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# Behavior, Neural Structure, and Metabolism in Adult Male Mice Exposed to Environmentally Relevant Doses of Di(2-ethylhexyl) Phthalate Alone or in a Phthalate Mixture

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**BACKGROUND:** We have previously shown that chronic exposure of adult male mice to low doses of di(2-ethylhexyl) phthalate (DEHP) altered male sexual behavior and induced down-regulation of the androgen receptor (AR) in the neural circuitry controlling this behavior.

**OBJECTIVES:** The cellular mechanisms induced by chronic exposure of adult male mice to low doses of DEHP alone or in an environmental phthalate mixture were studied.

**METHODS:** Two-month-old C57BL/6J males were exposed orally for 8 wk to DEHP alone (0, 5, or 50 µg/kg/d) or to DEHP (50 µg/kg/d) in a phthalate mixture. Behavior, dendritic density per 50-µm length, pre-/postsynaptic markers, synapse ultrastructure, and bioenergetic activity were analyzed.

**RESULTS:** Mice exposed to DEHP either alone or in a phthalate mixture differed in mating, emission of ultrasonic vocalizations, and the ability to attract receptive females in urinary preference tests from control mice. Analyses in the medial preoptic area, the key hypothalamic region involved in male sexual behavior, showed lower dendritic spine density and protein levels of glutamate receptors and differences in other postsynaptic components and presynaptic markers between the treated groups. Ultrastructural observation of dendritic synapses by electron microscopy showed comparable morphology between the treated groups. Metabolic analyses highlighted differences in hypothalamic metabolites of males exposed to DEHP alone or in a phthalate mixture compared to control mice. These differences included lower tryptophan and higher NAD<sup>+</sup> levels, respectively, a precursor and end product of the kynurenine pathway of tryptophan metabolism. The protein amounts of the xenobiotic aryl hydrocarbon receptor, one of the targets of this metabolic pathway and known negative regulator of the AR, were higher in the medial preoptic area of exposed male mice.

**DISCUSSION:** Differences in behavior of male mice exposed to environmental doses of phthalates were associated with differences in neural structure and metabolism, with possibly a key role of the kynurenine pathway of tryptophan metabolism in the effects mediated by these substances. <https://doi.org/10.1289/EHP11514>

## Introduction

Phthalates are widespread organic pollutants in the environment due to their massive use in the manufacture of several daily life products. Butyl benzyl phthalate (BBP), dibutyl phthalate (DBP), dicyclohexyl phthalate (DCHP), di(2-ethylhexyl) phthalate (DEHP), diisobutyl phthalate (DiBP), diisohexyl phthalate (DIHP), diisopentyl phthalate (DIPP), and dipentyl phthalate (DPP) were listed by the European Chemical Agency (ECHA) as substances of very high concern because of their toxicity for reproduction. BBP, DBP, DCHP, DEHP, and DiBP were also identified for their endocrine-disrupting properties. A tolerable daily intake (TDI) dose of 50 µg/kg/d and an oral reference dose of 20 µg/kg/d were established by the European Food Safety Authority<sup>1</sup> and the U.S. Environmental Protection Agency,<sup>2</sup> respectively, on the basis of the ability of phthalates to reduce fetal testosterone levels and increase liver weight. However, it is important to note that this risk assessment was based on only two end points and on a

majority of studies using very high doses of phthalates, which do not reflect environmental exposure.

The central nervous system (CNS) governs a wide variety of biological functions and behaviors. CNS structure and function undergo highly dynamic changes that occur from early developmental periods through late life stages, with a high sensitivity to both internal (e.g., hormones, cytokines) and external environmental factors (e.g., stressors, food, chemical pollutants). In this context, we have previously shown that chronic exposure of adult male mice to DEHP at doses equivalent to (50 µg/kg/d) or below the TDI dose (5 µg/kg/d) reduced the emission of courtship vocalizations and male attractiveness and delayed the initiation of mating.<sup>3</sup> These effects were associated with disruption of the blood–brain barrier (BBB) and increased astrocyte and microglial activation accompanied with enhanced expression of inflammatory molecules like inducible nitric oxide synthase and cyclooxygenase-2 in the medial preoptic area,<sup>4</sup> the key hypothalamic area controlling male sexual behavior. It is interesting to note that a selective reduction in the protein and mRNA levels of the androgen receptor (AR) was observed in the medial preoptic area in both the parenchyma<sup>3</sup> and cerebral capillaries.<sup>3,5</sup> No changes were observed in the expression of estrogen receptor (ER)  $\alpha$ , involved with the AR in the activation of male sexual behavior,<sup>6–8</sup> nor in testosterone levels or gonadotropic axis integrity. Down-regulation of the AR in the neural circuitry underlying male sexual behavior may underlie at least in part the observed behavioral and neural effects induced by adult exposure to DEHP. Indeed, chronic testosterone depletion of adult male mice by castration reduced neural AR expression, resulting in astrocyte and microglia activation, inflammatory molecule up-regulation, and BBB permeability increase in the medial preoptic area.<sup>9</sup>

The expression of male sexual behavior relies on the appropriate execution of neural processes related to synaptic plasticity

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and neurotransmission and prior genomic modulation of the signaling pathways involved in these processes by testosterone.<sup>10,11</sup> We have previously shown that the first sexual experience is sufficient to improve male sexual behavior and induce structural and molecular modifications in the medial preoptic area, with a rise in dendritic spine density and protein levels of excitatory synapse components such as postsynaptic density protein (PSD)-95 and glutamate receptors.<sup>10</sup> The potential effects of exposure to phthalates on these neural processes related to an optimal processing of male sexual behavior, in particular at low phthalate doses relevant to the environmental exposure, still remains to be investigated regarding the behavioral effects induced by such doses.<sup>3</sup> Structural and functional modifications related to synaptic plasticity requires energy provided by cellular metabolism and mitochondrial function.<sup>12</sup> It is interesting to note that a proteomic analysis conducted in the preoptic area showed differential expression of certain proteins involved in metabolic and mitochondrial functions between control and DEHP-treated male mice.<sup>3</sup> It is therefore possible that a potential alteration of such cellular functions may also impair structural and molecular processes in the neural circuitry underlying male sexual behavior.

We thus undertook the present study to investigate the cellular mechanisms underlying the behavioral effects of adult exposure to DEHP. Although DEHP is the most abundant phthalate, other phthalates are also present in the environment. We therefore compared the effects and mechanisms of exposure to DEHP alone at 5 or 50 µg/kg/d and to a phthalate mixture containing DEHP at 50 µg/kg/d, DBP at 0.5 µg/kg/d, BBP at 0.5 µg/kg/d, DiBP at 0.5 µg/kg/d, and DEP at 0.25 µg/kg/d. Adult C57BL/6J male mice were thus exposed orally for 8 wk to the vehicle (control), DEHP at 50 µg/kg/d, DEHP at 5 µg/kg/d, or to the phthalate mixture. The males were analyzed for their ability to mate, discriminate between pheromonal cues, attract females, and emit courtship ultrasonic vocalizations (USVs). Locomotor activity, general physiological parameters (body and androgen-sensitive organ weights), and protein amounts of sex steroid receptors AR and ER $\alpha$  and of the xenobiotic aryl hydrocarbon-receptor (AhR) in the medial preoptic area were characterized. Analyses of dendritic spines, pre- and postsynaptic components, synapse ultrastructure, and bioenergetic activity were also conducted in the medial preoptic area.

## Materials and Methods

### Animal and Treatments

Studies, performed according to the French and European legal requirements (Decree 2010/63/UE), were approved by the Charles Darwin Ethical Committee (project number 01490-01). The description of experiments was performed in accordance with the Animal Research: Reporting *in Vivo* Experiments (ARRIVE) guidelines.

The studied animals were obtained from the breeding of male and female C57BL/6J mice (Janvier Labs). Mice were housed in polysulfone cages and bottles, with a nest-enriched environment, at 22°C under an inverted light schedule with a 12 h:12 h light:dark cycle [the dark time began at 1330 hours (1:30 P.M.) and ended at 0130 (1:30 A.M.)]. They had *ad libitum* access to food (A03-10; SAFE) and water. Offspring from 15 litters for cohort 1, 18 litters for cohort 2, nine litters for cohort 3, and nine litters for cohort 4 were mixed at weaning to avoid potential litter effects, and all offspring were allowed to grow until 8 wk of age before treatment and analyses.

Oral exposure of 8-wk-old males was performed as previously described.<sup>3,4,13</sup> Phthalates (Sigma-Aldrich), dissolved in ethanol and water (1% and 40% of prepared food, respectively), were incorporated into food (A03-10; SAFE). Mice were fed *ad*

*libitum* with chow containing the vehicle (ethanol and water; control group), DEHP (CAS 117-81-7) at 50 or 5 µg/kg/d (DEHP-50 and DEHP-5 groups, respectively), or a phthalate mixture (Mix group) containing DEHP at 5 µg/kg/d, DBP (CAS 8474-2) at 0.5 µg/kg/d, BBP (CAS 85-68-7) at 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. Phthalate mixture composition was based on studies conducted in France and Europe showing an external coexposure to these substances<sup>14</sup> and the presence of their metabolites in urinary samples.<sup>15,16</sup> Phthalate doses were adjusted weekly to male mice body weights and calculated for a daily food intake of 5 g per animal. After 8 wk of exposure, the analyses started, and treatments were maintained until sacrifice.

Behavioral tests were conducted on male mice cohorts 1 and 2, which included each four treated groups. The first cohort was subjected to analyses of mating, olfactory preference, USV emission, and locomotor activity. At the end of analyses, males were sacrificed; body, testis, and seminal vesicle weights were measured; and the collected brains were processed for western blotting analyses of AR, ER $\alpha$ , and AhR proteins (Figure S1). The second cohort was subjected to urinary preference tests, performed after two mating tests as for the first cohort. The collected brains were used for analyses by Golgi staining, western blotting, and synapse ultrastructure (Figure S1). Two additional cohorts (3 and 4) were used for Seahorse and metabolomic analyses. All the analyses were performed by blind observers without any information on the assigned treatment group; males were identified by numbers attributed at weaning.

