to eight serial sections for each brain examined (n = 5 per treatment group). No significant difference was measured compared to vehicle-treated controls.

Scale bar: 10 µm. The quantifications of fluorescence density were measured over the entire surface of the images and the values represent mean percentages ± S.E.M of vehicle (100%).

**Figure S4. Exposure to DEHP alone or in a phthalate mixture did not affect ZO-1 immunoreactivity.**

(A, B) Representative images of immunodetection of ZO-1 in the mPOA of mice exposed to the vehicle (Veh), DEHP at 5 µg/kg/d (DEHP-5), DEHP at 50 µg/kg/d (DEHP-50), or a phthalate mixture (Mix) (A) and their corresponding quantitative analysis of the immunoreactivity density (B) of the six to eight serial sections for each brain examined (n=5 per group). No significant difference was measured compared to vehicle-treated controls. (C-H) Representative images of immunodetection of ZO-1 in the hippocampus (C: CA1; E: CA3; G: DG) and the corresponding quantitative analysis of the immunoreactivity density (D: CA1; F: CA3; H: DG) of the six to eight

serial sections for each brain examined (n = 5 per treatment group). No significant difference was measured compared to vehicle-treated controls.

Scale bar: 10  $\mu\text{m}$ . The quantifications of fluorescence density were measured over the entire surface of the images and the values represent mean percentages  $\pm$  S.E.M of vehicle (100%).

**Figure S5. Exposure to DEHP alone or in a phthalate mixture did not affect NDRG2 and S100 $\beta$  immunoreactivity in the hippocampus.**

Representative images of co-immunodetection of NDRG2 (green) and S100 $\beta$  (red) in the hippocampal CA1 (A), CA3 (B) and DG (C) of mice exposed to the vehicle (Veh), DEHP at 5  $\mu\text{g}/\text{kg}/\text{d}$  (DEHP-5), DEHP at 50  $\mu\text{g}/\text{kg}/\text{d}$  (DEHP-50), or phthalate mixture (Mix).

Scale bar: 10  $\mu\text{m}$ . L: Lumen delimited by the dotted lines.

**Table 1: List of primary antibodies**

<b>Antibody</b>	<b>Host</b>	<b>Manufacturer</b>	<b>Catalog no</b>	<b>Application</b>	<b>Working dilution</b>
NDRG2	Rabbit	Cell Signaling Technology	5667S	IHC	1/400
S100 $\beta$	Mouse	Sigma-Aldrich	s2532	IHC	1/1000
COX-2	Goat	Santa Cruz	Sc-1747	IHC - WB	1/200
Iba-1	Rabbit	WAKO	016-20001	WB	1/500
Iba-1	Rabbit	Biocare Medical	CP-290	IHC	1/300
ZO-1	Rabbit	Invitrogen	61-7300	IHC - WB	1/125 - 1/500
Claudin-5	Rabbit	Invitrogen	34-1600	IHC - WB	1/500
Cav-1	Mouse	Biosciences	610407	IHC	1/200
Cav-1	Mouse	Santa Cruz	sc-53564	WB	1/200
iNOS	Mouse	Santa Cruz	sc-7271	WB	1/200
iNOS	Mouse	Sigma-Aldrich	n9657	IHC	1/1000
GFAP	Mouse	Sigma-Aldrich	G3893	WB	1/500
GFAP	Rabbit	DAKO	Z0334	IHC	1/500
Laminin	Rabbit	Sigma-Aldrich	L9393	IHC - WB	1/200 - 1/100
Occludin	Rabbit	Invitrogen	40-4700	IHC - WB	1/500 - 1/250
GAPDH	Mouse	Santa Cruz	sc-32233	WB	1/10000

IHC: Immunohistochemistry; WB: Western blotting

**Table 2: Synthesis of the effects of an oral exposure during adulthood to low doses of DEHP alone or in phthalate mixture in male mice on BBB integrity and associated inflammation in the hypothalamus and hippocampus**

		Immunohistochemistry analysis				Western blot analysis	
		mPOA	CA1	CA3	DG	Hypothalamus	Hippocampus
<b>BBB integrity</b>	<b>Permeability</b>						
	<i>Evans blue extravasation</i>	increased: DEHP-5, DEHP-50 and Mix	increased: DEHP-5 and DEHP-50	increased: DEHP-50	not affected	n.d.	n.d.
	<i>Endogenous IgG extravasation</i>	increased: DEHP-5, DEHP-50 and Mix	increased: DEHP-5, DEHP-50 and Mix	increased: DEHP-5, DEHP-50 and Mix	not affected	n.d.	n.d.
	<b>Endothelial tight junctions</b>						
	<i>Claudin-5</i>	not affected	not affected	not affected	not affected	not affected	not affected
	<i>Occludin</i>	not affected	not affected	not affected	not affected	not affected	not affected
	<i>ZO-1</i>	not affected	not affected	not affected	not affected	decreased: DEHP-5, DEHP-50 and Mix	increased: DEHP-50
	<b>Trans-endothelial vesicular transport</b>						
	<i>Cav-1</i>	decreased: DEHP-50 and Mix	decreased: DEHP-50 and Mix	decreased: DEHP-50 and Mix	not affected	decreased: DEHP-50 and Mix	decreased: DEHP-50 and Mix
<b>Inflammation</b>							
	<b>Astrocyte activation</b>						
	<i>NDRG2</i>	increased: Mix	not affected	not affected	not affected	n.d.	n.d.
	<i>S100 β</i>	increased: DEHP-50 and Mix	not affected	not affected	not affected	n.d.	n.d.
	<i>GFAP</i>	increased: DEHP-50	not affected	not affected	not affected	decreased: DEHP-5, DEHP-50 and Mix	not affected
	<b>Microglia activation</b>						
	<i>Iba-1</i>	increased: DEHP-50	increased: DEHP-5, DEHP-50 and Mix	increased: DEHP-5, DEHP-50 and Mix	not affected	not affected	not affected
	<b>Inflammatory molecules</b>						
	<i>iNOS</i>	increased: DEHP-5, DEHP-50 and Mix	increased: DEHP-5, DEHP-50 and Mix	not affected	increased: DEHP-5	not affected	not affected
	<i>COX-2</i>	increased: DEHP-50	not affected	not affected	not affected	not affected	not affected

n.d.: not determined; DEHP-5: DEHP at 5µg/kg/d; DEHP-50: DEHP at 50 µg/kg/d; Mix: phthalate mixture