



Exposure of Adult Female Mice to Low Doses of di(2-ethylhexyl) Phthalate Alone or in an Environmental Phthalate Mixture: Evaluation of Reproductive Behavior and Underlying Neural Mechanisms

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1 **Title**

2 Exposure of adult female mice to low doses of di(2-ethylhexyl)phthalate alone or in an
3 environmental phthalate mixture: Evaluation of reproductive behavior and underlying neural
4 mechanisms

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19

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25

26 **CFID declaration**

27 The authors declare they have no actual or potential competing financial interests.

28

29 **Abstract**

30 **Background.** We have previously shown that adult male mice exposure to low doses of an
31 ubiquitous endocrine disruptor, di(2-ethylhexyl)phthalate (DEHP), alters courtship behavior.

32 **Objective.** The effects of adult exposure to low doses of DEHP alone or in an environmental
33 phthalate mixture on estrous cyclicity, reproductive behavior and underlying neural structures were
34 analyzed in female mice.

35 **Methods.** Two-month-old C57BL/6J females were exposed orally for 6 weeks to DEHP alone (0, 5
36 or 50 µg/kg/d) or to DEHP (5 µg/kg/d) in a phthalate mixture. Estrous cyclicity was analyzed in
37 intact mice, and behavior (lordosis, olfactory preference, partner preference, ability to stimulate
38 male ultrasonic vocalizations) was measured in ovariectomized mice primed with estradiol and
39 progesterone. Immunohistochemical studies were conducted in the neural structures involved in
40 behavior for estrogen (ER) α and progesterone (PR) receptors.

41 **Results.** Exposure to DEHP alone or in mixture lengthened the estrous cycle duration, with a
42 shorter proestrus and longer estrus and metestrus stages. Under normalized hormonal levels,
43 females exposed to DEHP alone or in mixture exhibited altered olfactory preference. A lower
44 lordosis behavior and ability to attract and stimulate male emission of courtship ultrasonic
45 vocalizations was observed, probably due to modifications of pheromonal emission in exposed
46 females. The behavioral alterations were associated with a lower number of PR-expressing neurons,
47 without changes in ER α , in the neural circuitry underlying sexual behavior. The majority of effects
48 observed was comparable between the two DEHP doses and were driven by DEHP in the mixture.

49 **Conclusions.** Exposure to environmental doses of DEHP alone or in mixture altered several
50 components of female sexual behavior in mice, probably through selective disruption of neural PR
51 signaling. Together with the previously reported vulnerability of male mice, this suggests a major
52 impact of exposure to phthalates on sexual reproduction, including in other species with similar
53 neural regulatory processes.

54

55 **Introduction**

56 Phthalates are among the most frequently detected organic pollutants in the environment
57 worldwide (Gao and Wen 2016), due to their extensive use as plasticizers in several daily life
58 products. Di(2-ethylhexyl)phthalate (DEHP) is the most abundant molecule of this family (Gao and
59 Wen 2016). Previous studies in humans reported associations between phthalate metabolites in
60 urine and reduced anogenital distance in boys (Bornehag et al. 2015) and interest in sexual activity
61 in women (Barrett et al. 2014), or altered age of pubertal onset in girls (Berger et al. 2018).
62 Experimental studies using rodents described adverse effects of developmental exposure to DEHP
63 on sexual differentiation of the urogenital tract in males, age of puberty and testicular and ovarian
64 functions (for review: Hannon and Flaw 2015; Howdeshell et al. 2008; Rowdhwal and Chen 2018).
65 However, the potential effects of adult exposure to DEHP on the neural regulation of reproductive
66 behavior have received less attention. In this context, we previously showed that exposure of adult
67 male mice to DEHP at the tolerable daily intake dose (TDI) of 50 µg/kg/d (EFSA 2005; 2019) or at
68 lower doses close to the environmental exposure altered courtship behavior (Dombret et al. 2017).
69 In particular, DEHP exposure lowered the emission of ultrasonic vocalizations and the ability to
70 attract females, and delayed the initiation of mating. In female rodents, previous studies reported
71 that acute exposure to DEHP during adulthood alters estrous cyclicity and ovarian function (Chiang
72 et al. 2020; Davis et al. 1994; Hannon et al. 2014; Herreros et al. 2013; Li et al. 2012). Whether and
73 how adult exposure to low environmental doses of DEHP affects female behavior and underlying
74 neural structures remains to be investigated. Indeed, the effects of exposure to phthalates on female
75 sexual behavior were only analyzed for perinatal exposure (Guerra et al. 2010; Lee et al. 2006).