### Behavioral Tests

Tests were conducted under red light 2 h after lights were turned off and were videotaped for later analysis (LabWatcher, ViewPoint) as previously described.<sup>3,13,17</sup> After 8 wk of exposure to DEHP alone or in a phthalate mixture, male mice were housed individually for 3 d and then subjected to behavioral experiments as described below and in Figure S1. Except for mating, which was tested in the home cage of male mice, the mazes or equipment used in olfactory preference and urinary preference tests, USV recording, and locomotor activity were cleaned after the passage of each animal with ethanol 10%.

**Preparation of sexually receptive females.** C57BL/6J female mice were ovariectomized under general anesthesia (xylazine/ketamine) and implanted with SILASTIC implants (Dow Corning) filled with 50 µg of estradiol-benzoate (Sigma-Aldrich) in 30 µL of sesame oil. Two weeks after surgery and 4–5 h before the first sexual experience and before each behavioral test where females were used as stimuli, females received a subcutaneous injection of progesterone (Sigma-Aldrich) at 1 mg per 100 µL of sesame oil. Female receptivity was verified using sexually experienced males before experiments.

**Mating.** Three days before the first mating (Test 1), males were individually housed. They were paired in their home cages with sexually receptive females for 10 h and let to reach ejaculation. This first mating was conducted to improve olfactory preference toward receptive females, USV emission, and mating.<sup>3,10,18</sup> Behavioral analyses were conducted 2 wk later on sexually experienced males in Test 2. The percentage of males reaching ejaculation, the numbers of mountings with or without intromissions and thrusts, and the latency to reach ejaculation were scored.

### Olfactory Preference Test

The test was performed in an enclosed plexiglass Y-maze with two empty perforated goal boxes placed at each end as previously described.<sup>3,13,17,18</sup> Animals were allowed to explore the maze for

10 min over 2 consecutive days. On the day of the test, male mice had the choice between an anesthetized sexually receptive female and an anesthetized gonadally intact male, which were placed in the goal boxes. The time spent in chemo-investigation of each was scored over 10 min.

### **Urinary Preference Test**

The test was performed in the same Y-maze as previously described.<sup>13</sup> During 2 consecutive days, sexually receptive females explored the empty maze for 10 min. One hour before the test, an equivalent volume of urine collected from all males from each treatment group was mixed, and 10  $\mu$ L of this mix was put on a piece of filter paper. The urine was collected the day of the test, and  $\sim$ 13 wk after the beginning of treatment as illustrated in Figure S1. A filter paper containing the urine from the control group and a filter paper containing the urine from the DEHP-5, DEHP-50, or Mix-treated groups were placed inside perforated goal boxes at each end of the maze. Females had the choice between the filter paper containing the control urine and the filter paper containing the DEHP- or Mix-exposed urine. The time spent by sexually receptive females in chemo-investigation of each stimulus was scored during the 10 min test.

### **USVs**

Each male from one of the four treatment groups was tested in its home cage in the presence of a sexually receptive female as previously described.<sup>3,13,17,19,20</sup> The 4-min recording of vocalizations started after the introduction of the female, using an UltraSoundGate microphone (Avisoft Bioacoustics), which was connected to a computer equipped with the Avisoft-SASLab Pro (Avisoft Bioacoustics) recording software. Avisoft-SASLab Pro (Avisoft Bioacoustics) was used to analyze USVs and generate spectrograms for each detected call. Emitted syllables were grouped into simple, complex, and frequency-jump categories based on their shape and frequency.<sup>3,17</sup> The total number and duration of USVs and the number and duration of each syllable were quantified.

### **Locomotor Activity**

The activity was measured in a computed circular corridor (4.5-cm width, 17-cm external diameter) crossed by four diametrically opposite infrared beams (Imetronic) as previously described.<sup>7</sup> The activity was counted when male mice interrupted two successive beams and had traveled a quarter of the circular corridor. Activity was recorded for 120 min.

### **Golgi-Cox Staining**

Mice were sacrificed by decapitation and the removed brains were immersed for 8 d in Golgi-Cox solution (NovaUltra Golgi-Cox Stain kit; Interchim) as previously described.<sup>10</sup> Coronal slices of 30- $\mu$ m thickness were cut using a vibratome (Leica Microsystems VTC1000S). The anterior commissure was used as a landmark to identify hypothalamic slices including the medial preoptic area. These slices were immersed in postimpregnation solution for 10 min, washed in phosphate-buffered saline (PBS), and mounted with Mowiol. Sections were first scanned with a high-resolution NanoZoomer Hamamatsu scanner (Hamamatsu Corporation). For each animal, 10 neurons homogeneously distributed throughout the medial preoptic area, within an area of 0.80 mm<sup>2</sup> in anatomically matched sections identified using the Mouse Brain Atlas (Bregma 0.14, plate 30), were selected for further analyses as illustrated in Figure S2A. For each neuron, the

analyses were performed using Zeiss Axiovert 200 M standard light microscope on a dendritic length of 50  $\mu$ m and from 30  $\mu$ m of the beginning of the dendrite as shown in Figure S2B. Subtypes of dendritic spines were determined according to their maturity level by applying previously described criteria.<sup>21</sup> Thin spines had a length greater than the neck diameter and similar head and neck diameters. Stubby spines had similar neck diameter and total length of the spine, whereas mushroom spines corresponded to those with head diameter greater than neck diameter.

### **Western Blotting for Immunodetection of Presynaptic and Postsynaptic Markers and AR and ER $\alpha$**

Male mice were sacrificed by decapitation, and the brains were sliced with a Vibroslice (World Precision Instrument) in cold 0.1 M phosphate buffer. The medial preoptic area was punched out from sections of 400- $\mu$ m thickness using 1 mm diameter canula. Punched samples were homogenized in a solution containing Tris, NaCl, sodium dodecyl sulfate (SDS), sodium deoxycholate, EDTA, EGTA, Triton-x100, and protease inhibitors, and proteins were extracted and quantified as previously described for pre- and postsynaptic markers (Coomassie assay; Thermo Fisher Scientific),<sup>10,22</sup> or AR and ER $\alpha$  immunodetection (Bradford assay; Thermo fisher).<sup>7</sup> Proteins (20 to 30  $\mu$ g) were then separated on a 4%–12% polyacrylamide gel (Invitrogen) and transferred onto a nitrocellulose membrane. Because limited protein amounts were collected after punches extraction, nitrocellulose membranes were stained with Ponceau S and cut into several sheets based on the loaded molecular weight scale and known molecular weights of studied proteins as routinely used in the laboratory. This methodological approach allowed performing several western blots from the same samples.

Blots were probed overnight at 4°C with polyclonal anti-AR, anti-ER $\alpha$ , anti-AhR, anti-spinophilin, anti-GluN2A or monoclonal anti-glyceraldehyde 3-phosphate dehydrogenase gene (GAPDH), anti-PSD-95, anti-GluN1, anti-GluN2B, and anti-synaptophysin as described in Table S1 and previously reported.<sup>7,10</sup> Then, incubation with peroxidase-conjugated second monoclonal or polyclonal antibodies diluted at 1:10,000 (Amersham Bioscience) was performed at room temperature for 1 h. Signals were detected with the Pico or Super Signal (Femto) detection kits (Thermo Scientific) as described in Table S1, visualized using Amersham Imager 600 (GE Healthcare Bio-Sciences AB), quantified with ImageJ software (National Institutes of Health), and normalized with respect to GAPDH.

### **Ultrastructural Observation**

Mice were deeply anesthetized with pentobarbital (120 mg/kg, intraperitoneal injection) and were transcardially perfused with an 0.9% saline solution followed by a 2.5% glutaraldehyde solution diluted in 0.1 M cacodylate buffer pH 7.4. Brains were collected and postfixed in the same fixative solution overnight at 4°C. After washes in cacodylate buffer, four coronal sections including the medial preoptic area (100- $\mu$ m thickness) were cut from each brain with a vibratome (Leica VT1000S, Leica Biosystems). Sections were postfixed for 1 h at room temperature in 2% osmium tetroxide diluted in cacodylate buffer (v/v), dehydrated through an alcohol ascending series, and then embedded in Epon resin (Epoxy-Embedding Kit, catalog no. 45359; Sigma Aldrich) and polymerized at 60°C for 48 h between two silicone-coated glass slides. Ultrathin sections (70 nm) of medial preoptic areas were cut with an ultramicrotome (Reichert/Leica Ultracut S ultramicrotome; Leica Microsystems), collected into copper mesh grids, and contrasted for observation with 2% uranyl acetate for 5 min and Reynold's lead citrate for 2 min. Observations

were made in STEM mode in a Field Emission SEM GeminiSEM 500 (Carl Zeiss Microscopy) operating at 20 kV with a 20- $\mu$ m aperture. Transmitted electrons were collected in bright field mode with the STEM detector located beneath the sample.

### Metabolic Analyses

**Seahorse analyses.** Animals were sacrificed by pentobarbital injection (120 mg/kg), and brains were collected and sliced using a Vibroslice (World Precision Instrument) in cold artificial cerebrospinal fluid (aCSF: 120 mM NaCl, 3.5 mM KCl, 0.4 mM  $\text{NaH}_2\text{PO}_4$ , 1.3 mM  $\text{CaCl}_2$ , 1 mM  $\text{MgCl}_2$ , 5 mM HEPES, pH 7.4) as previously described.<sup>10</sup> Tissue punches were recovered through the medial preoptic area with a 1-mm diameter canula from 300- $\mu$ m-thick brain slices corresponding to plates 30–31 of the Mouse Brain Atlas and placed on XF Islet Capture Microplate wells. Punches were covered by nylon inserts and immersed into 500  $\mu$ L of prewarmed aCSF supplemented with 4 mg/mL of bovine serum albumin (BSA) and 10 mM glucose. After 1 h incubation in a  $\text{CO}_2$ -free incubator at 37°C to allow temperature and pH equilibration, Oxygen Consumption Rate (OCR) and ECAR (Extracellular Acidification Rate) were measured using a Seahorse XFe24 extracellular flux analyzer (Agilent Technologies). OCR was measured in basal conditions (three measurement cycles) and after sequential injections of oligomycin (20  $\mu$ M), carbonyl cyanide 4-(trifluoromethoxy) phenylhydrazone (FCCP at 10  $\mu$ M) and a mix of rotenone/antimycin (10  $\mu$ M) with six measurement cycles after each injection and three final measurement cycles. For ECAR measurement, punches were covered by nylon inserts and immersed into 500  $\mu$ L of prewarmed aCSF supplemented with 4 mg/mL of BSA. ECAR was measured in basal conditions (three measurement cycles) and after sequential injections of glucose (10 mM), oligomycin (40  $\mu$ M), and the glucose analog 2-deoxy-glucose (2-DG) (25 mM) with six measurement cycles after each injection and three final measurement cycles.