76
77 In female rodents, the expression of sexual behavior is limited to the estrus phase of the cycle,
78 coinciding with ovulation (for review: Mhaouty-Kodja et al. 2018). This behavior is induced by a
79 hormonal sequence involving the preovulatory surge of estradiol, which triggers both the ovulatory
80 surge of pituitary luteinizing hormone (LH) and, via an estrogen receptor (ER) α mediated action,

81 the up-regulation of progesterone receptors (PR). Progesterone liberated following ovarian
82 stimulation by LH induces female receptivity. Female sexual behavior includes an attractivity phase
83 during which the female stimulates male behavior by emitting pheromones, and a copulatory phase
84 with the female adopting a receptive posture called lordosis while being approached from behind
85 for insemination by the courting male. All these behavioral patterns are controlled by a neural
86 circuitry involving the olfactory bulb, which transmits chemosignals to the medial and
87 posteromedial cortical amygdala and then to the bed nucleus of the stria terminalis and the
88 ventromedial hypothalamus. This principal facilitatory system for lordosis behavior is activated by
89 estradiol and progesterone during the estrus phase, as mentioned above. Inversely, the constraints
90 exerted by the inhibitory system involving the hypothalamic preoptic and arcuate nuclei are lifted
91 during this period.

92
93 In the present study, we characterized the effects of chronic exposure of adult female mice to
94 DEHP alone or in an environmental phthalate mixture on reproductive behavior and underlying
95 neural structures. For this purpose, adult C57BL/6J female mice were assigned to one of four
96 exposure groups. The first three groups included females exposed orally for 6 weeks to the vehicle
97 (control), DEHP at the TDI dose of 50 µg/kg/d, or DEHP at 5 µg/kg/d. The DEHP dose of 5
98 µg/kg/d is within the environmental exposure range; this dose induced behavioral alterations in
99 male mice following adult or pubertal exposure (Capela and Mhaouty-Kodja 2021; Dombret et al.
100 2017). In order to mimic the environmental coexposure to phthalates (Martine et al. 2013; Anses
101 report 2015), the fourth group of females was exposed to a phthalate mixture containing DEHP at 5
102 µg/kg/d, dibutyl phthalate (DBP) at 0.5 µg/kg/d, butylbenzylphthalate (BBP) at 0.5 µg/kg/d, di-
103 isobutylphthalate (DiBP) at 0.5 µg/kg/d and di-ethylphthalate (DEP) at 0.25 µg/kg/d. A first cohort
104 of female mice including the four treated groups was analyzed for estrous cyclicity, body and
105 uterine weights. A second cohort of females was ovariectomized and primed with estradiol and
106 progesterone to induce their receptivity (acceptance of male mounting and display of lordosis

107 behavior in response to mounts) under similar hormonal conditions. These females were analyzed
108 for their lordosis and rejection behaviors as well as for olfactory preference in the presence of
109 sexually-experienced males. The ability of females and their pheromonal cues to attract male
110 partners and induce the male emission of courtship ultrasonic vocalizations was also investigated.
111 In this second cohort, locomotor activity was measured and body weight was monitored during the
112 whole period of treatment and behavioral analyses. The neural structures involved in the expression
113 of sexual behavior and belonging to the facilitatory and inhibitory systems of lordosis behavior
114 were studied for the number of ER α and PR-immunoreactive neurons and mean fluorescence
115 density.
116

117 **Methods**

118 *Animals and Treatments*

119 Studies were performed in accordance with the French and European legal requirements (Decree
120 2010/63/UE) and were approved by the “Charles Darwin” Ethical committee (project number
121 01490-01). The experiments were reported following the Animal Research: Reporting in Vivo
122 Experiments (ARRIVE) guidelines.