**Protein extraction and western blots.** Punches of the medial preoptic area from males exposed to the vehicle, DEHP at 5 or 50  $\mu$ g/kg/d or to a phthalate mixture (Mix) were homogenized on ice in lysis buffer containing Tris 25 mM pH 7.5, NaCl 150 mM, NP40 1%, and 1% sodium deoxycholate 0.5% using a tissue grinder. After sonication and centrifugation at 14,000  $\times$  g at 4°C for 20 min, supernatants containing proteins were collected and quantified using BCA Protein Assay Kit (Pierce). Proteins (25  $\mu$ g) were separated on Any-kD gels (Bio-Rad) and transferred onto nitrocellulose membranes (Bio-Rad). Membranes were incubated with primary antibodies overnight at 4°C and with secondary antibodies for 1.5 h at room temperature (antibodies are listed in Table S2). Bands were revealed by Enhanced ChemiLuminescence using ChemiDoc (Bio-Rad).

### Targeted Liquid Chromatography–Mass Spectrometry (LC–MS) Metabolite Analyses

Metabolomic analysis was conducted as previously detailed.<sup>23</sup> Briefly, hypothalamic samples were extracted (1 mL of solution per 50 mg of tissue) and then vortexed for 5 min at 4°C before centrifugation at 16,000  $\times$  g for 15 min at 4°C. Supernatants (5  $\mu$ L) have then been further analyzed by LC/MS using chromatographic device (Zic-pHilic with a guard column; Millipore). Buffers used for chromatographic separation were used as followed: 20 mM ammonium carbonate and 0.1% ammonium hydroxide (pH 9.2) (buffer A); acetonitrile (buffer B). The flow rate for chromatographic gradient was 0.2  $\mu$ L/min as follows: linear gradient from 80% to 20% B during 0–20 min; linear gradient from 20% to 80% B during 20–20.5 min; hold at 80% B during 20.5–28 min. The metabolite detection was done in a mass range of 75–1,000  $m/z$ . Thermo Fisher Scientific (Thermo) Xcalibur

software (version 4.3; Thermo Scientific) was used to get the data. For each metabolite, the peak area was determined using Thermo TraceFinder software. Metabolite determination was done considering two parameters: mass of the single charged ion and known retention time on the chromatographic column. MetaboAnalyst 5.0 software was used to analyze raw data (version 5.0; MetaboAnalyst). For each metabolite, the peak area was normalized to the total peak areas of all metabolites detected for each sample. The 40 most significantly different metabolites between the treated groups were determined using the top 40 heat map. Metabolites presenting similar abundance patterns were positioned closer together.

### Body, Seminal Vesicle and Testis Weight Measurements

The body weight of the male mice was assessed weekly from the beginning until the end of treatments and analyses. Animals were euthanized by pentobarbital injection (120 mg/kg), and the seminal vesicle and testes were collected and weighed. The data were expressed as relative seminal vesicle and testis weights (percentage of body weight).

### Statistics

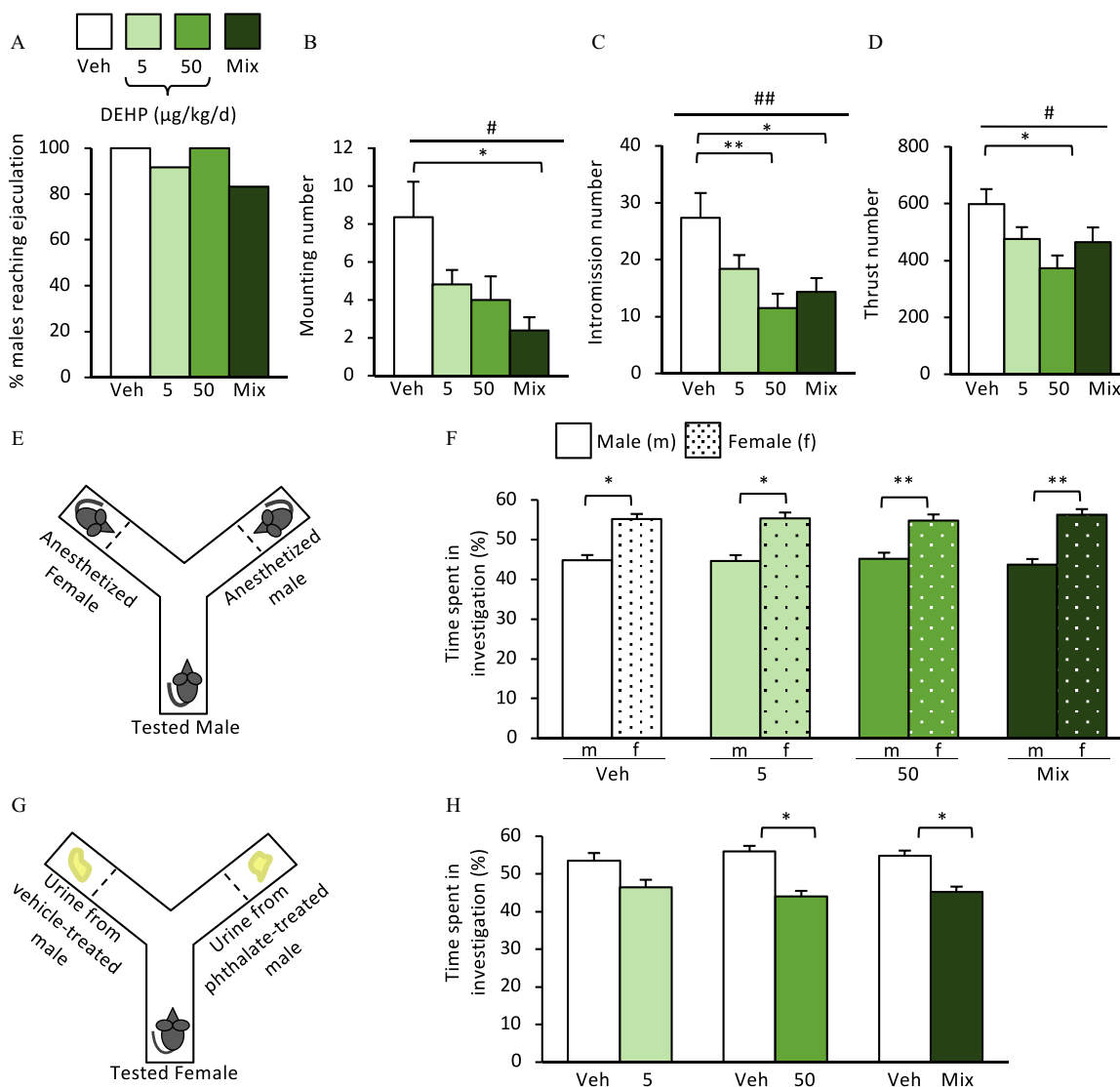
Data were expressed as means  $\pm$  SEM, and analyzed by GraphPad Prism 7 (version 7.00; GraphPad Software). Normality tests were performed (Kolmogorov–Smirnov and Shapiro–Wilks tests). Two-way analysis of variance (ANOVA) was used to analyze the main effect of exposure and stimulus on the time of investigation for olfactory preference. Bonferroni's post hoc tests were used to determine group differences. The Kruskal–Wallis was used to analyze the effect of exposure on the number of mounts without intromission in the mating behavior, the latency to ejaculation, the number of syllables (total, short, flat, downward, modulated, complex, one-jump, and frequency-jump) the duration of syllables (short, flat, upward, downward and one-jump), the testis and seminal vesicle weights, and Seahorse analyses. Dunn's post hoc tests were used to determine group differences. Significance for metabolomic analyses was analyzed using ANOVA, and a false discovery rate (FDR)–adjusted  $p$ -value of  $<0.05$  was considered as significant. Fisher's least significant difference (LSD) was used as a post hoc test.

One-way ANOVA was used to analyze the effect of exposure on the remaining data. Bonferroni's post hoc tests were used to determine group differences.  $p$ -Values that were  $<0.05$  were considered to be significant.

## Results

### Effects of Adult Exposure to DEHP Alone or in a Phthalate Mixture on Male Sexual Behavior

Sexually experienced male mice exposed to the vehicle or to DEHP alone or in a phthalate mixture were assessed for mating with receptive females. Figure 1A shows that all males of the vehicle (10) and DEHP-50 (12) groups and the majority of mice from the DEHP-5 (92%; 11/12) and Mix (83%; 10/12) groups reached ejaculation. Analyses of the behavioral components show that there was an effect of treatment on the number of mounts ( $p=0.047$ ), intromissions ( $p=0.004$ ) and thrusts ( $p=0.022$ ) (Figure 1B–D). Post hoc analyses showed that male mice demonstrated significantly fewer mounts in the mix group (–71% vs. the vehicle), fewer number of intromissions in the DEHP-50 and Mix groups (–58% and –48% vs. the vehicle, respectively), and fewer thrusts in the DEHP-50 group (–38% vs. the vehicle) in comparison with vehicle control. Although not significant, the tendency for fewer mounts, intromissions, and thrusts was also seen for the DEHP-5 group. There was also an effect of treatment on the latency to reach