123 Animals were obtained from breeding of male and female C57BL/6J mice (Janvier Labs, Le
124 Genest-Saint-Isle, France) and housed in nest-enriched polysulfone cages with polysulfone bottles.
125 Mice were kept at 22°C under an inverted light schedule, i.e. the dark time began at 1:30 PM with a
126 12:12 h light-dark cycle, and fed a standard diet (A03–10; Safe-diets, Augy, France) with free
127 access to food and water. Offspring were mixed at the weaning to avoid potential litter effects (no
128 more than one female per litter per cage, and one to two females per litter in each treatment group),
129 and allowed to grow until 8 weeks of age.

130 Oral exposure was performed as previously described (Dombret et al. 2017). Phthalates (Sigma-
131 Aldrich, Saint-Quentin Fallavier, France) were dissolved in ethanol and water (1% and 40% of
132 prepared food, respectively) and incorporated by the experimenter into powdered food (A03–10;
133 Safe-diets) that was then reconstituted into pellets. Eight-week-old females were fed ad libitum with
134 chow containing the vehicle i.e. ethanol and water (control group), DEHP (CAS 117-81-7) at 50 or
135 5 µg/kg/d (DEHP-50 and DEHP-5 groups, respectively), or a phthalate mixture (Mix group)
136 containing DEHP at 5 µg/kg/d, DBP (CAS 84-74-2) at 0.5 µg/kg/d, BBP (CAS 85-68-7) at 0.5
137 µg/kg/d, DiBP (CAS 84-69-5) at 0.5 µg/kg/d and DEP (CAS 84-66-2) at 0.25 µg/kg/d. The
138 composition of the phthalate mixture was based on French and European studies showing an
139 external co-exposure to these molecules (Martine et al. 2013) and the presence of their metabolites
140 in urinary samples (Anses 2015; Dewalque et al. 2014). The ratio of DEHP over the other
141 phthalates was determined on the basis of the estimated daily intake in France and Europe
142 (Dewalque et al., 2014; Martine et al., 2013). Mice were weighed weekly and phthalate doses

adjusted to their body weights and calculated for a daily food intake of 5 g per animal. Reconstituted pellets were prepared every week immediately after animal weighing, stored at 4°C and changed twice a week. Analyses started after 6 weeks of exposure and treatments were maintained during the whole period of the study. Experiments were conducted on two cohorts of female mice each including 4 treated groups. The first cohort including 15 intact females per treatment group was subjected to analyses of estrous cyclicity; body and uterine weights were collected from all these females at necropsy (Figure S1). Behavioral analyses were conducted on a second cohort including 11 females from the control group, 12 from the DEHP-5 group, 13 from the DEHP-50 group and 13 females from the Mix group, which were all ovariectomized and hormonally primed (Figure S1). At the end of behavioral analyses, females were sacrificed and body and uterine weights were measured and the brains collected from 6 females per treatment group were processed for immunohistochemical analyses. All analyses were performed by blind observation, since females were identified by numbers attributed at weaning without any information concerning their treatment details.

Estrous cyclicity

Six weeks after the beginning of exposure, analyses of the estrous cycle were started while maintaining the treatment. Vaginal smears flushed with physiological saline were taken daily from females for seven weeks. The estrous cycle phases were identified by microscopy after hematoxylin / eosin coloration of the vaginal smears. The cycle duration was calculated as the average mean of days spent in seven complete cycles. The number of days spent in each stage of the estrous cycle was also determined. Body weight was examined during the whole period of treatment and estrous cycle analyses. The estrous cycle was monitored until sacrifice by pentobarbital injection (120 mg/kg) in order to measure body and uterine weights of females at the metestrus stage.