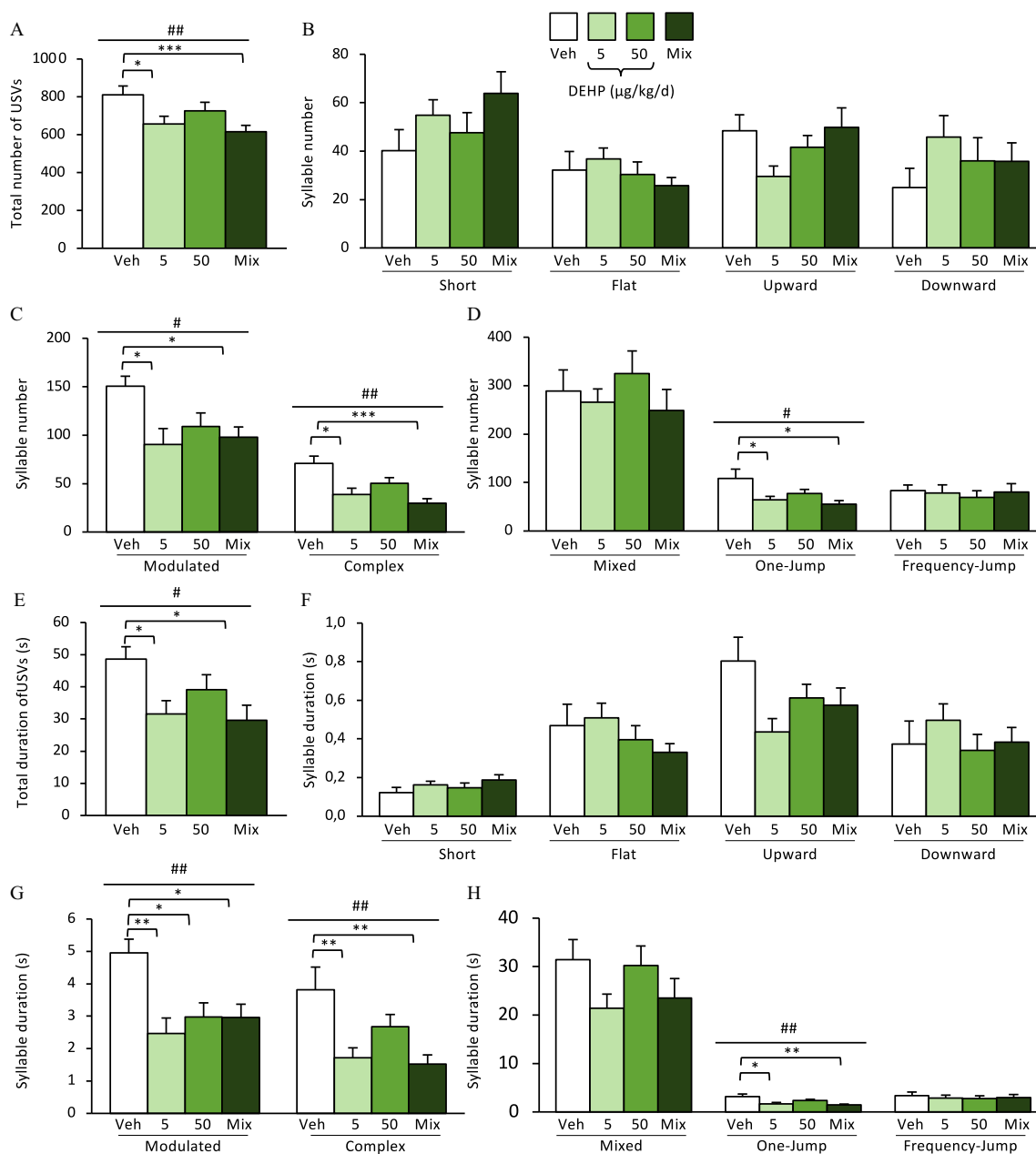
**Figure 1.** Effects of adult exposure to DEHP alone or in a phthalate mixture on mating, olfactory, and urinary preferences in male mice. (A) Percentage of males exposed to the vehicle (Veh: 10 out of 10), DEHP alone at 5 (11 out of 12) or 50  $\mu\text{g}/\text{kg}/\text{d}$  (12 out of 12) or in a phthalate mixture (Mix: 10 out of 12) reaching ejaculation. (B–D). Total number of mounts (B), intrusions (C), and thrusts (D) displayed throughout the mating test. Effects of treatment ( $\#p < 0.05$ ,  $\#\#p < 0.01$ ), and post hoc comparisons ( $*p < 0.05$ ,  $**p < 0.01$ ) are shown. (E–F). Olfactory preference of males toward an anesthetized male and female was measured in a Y-maze paradigm (E). The time spent in investigation of male vs. female stimuli, expressed as percentage of total time of investigation, is shown as means  $\pm$  SEM. (F). Post hoc analyses ( $*p < 0.05$ ,  $**p < 0.01$ ) are shown. (G–H). In a Y-maze paradigm, a sexually receptive female had the choice between urine from vehicle-exposed males and urine from males exposed to DEHP alone or in mixture (G). The percentage of time spent investigating the Veh vs. DEHP- or Mix-exposed female urine (H) are presented as means  $\pm$  SEM.  $*p < 0.05$  vs. the control group. Ten to 12 males per treatment group were used for (A) to (H). Summary data for panels A–H can be found in Excel Tables S4–6. Note: DEHP, di(2-ethylhexyl) phthalate; SEM, standard error of the mean; Veh, vehicle.

ejaculation ( $p = 0.019$ ), with males from the DEHP-5, DEHP-50, and Mix groups exhibiting this behavior later than the vehicle group ( $110.27 \pm 56.23$  min,  $81.60 \pm 52.05$  min, and  $134.01 \pm 69.54$ , respectively, vs. the vehicle group  $22.74 \pm 2.81$  min).

During mating, males and females emit pheromones that are mandatory for mutual sexual stimulation. Males exposed to the vehicle or DEHP alone or in a phthalate mixture were thus tested for their olfactory preference toward receptive females in a Y-maze test (Figure 1E). Two-way ANOVA showed an effect of stimulus ( $F_{(1,42)} = 40.05$ ,  $p < 0.0001$ ) but not of treatment ( $F_{(3,42)} = 0.77$ ,  $p = 0.52$ ). All males, regardless of their treatment group, exhibited a preference for females (Figure 1F). To further assess whether these males were able to attract females, urine from the four treatment groups was collected and used in a Y-maze preference test where sexually receptive females were

given the choice between urine from vehicle vs. phthalate-treated males (Figure 1G). Female mice spent significantly less time investigating the males from the DEHP-50 or the Mix group ( $-20\%$ ) than those from the vehicle group (Figure 1H).

Olfactory stimulation of males by female pheromones triggers the emission of USVs by males. Males were then analyzed for their ability to emit USVs in the presence of receptive females. Data illustrated in Figure 2A show that treatment had an effect on the total number of USVs ( $p = 0.002$ ), with significantly fewer observed in the DEHP-5 and Mix groups ( $-22\%$  and  $-27\%$ , respectively) in comparison with the vehicle group. Detailed analyses of emitted syllables (Figure 2B–D) show that there was a significant effect of treatment on the number of modulated ( $p = 0.024$ ), complex ( $p = 0.002$ ), and one-jump syllables ( $p = 0.013$ ), with significantly fewer vocalizations in the