Behavioral tests

Four weeks after the beginning of exposure, female mice were ovariectomized under general anesthesia (xylazine 10 mg/kg and ketamine 100 mg/kg). At the time of ovariectomy, all females received 1 cm subcutaneous SILASTIC implants (3.18 mm outer diameter X 1.98 mm inner diameter; Dow Corning, Midland, MI) filled with 50 µg of estradiol benzoate (Sigma-Aldrich) in 30 µl sesame oil and sealed at each end with SILASTIC adhesive as previously described (Dombret et al. 2017; Naulé et al. 2014; Naulé et al. 2015; Raskin et al. 2012). Two weeks later, behavioral analyses were started and each female was given a subcutaneous injection of 1 mg / 100 µl progesterone (Sigma-Aldrich) dissolved in sesame oil 4-5 h before each test to induce female receptivity. Tests were conducted under red-light illumination, 2 h after lights off and were videotaped for later analyses. They were conducted following the order indicated below and in Figure S1, starting with lordosis, then olfactory preference, partner preference, USV analysis, and ending with locomotor activity. All females of the second cohort were analyzed in the behavioral tests. Control untreated males were sexually experienced before the beginning of tests, which were performed with a different male per female (lordosis and ultrasonic vocalizations tests) or per a pair of females or urine (partner preference tests). The devices used in the tests, at the exception of home cage animals, were cleaned with 10% ethanol between trials.

Lordosis. Females were tested twice: in a first test (naive) and two weeks later after this first sexual experience in a second test (sexually experienced). Each female was introduced into the cage of a sexually experienced male used as a partner. Tests ended after 20 min. The percentage of females exhibiting lordosis behavior, lordosis quotient (lordosis number/number of mounts) and rejection quotient (rejection number/number of mounts) were calculated for each subject in response to male mounting (Naulé et al. 2014; Naulé et al. 2015).

Olfactory preference. Olfactory preference was assessed in an enclosed plexiglass Y-maze as previously described (Capela et al. 2018; Dombret et al. 2017; Picot et al. 2014). Female mice were

195 allowed to become familiar with the maze, where two empty perforated goal boxes were placed at
196 each end, for 10 min over two consecutive days. On the day of the test, females were offered the
197 choice between a sexually receptive female and a gonadally intact male, which were placed in the
198 goal boxes. Stimuli were anesthetized to avoid any social interaction. Exposed females did not have
199 direct access to these stimuli but the perforated walls of goal boxes allowed air to flow from the
200 boxes into the maze. The total time spent in chemo-investigation and the number of entries into
201 each arm of the maze were scored during the 10-min test. The discrimination index was calculated
202 as the time spent by exposed females in male investigation (M) minus the time spent in female
203 investigation (F) divided by the total time of investigation (M-F/F+M).

204

205 *Partner preference tests*

206 *Three-chamber test.* Sexually experienced males were allowed to become familiar, for 10
207 min over two consecutive days, with the testing arena where two perforated goal boxes were placed
208 in the side chambers as previously described (Dombret et al. 2017). On the day of the test, each
209 male was placed in the neutral chamber and allowed to freely explore each chamber of the testing
210 arena for 10 min. A female treated with DEHP alone or in mixture was placed inside a goal box and
211 randomly assigned to the left or right chamber, while a vehicle-treated female was placed inside a
212 goal box in the other chamber. The number of entries into each compartment and the time spent
213 sniffing each female by the male over the 10-min test were scored.