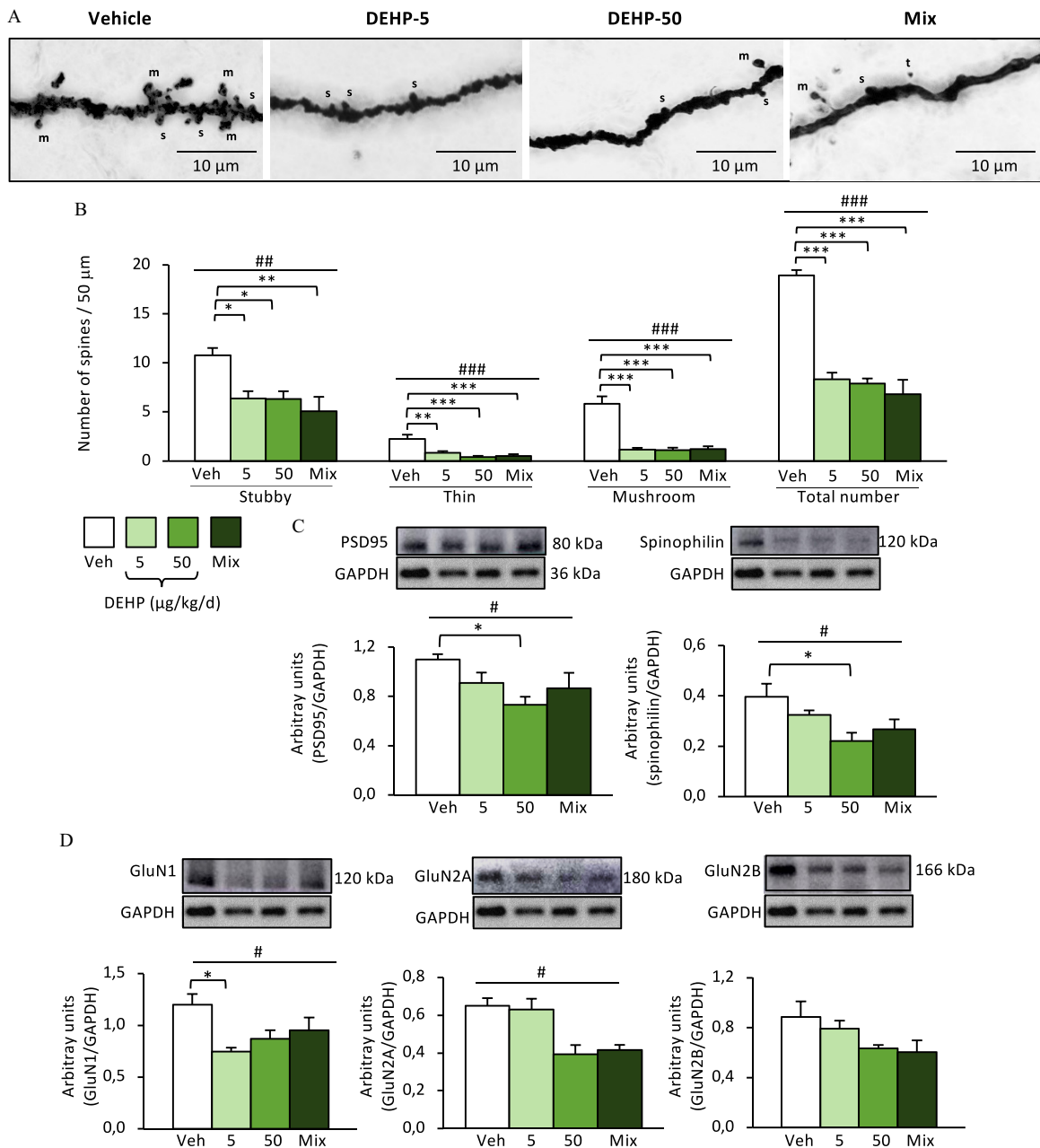


**Figure 2.** Effects of adult exposure to DEHP alone or in phthalate mixture on the emission of USVs in male mice. (A) Total number of USVs emitted by males exposed to the Veh, DEHP at 5 or 50 µg/kg/d, or to a phthalate mixture (Mix) in the presence of sexually receptive females during the 4-min recording. Data are expressed as means ± SEM. Effect of treatment (## $p < 0.01$ ) and post hoc comparisons ( $*p < 0.05$ ,  $***p < 0.001$ ) are shown. (B–D). Total number (means ± SEM) of syllables of the simple (B), complex (C), and frequency-jump (D) categories. Significant treatment effect [(# $p < 0.05$ , ## $p < 0.01$ ), and post hoc comparisons ( $*p < 0.05$ ,  $**p < 0.01$ ,  $***p < 0.001$ )] are indicated. (E) Total duration (means ± SEM) of USVs emitted by males. Effect of treatment (# $p < 0.05$ ) and post hoc analyses ( $*p < 0.05$ ) are shown. (F–H) Total duration (means ± SEM) of syllables of the simple (F), complex (G) and frequency-jump (H) categories. Treatment effects (## $p < 0.01$ ) and post hoc analyses ( $*p < 0.05$ ,  $**p < 0.01$ ) are indicated. Ten to 12 males per treatment group were used for (A)–(H). Summary data for panels (A)–(H) can be found in Excel Table S7. Note: DEHP, di(2-ethylhexyl) phthalate; SEM, standard error of the mean; USV, ultrasonic vocalization; Veh, vehicle.

DEHP-5 (–40%, –45%, and –41%, respectively) and Mix groups (–35%, –58%, and –49%, respectively) in comparison with the vehicle group. The treatment also affected the total duration of USVs ( $p = 0.037$ ), with significantly shorter duration in the DEHP-5 and Mix-groups (–35% and –31% vs. the vehicle group, respectively) as shown in Figure 2E. Significant treatment effects were seen on the duration of modulated, complex, and one-jump syllables ( $p = 0.002$  for the three syllables) as shown in Figure 2F,H. The duration was shorter in the three treated groups relative to the vehicle group for modulated

syllables (–50%, –39.8%, and –40%, respectively), and in the DEHP-5 and Mix groups for complex (–55% and –60%, respectively) and one-jump syllables (–48% and –56%, respectively).

General behavior, including locomotor activity, was also monitored (Figure S3). There was an effect of time ( $F_{(5,215)} = 101.1$ ,  $p < 0.0001$ ), but not of treatment ( $F_{(3,43)} = 1.420$ ;  $p = 0.250$ ) on the activity measured during 2 h (Figure S3A). No significant differences were observed in the cumulative activity between the treatment groups (Figure S3B).



**Figure 3.** Effects of adult male mice exposure to DEHP alone or in a phthalate mixture on neural structure in the medial preoptic area. (A) Representative images of dendritic spines in the medial preoptic area of males exposed to the Veh, DEHP at 5 (DEHP-5) or 50 μg/kg/d (DEHP-50), or to a phthalate mixture (Mix). Thin (t), stubby (s) and mushroom (m) dendritic spines are indicated. (B) Quantification of total and subtype dendritic spines (10 neurons per mouse). Data are presented as means ± SEM from four males per treatment group. Treatment effects (## $p < 0.01$ , ### $p < 0.001$ ) and post hoc comparisons (\* $p < 0.05$ , \*\* $p < 0.01$ , \*\*\* $p < 0.001$ ) are indicated. (C–D). Quantification of protein amounts for PSD-95 (left) and spinophilin (right) in (C), and for GluN1 (left), GluN2A (middle), and GluN2B (right) in (D) are normalized to GAPDH in the medial preoptic area. Data are expressed as the means ± SEM of four to six males per treatment group. Treatment effects (# $p < 0.05$ ) and post hoc comparisons (\* $p < 0.05$ , \*\* $p < 0.01$ ) are indicated. Summary data for panels (A) to (H) can be found in Excel Table S8. Note: DEHP, di(2-ethylhexyl) phthalate; GAPDH, glyceraldehyde 3-phosphate dehydrogenase; PSD, postsynaptic density protein; SEM, standard error of the mean; Veh, vehicle.

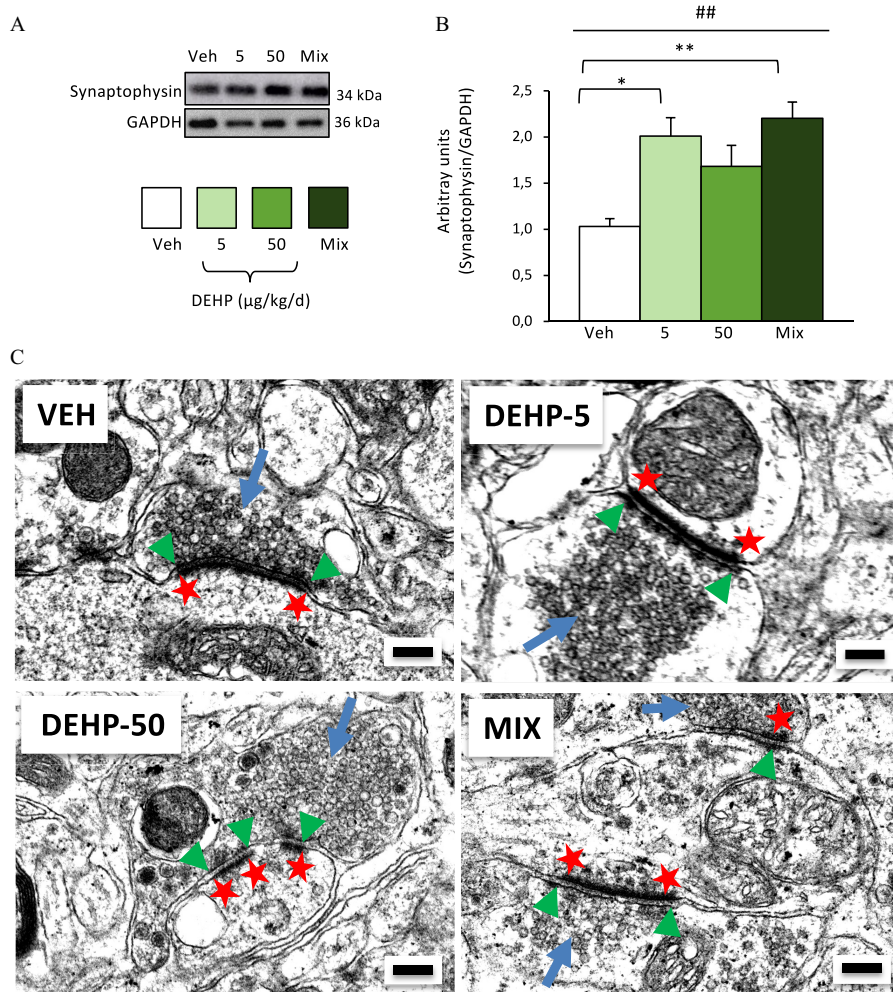
### Dendritic Spines, Pre- and Postsynaptic Components and Synapse Ultrastructure in the Medial Preoptic Area of Adult Male Mice Exposed to DEHP Alone or in a Phthalate Mixture

The expression of a fully mature sexual behavior relies on long-lasting structural and functional modifications that occur after a first sexual experience in the medial preoptic area and involves an increase in dendritic spine density and expression of postsynaptic components.<sup>10</sup> We thus assessed the effects of adult exposure to

DEHP alone or in a phthalate mixture on dendritic spine density per 50 μm of length and protein levels of postsynaptic components in sexually experienced male mice.

The number and morphology of spines located on neuronal dendrites and corresponding to excitatory post synapses were analyzed in brains, which were processed in Golgi-Cox staining. Dendritic spines were analyzed in the medial preoptic area and classified into stubby, thin, and mushroom types according to their level of maturation as previously described.<sup>10</sup> Figure 3A,B shows that there was an effect on the number of stubby ( $p = 0.007$ ), thin





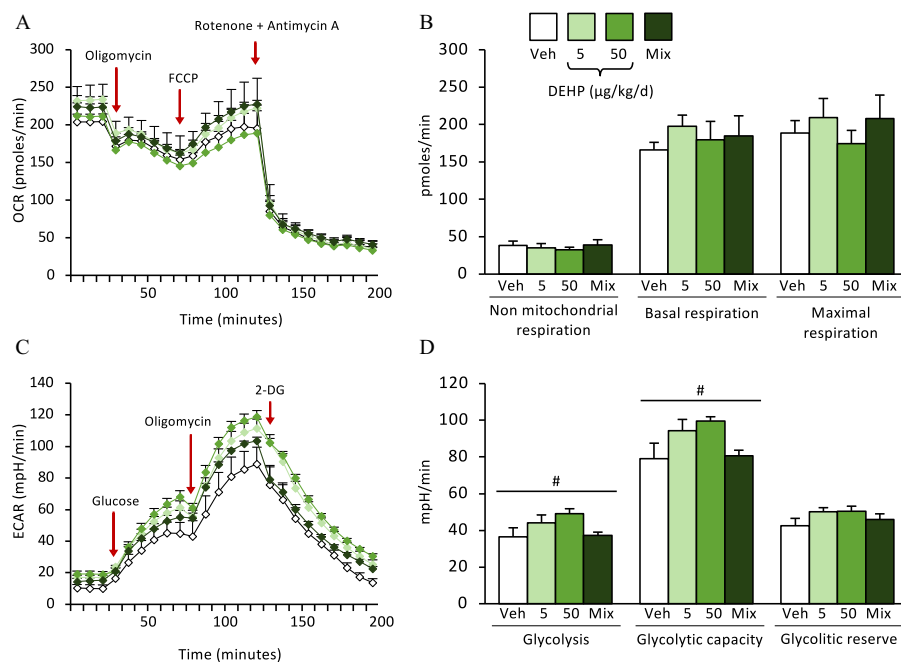
**Figure 4.** Effects of adult male mice exposure to DEHP alone or in a phthalate mixture on synaptophysin protein levels in the medial preoptic area. (A–B). Quantification of protein amounts of synaptophysin normalized to GAPDH in the medial preoptic area of males exposed to the Veh, DEHP at 5 or 50  $\mu\text{g}/\text{kg}/\text{d}$ , or to a phthalate mixture (Mix). Data are expressed as the means  $\pm$  SEM of five to seven males per treatment group. Treatment effects ( $##p < 0.01$ ) and post hoc comparisons ( $*p < 0.05$ ,  $**p < 0.01$ ) are indicated. (C) Representative electron micrographs of three independent experiments per treatment group showing synapses associated with dendrites in the medial preoptic area of male mice from the Veh, DEHP-5, DEHP-50, and Mix groups. Scale bar = 200 nm. Red stars indicate the postsynaptic membrane; green arrowheads indicate the presynaptic membrane; blue arrows show vesicles in the pre-synaptic compartment. Summary data for panel (B) can be found in Excel Table S9. Note: DEHP, di(2-ethylhexyl) phthalate; GAPDH, glyceraldehyde 3-phosphate dehydrogenase; SEM, standard error of the mean; Veh, vehicle.

( $p = 0.0006$ ), and mushroom spines ( $p = 0.0001$ ). The density of spines on the dendritic segments selected for analysis was lower in all treatment groups in comparison with vehicle-treated males ( $-41\%$  to  $-53\%$  for stubby spines,  $-64\%$  to  $-77\%$  for thin spines, and  $-79\%$  to  $-81\%$  for mushroom spines). This lower density resulted in a lower ( $-56\%$  to  $-64\%$  vs. the vehicle group) total number of dendritic spines.

Quantification of some of the major components of dendritic spines, including the postsynaptic PSD-95, spinophilin, and glutamate receptors GluN1, GluN2A, and GluN2B, was performed by western blotting of proteins extracted from the medial preoptic area. Figure 3C,D shows a significant effect of treatment on the protein amount of PSD-95 ( $p = 0.031$ ), spinophilin ( $p = 0.019$ ), and GluN1 ( $p = 0.011$ ) and GluN2A receptors ( $p = 0.010$ ). No significant treatment effect was found on the protein amount of GluN2B ( $p = 0.106$ ), although a tendency of a lower level was also observed. Post hoc analyses showed that the protein levels were lower for PSD-95 ( $-34\%$ ) in the DEHP-50 group, spinophilin ( $-44\%$ ) in the DEHP-50 group, and GluN1 ( $-38\%$ ) in the DEHP-5 group in comparison with the vehicle group as shown in Figure 3C,D.

The protein levels for the presynaptic synaptophysin, a synaptic vesicle membrane protein, were also quantified in the medial preoptic area. Figure 4A,B shows an effect of treatment ( $p < 0.01$ ), with a higher amount in DEHP-5 and Mix-groups ( $+95\%$  to  $+113\%$  vs. the vehicle group).

We also conducted a preliminary qualitative ultrastructural analysis of synapses corresponding to dendritic spines in the medial preoptic area. For this, 16–23 synapses for each experimental group associated with dendrites were randomly observed on the entire surface of the sections present on the grids by transmission electron microscopy. Representative images in Figure 4C and Figure S4 show a clean homogeneous, compact, and electron-dense postsynaptic membrane (red stars) in the vehicle group. Vesicles (40 nm in diameter) with low dense electron density content were visible in the presynaptic compartment (green arrowheads), grouped in clusters (blue arrows). The vesicles were closely associated with the presynaptic plasma membrane along its entire length. For DEHP-5, DEHP-50 and Mix, the ultrastructure of the synapses, including the presynaptic membrane, postsynaptic membrane, and synaptic cleft, was comparable to that observed for the vehicle group. The vesicle clusters in



**Figure 5.** Metabolic effects of adult male mice exposure to DEHP alone or in a phthalate mixture in the medial preoptic area. (A–D). Seahorse metabolic analysis in the medial preoptic area of males exposed to the Veh, DEHP at 5 or 50  $\mu\text{g}/\text{kg}/\text{d}$ , or to a phthalate mixture (Mix). OCR and ECAR data were determined for each of the mitochondrial respiration steps after oligomycin, FCCP, and rotenone/antimycin treatment (A). Nonmitochondrial, basal and maximal respiration were derived from OCR measurements (B). ECAR values were determined after sequential treatment with glucose, oligomycin and 2-DG (C). Glycolysis, glycolytic capacity, and reserve were derived from ECAR measurements (D). Data are shown as the means  $\pm$  SEM of six to eight males per treatment group. Treatment effects ( $\#p < 0.05$ ) are indicated. Summary data for panels (B) and (D) can be found in Excel Tables S10–11. Note: DEHP, di(2-ethylhexyl) phthalate; ECAR, extracellular acidification rate; FCCP, carbonyl cyanide 4-(trifluoromethoxy)phenylhydrazone; OCR, oxygen consumption rate; SEM, standard error of the mean; Veh, vehicle.

the presynaptic element seemed to be more distant from the presynaptic membrane, with very, if any, vesicles observed in contact with the presynaptic membranes, as illustrated by representative images presented in Figure 4C and Figure S4.

### Bioenergetic Activity of the Medial Preoptic Area of Male Mice Exposed to DEHP Alone or to a Phthalate Mixture

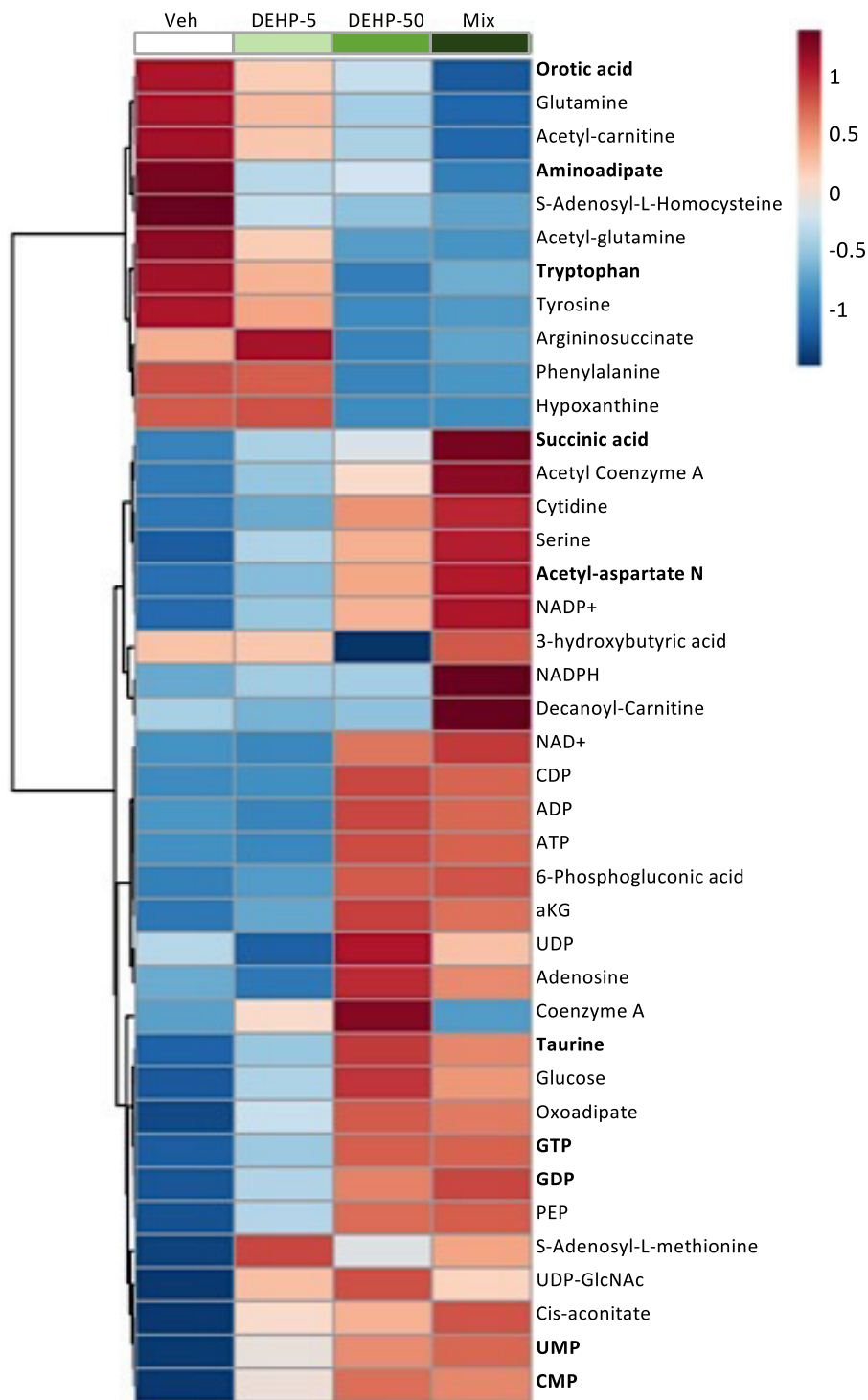
There is a tight link between neural function and cellular bioenergy. A potential bioenergetic dysregulation and notably mitochondrial dysfunction may lead to neural alterations and neurological disorders.<sup>12</sup> It is interesting to note that our previous comparative analysis of the medial preoptic area proteome between the vehicle and DEHP-exposed groups identified differentially expressed proteins involved in mitochondrial and cellular metabolism processes.<sup>3</sup> We thus assessed the effect of DEHP alone or in a phthalate mixture on the bioenergetic profile of the medial preoptic area. We did not observe any significant differences in OCR parameters between exposed and vehicle mice (Figure 5A,B), nor did we observe any difference in the expression of oxidative phosphorylation complexes and the central inducer of mitochondrial biogenesis PGC1- $\alpha$  (Figure S5). This absence of observed differences suggests that mitochondrial function was not affected by exposure. However, there was an effect of treatment on glycolysis ( $p = 0.02$ ) and glycolytic capacity ( $p = 0.04$ ) with a moderately higher level of each in exposed groups (Figure 5C,D), whereas no differences of the neural glucose transporters GLUT1 and GLUT3 nor the glycolytic enzymes hexokinases 1 and 2 (HK1 and HK2) were observed (Figure S5).

To further investigate the effect of exposure, a metabolic analysis by LC/MS was conducted on the hypothalamus from adult mice exposed to the vehicle, to DEHP alone, or to DEHP in a phthalate mixture. The heat map of the top 40 modified

metabolites is shown in Figure 6. Among these metabolites, ANOVA and Fisher's LSD as a post hoc test allowed identifying 10 metabolites that were significantly modified by exposure to DEHP alone or to phthalate mixture (FDR  $p$  adjusted value  $< 0.05$ ). These included taurine ( $p = 0.0026$ ), *N*-acetylaspartate ( $p = 0.0029$ ), and succinate ( $p = 0.00043$ ), which have been described as important in neuronal physiology and physiopathology.<sup>24,25</sup> Nucleotide metabolism was also altered with a down-regulation of orotic acid ( $p = 0.0018$ ), a precursor of pyrimidine synthesis, and a concomitant up-regulation of uridine monophosphate (UMP) ( $p = 0.0003$ ) and cytosine monophosphate (CMP) ( $p = 0.0004$ ). Guanosine-derived nucleotides guanosine diphosphate (GDP) ( $p = 0.0016$ ) and guanosine triphosphate (GTP) ( $p = 0.003$ ), which are important in neurotransmission, were also affected. Finally, tryptophan and amino adipate, which are implicated in glutamatergic neurotoxicity,<sup>26,27</sup> were significantly lower in exposed mice than in vehicle-treated mice ( $p = 0.0026$  and  $p = 2.7 \times 10^{-7}$ , respectively). Tryptophan can be metabolized via the kynurenine pathway in neuroactive compounds. An interesting finding was that higher level of NAD, the end product of the kynurenine pathway of tryptophan metabolism,<sup>27–29</sup> was observed for DEHP-50 ( $p = 0.043$ ) and Mix groups ( $p = 0.036$ ) in comparison with controls (Excel Table S3).

### Protein Amounts of AR, ER $\alpha$ , and AhR in the Preoptic Area of Adult Male Mice Exposed to DEHP Alone or to DEHP in a Phthalate Mixture

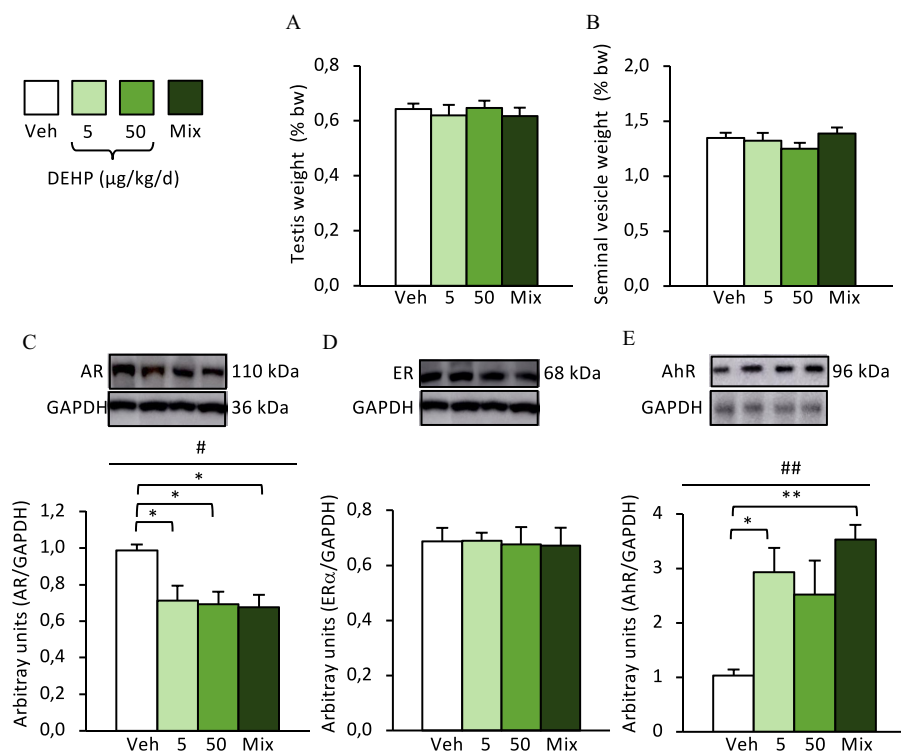
We previously showed that adult exposure to DEHP alone reduced the protein amount and mRNA levels of the AR in the preoptic area, without any effect on the gonadotropic axis or circulating levels of testosterone.<sup>3,20</sup> We therefore analyzed whether DEHP in the phthalate mixture behaves in the same way as DEHP alone. For this purpose, we assessed the weight of



**Figure 6.** Pan-metabolomic analysis in the hypothalamus of mice exposed to the Veh, DEHP at 5 (DEHP-5), or 50 µg/kg/d (DEHP-50), or to a phthalate mixture (Mix). Data are means of seven to eight males per treatment group. Relative levels vary from high (red) to low (blue). The ten metabolites that were significantly different after exposure to DEHP or phthalate mixture (FDR-p adjusted value <0.05) are bolded. The metabolomic data can be found in Excel Table S3. Note: ADP, adenosine diphosphate; aKG, α-ketoglutarate; ATP, adenosine triphosphate; CDP, cytosine diphosphate; CMP, cytosine monophosphate; DEHP, di(2-ethylhexyl) phthalate; GDP, guanosine diphosphate; GLN, glutamine; GTP, guanosine triphosphate; NADPH, NAD phosphate; NAD+, nicotinamide adenine dinucleotide; PEP, phosphoenolpyruvate; UDP, uridine diphosphate; UMP, uridine monophosphate; Veh, vehicle.

androgen-sensitive organs (seminal vesicles and testis) and quantified the protein amount of the AR and ERα in the medial preoptic area. Analyses of body weight throughout the treatment period did not show any effect of exposure to DEHP alone or in a phthalate mixture (Figure S6). No effects were observed on the relative weights of testis or seminal vesicles expressed as percentage of body

weight (Figure 7A,B). Quantification by western blotting indicated an effect of treatment on the protein amounts of the AR ( $p=0.01$ ) but not on protein amounts of the ERα ( $p=0.99$ ) as shown in Figure 7C,D. A significantly lower AR protein amount was found for DEHP-5, DEHP-50, and Mix groups (−28%, −30%, and −32%, respectively) in comparison with vehicle-treated males.



**Figure 7.** Effects of adult male mice exposure to DEHP alone or in a phthalate mixture on the weight of androgen-sensitive tissues and protein amounts of androgen (AR) and estrogen receptor (ER)  $\alpha$ , and AhR in the medial preoptic area. (A–B). The weight of testes and seminal vesicles of males exposed to the Veh, DEHP at 5 or 50  $\mu\text{g}/\text{kg}/\text{d}$ , or to a phthalate mixture (Mix) are expressed as percentage of body weight (bw). Data are expressed as the means  $\pm$  SEM of 11 males per treatment group. (C–E). Quantification of the protein amounts for the AR (C), ER $\alpha$  (D), and AhR (E) normalized to GAPDH in the medial preoptic area. Data are expressed as the means  $\pm$  SEM of four to six males per treatment group. Treatment effect (# $p < 0.05$ , ## $p < 0.01$ ) and post hoc analyses (\* $p < 0.05$ , \*\* $p < 0.01$ ) are indicated. Summary data for panels (A) to (E) can be found in Excel Table S12. Note: AhR, aryl hydrocarbon receptor; DEHP, di(2-ethylhexyl) phthalate; GAPDH, glyceraldehyde 3-phosphate dehydrogenase; SEM, standard error of the mean; Veh, vehicle.

The kynurenine pathway of tryptophan metabolism can activate or antagonize several signaling pathways.<sup>29</sup> It is interesting to note that one of the activated pathways involves the xenobiotic receptor AhR, which has been shown to interact with the AR<sup>30</sup> and reduce its expression.<sup>31</sup> We thus investigated whether the observed lower AR expression in males exposed to DEHP alone or in a phthalate mixture was associated with modifications in AhR protein amount in the medial preoptic area. Analysis by western blotting shows an effect of treatment on AhR protein amounts ( $p = 0.005$ ; Figure 7E), with a significant higher level of AhR protein in the DEHP-5, DEHP-50, and Mix groups in comparison with the vehicle group (+184%, +144%, and +242%, respectively).

## Discussion

Adult male mice exposed to an environmental phthalate mixture exhibited impaired sexual behavior, similar to those exposed to DEHP alone. To our knowledge, the present study shows for the first time that these behavioral alterations were associated with structural and molecular modifications in the preoptic area and a disrupted metabolomic profile in the hypothalamus. In particular, the kynurenine pathway of tryptophan metabolism seemed to be activated, as evidenced by the observed lower levels of tryptophan and a higher level of NAD<sup>+</sup> the end product metabolite of this pathway. This activation, together with the higher protein level of AhR, a xenobiotic target of the kynurenine pathway<sup>32</sup> and known modulator of AR expression, may participate at least in part in the observed AR down-regulation and altered neuroplasticity in the preoptic area and sexual behavior in male mice exposed to DEHP alone or in a phthalate mixture.

The behavioral analyses showed that mice exposed to the phthalate mixture at environmental doses behaved as mice exposed to DEHP alone. This finding confirms our previous data reporting altered emission of USVs and mating without changes in olfactory preference following chronic exposure to DEHP at 5 or 50  $\mu\text{g}/\text{kg}/\text{d}$ .<sup>3</sup> Furthermore, it suggests that DEHP probably drove the effects in the mixture. This observation could be explained by the higher ratio of DEHP (10- to 20-fold higher than the other phthalates) in the used mixture, which was based on environmental coexposure to these substances in France.<sup>13,14,33</sup> Other reported phthalate mixtures contained more DEP<sup>34,35</sup> based on U.S. cohorts showing a higher exposure to this phthalate.<sup>36,37</sup>

In this study, we also showed, using the three-chamber test, that receptive females spent less time investigating awake DEHP-treated males than males of the vehicle group.<sup>3</sup> We thus suggested that this lower ability of DEHP-exposed males to attract receptive females was probably due to at least partly to their reduced emission of USVs. In the present study, when receptive females were given the choice between urine from vehicle- or DEHP-Mix treated males, they spent less time investigating the urine from males exposed to DEHP alone or in mixture than the urine from the control group. Urinary excretions play an essential role during mating; some sociosexual pheromones such as the exocrine gland-secreting peptide 1<sup>38</sup> or major urinary proteins<sup>39</sup> have been identified in males. It is thus possible that the lower ability of DEHP- or Mix-exposed males to attract females was due both to altered production of courtship vocalizations and to emission of pheromonal cues.

The effects of exposure to DEHP alone or in a phthalate mixture on behavior were associated with a lower density of postsynaptic dendritic spines in the medial preoptic area. Effects were

observed on spines at all maturation stages, from the transient spine (thin) to the most stable and persistent ones (mushroom). Analyses of known postsynaptic markers of mature excitatory synapses showed lower protein amounts of glutamate receptors, spinophilin, and PSD-95 in comparison with control males. Altogether, these data show low dendritic spine density per 50  $\mu\text{m}$  length, possibly caused by reduced protein levels in key postsynaptic components in the preoptic area of male mice exposed to DEHP alone or in a phthalate mixture. We have previously shown that the first sexual experience, which is required and sufficient for a fully mature sexual behavior, induces a rise in dendritic spine density and protein levels of excitatory synapse components such as PSD-95 and glutamate receptors in the medial preoptic area.<sup>10</sup> Because naive male mice were exposed to DEHP alone or in a phthalate mixture for 8 wk before the first mating test, we suggest that such exposure possibly prevented structural and molecular changes required for dendritic spine formation, maturation, and/or maintenance in the medial preoptic area, thus interfering with neuronal connectivity and leading to a low expression of male sexual behavior.

The ultrastructural analysis of synapses corresponding to dendritic spines in the medial preoptic area showed comparable morphology of synapses between the vehicle and DEHP- and Mix-exposed groups. This comparable morphology, together with the apparent similar distribution of the types of dendritic spines between the treated groups, suggests that exposure to DEHP alone or in a phthalate mixture impacted more the dendritic spine density than spine morphology. However, whether such exposure interfered or not with neurotransmission still needs to be determined. Higher protein levels of synaptophysin, a presynaptic vesicle membrane protein, were observed in the medial preoptic area of male mice exposed to DEHP alone or in a phthalate mixture. The role of synaptophysin in synapse morphology and synaptic transmission is still not clear, but a recent study using quadruple knockout mice lacking all four members of synaptophysin/gyrin family showed that mutants presented increased efficiency of the release machinery.<sup>40</sup> In addition, it seemed from randomly observed synapses corresponding to dendritic spines in three independent experiments that the vesicles were more distant from the presynaptic membrane in male mice exposed to DEHP alone or in a phthalate mixture in comparison with the vehicle group. Because secretion of neurotransmitters into the synaptic cleft requires fusion of vesicles with the presynaptic membrane, it remains to be determined whether exposure to DEHP alone or in a phthalate mixture also impacted neurotransmitter release, in addition to reducing dendritic spine number per length.

The bioenergetic activity of the preoptic area was also affected by exposure to DEHP alone or in a phthalate mixture. Using the Seahorse analyzer on punches of the preoptic area, similar mitochondrial function and a moderately higher level of glycolysis were found in male mice exposed to DEHP alone or in a phthalate mixture. The latter increase suggests an enhancement of activity as a possible adaptive response to maintain normal adenosine triphosphate production. Different effects were observed in glycolytic activity between DEHP alone or a phthalate mixture, suggesting that specific pathways could be involved regarding the phthalate structure and concentration. Furthermore, it has been shown that phthalate esters may differentially interact and inhibit some metabolic enzymes depending on the phthalate structure.<sup>41,42</sup>

A further metabolomic approach showed altered hypothalamic levels of metabolites including nucleotides involved in neurotransmission or metabolites involved in neurotoxicity, such as amino adipate and tryptophan. Tryptophan metabolism is of great interest in the context of exposure to DEHP alone or in a

phthalate mixture for the following reasons: Tryptophan is an essential amino acid provided by the diet. Once absorbed, it can be used in the periphery or the brain in the serotonin synthesis pathway or the kynurenine pathway, which plays a key role in the generation of NAD<sup>+</sup>, a source of energy in particular in the context of the immune response.<sup>28</sup> Dysregulation of the kynurenine pathway of tryptophan metabolism has been suggested to be involved in several psychiatric and neurodegenerative diseases.<sup>27,29</sup> The intermediate metabolites of this pathway are indeed neuroactive and modulate neuroplasticity through effects on glutamatergic neurotransmission toxicity.<sup>27,43</sup> They are also widely known as inflammatory mediators that induce production of proinflammatory factors in astrocytes and activate microglia, the brain immune cells, through a mechanism involving activation of the xenobiotic receptor AhR.<sup>29</sup> An interesting aspect is that exposure to phthalates was previously reported to increase the kynurenine pathway of tryptophan metabolism. In male rats, oral exposure to DEHP, DBP, or other phthalate esters at 0.01%–3% of the diet for 21 d increased the liver conversion ratio of tryptophan to niacin, a by-product of the kynurenine pathway, and urinary excretion of kynurenine pathway metabolites.<sup>41,44,45</sup> In men, high DBP exposure derived from mesalamine intake increased the urinary concentrations of neurotoxic intermediate metabolites of the kynurenine pathway.<sup>46</sup> Moreover, urinary concentrations of several phthalate metabolites were positively associated with kynurenine-derived neuroactive metabolites in women.<sup>47</sup> Because of their structural similarities with tryptophan metabolites, phthalates bind and inhibit the enzyme that prevents the accumulation of kynurenine pathway metabolites.<sup>48</sup> These effects of phthalates have been described to occur in peripheral tissues. However, to our knowledge our study shows for the first time that an imbalance in the kynurenine pathway of tryptophan metabolism following exposure to DEHP alone or in a phthalate mixture can take place in the brain given the observed decrease of tryptophan, the initiating precursor, and increased levels of NAD<sup>+</sup>, the end product of this metabolic pathway. We recently showed that exposure of adult male mice to DEHP alone or in a phthalate mixture, under similar experimental conditions, induced neuroinflammation with astrocyte and microglial activation as well as increased BBB permeability in the medial preoptic area.<sup>4,5</sup> In this context, and in view of all the alterations induced by exposure of adult male mice to DEHP alone or in a phthalate mixture, including neuroinflammation, BBB disruption, and altered behavior and neural structure evidenced by lower dendritic spine density per 50- $\mu\text{m}$  length and protein levels of glutamate receptors and other key postsynaptic and presynaptic components<sup>3–5</sup> (as seen in the present study), we suggest that chronic exposure to phthalates first induced a metabolic perturbation, including a dysregulation of tryptophan metabolism, which then impacted glial and neuronal structure and function, thus leading to behavioral impairment.

As in our previous work on DEHP,<sup>3</sup> we show in this study that the behaviors exhibited by mice exposed to DEHP alone or to a phthalate mixture were not associated with different levels of testosterone and/or weight of androgen-sensitive tissues in comparison with mice exposed to vehicle. A selective lower expression of AR, the key receptor involved in the expression of sexual behavior, was observed in cerebral parenchyma in our previous<sup>3</sup> and present studies and in capillaries<sup>5</sup> of male mice exposed to DEHP alone or in a phthalate mixture. A possible link between tryptophan metabolism perturbation and AR down-regulation could be the above-mentioned AhR because exposure to DEHP alone or in a phthalate mixture increased the protein amounts of this xenobiotic receptor. We thus hypothesize that exposure to DEHP alone or in a phthalate mixture activated and/or increased

the expression levels of AhR, which then reduced AR expression as previously reported in LNCaP cells.<sup>31</sup> However, additional molecular mechanisms underlying the effects of phthalates on AR expression cannot be excluded. Down-regulation of the neural AR may participate not only in reduced sexual behavior but also in increased neuroinflammation and impaired BBB induced by exposure to DEHP alone or in a phthalate mixture<sup>3–5</sup> (present study). First, the role of neural AR in the expression of male sexual behavior has been well proven by the use of conditional transgenic male mice.<sup>7,49</sup> Second, chronic testosterone depletion in castrated adult male mice induced neural AR down-regulation, resulting in the activation of astrocytes and microglia and up-regulation of inflammatory molecules.<sup>9</sup> These males also exhibited greater BBB permeability for different tracers and endogenous immunoglobulins, altered organization of tight junction ultrastructure (as revealed by electron tomography), and lower expression levels of tight junction proteins.

## Conclusion

The data obtained in the context of this thorough investigation combining complementary approaches show that low, environmentally relevant doses targeted the brain and impacted structural, molecular, and cellular events essential for neural functioning and processing of male reproductive behavior. The studied behaviors and other outcomes in mice exposed to DEHP at 5 and 50 µg/kg/d were similar in the direction that they trended in comparison with those in control mice, suggesting that the effect of this phthalate either started or reached a maximal level after the dose of 5 µg/kg/d.

We suggest that exposure to DEHP alone or in a phthalate mixture possibly led to brain metabolic perturbations, which included a dysregulation of the kynurenine pathway of tryptophan metabolism. The intermediate metabolites of this pathway might trigger AR down-regulation either directly or indirectly through AhR activation. Then, the reduced androgen sensitivity and the activated kynurenine/AhR pathway might act together to induce neuroinflammation, BBB permeability, and altered neural structure and connectivity. All these cellular alterations might then result in impaired behavioral processing in the medial preoptic area. Future studies will assess whether these effects are specific to this hypothalamic subregion or can also occur in other brain areas.

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S.M.-K. and I.P. designed the study. S.D. and F.T. carried out the behavioral tests, characterization of dendritic spine density and synaptic components, and the analyses of physiological parameters. V.G.-M. performed the electron microscopy study. E.D., L.P.-M., A.L'h., C.C.-C., and I.N. carried out the metabolic analyses. S.M.-K. wrote the manuscript; V.G.-M., E.D., and I.P. corrected the manuscript; and all authors read and approved the final version.

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