214 *Y-maze test.* Sexually experienced males were allowed to become familiar with the maze for
215 10 min over two consecutive days. On the day of the test, male mice were offered the choice
216 between an anesthetized female from the vehicle group and an anesthetized female from the groups
217 exposed to DEHP alone or in mixture, placed at each end of the maze inside perforated goal boxes.
218 In the second version of this paradigm, female mice were replaced by their urine collected 1 h
219 before the test. For this purpose, an equivalent volume of urine collected from all females of each
220 treatment group was mixed, and 10 µl of this mix was applied on a piece of filter paper. On the day

221 of the test, male mice were offered the choice between a filter paper containing the urine from the
222 vehicle group and a filter paper containing the urine from the groups exposed to DEHP alone or in
223 mixture, placed at each end of the maze inside perforated goal boxes.

224 For both tests, the time spent by males in chemo-investigation of each stimulus and the number of
225 entries into each arm were scored during the 10-min test.

226

227 *Ultrasonic vocalizations.* Each male was tested in its home cage in the presence of a female from
228 one of the four treatment groups as previously described (Capela et al. 2018; Capela et al. 2019;
229 Dombret et al. 2017). After the introduction of a female, vocalizations were recorded for 4 min with
230 an UltraSoundGate microphone (AvisoftBioacoustics, Glienicke/Nordbahn, Germany), which was
231 connected to an Ultrasound recording interface plugged into a computer equipped with the Avisoft-
232 SASLab Pro 5.2.09 recording software. Vocalizations were analyzed using SASLab Pro (Avisoft
233 Bioacoustic). Spectrograms were generated for each detected call (frequency resolution: FFT-
234 length: 512; frame size: 100%; overlap: 50%). The parameters used for the automatic quantification
235 of the vocalizations were: cut-off frequency of 30 kHz, element separation based on an automatic
236 single threshold with a hold time of 15 ms. Syllables were identified and grouped into three main
237 categories (simple, complex, frequency-jump). The total number and duration of ultrasonic
238 vocalizations were analyzed, as well as the number and duration of each syllable.

239

240 *Locomotor activity.* The computed circular corridor used to measure activity was made of two
241 concentric cylinders crossed by four diametrically opposite infrared beams (Dombret et al. 2017;
242 Raskin et al. 2009). The locomotor activity was counted when animals interrupted two successive
243 beams and had thus traveled a quarter of the circular corridor. Activity was recorder for 120 min
244 and was expressed as cumulative activity over the whole 120 min test.

245

246 *Body and uterine weight measurements.* Body weight of female mice of the second cohort was
247 monitored during the whole period of treatment and behavioral analyses. At the end of behavioral
248 experiments, animals were sacrificed by pentobarbital injection (120 mg/kg) and the uterus was
249 collected and weighed. The results were expressed as absolute body and uterine weights, and as
250 relative uterine weight (percentage of body weight).

251

252 ***Immunohistochemistry***

253 Brains from perfused animals were post-fixed overnight in 4% paraformaldehyde in phosphate
254 buffered saline (PBS). Brains were then sliced into coronal sections of 30 μm in a vibratome and
255 processed for immunolabeling. Sections were blocked for 2 h with 2% normal donkey serum
256 (Sigma-Aldrich) in PBS that contained 0.3% Triton-X100, then incubated with polyclonal anti-ER α
257 antibody diluted at 1:400 (Santa Cruz Biotechnology, Heidelberg, Germany) or polyclonal anti-PR
258 antibody diluted at 1:400 (Dako-Agilent, Santa-Clara, USA) for 72 h at 4 °C. Immunofluorescence
259 was performed with an Alexa Fluor 488-conjugated chicken anti-rabbit secondary antibody diluted
260 at 1:500 (Life Technologies-Invitrogen, Carlsbad, USA) for 2 h at room temperature in the dark.
261 After several rinses with PBS, sections were rinsed in water, mounted in Mowiol and stored at 4 °C
262 in the dark. Sections were scanned using a high-resolution Nanozoomer Hamamatsu scanner
263 (Hamamatsu Corporation, Massy, France). The number of labeled cells and mean fluorescence per
264 section were counted by NDP.view (Hamamatsu Corporation) and ImageJ software (NIH;
265 Abràmoff et al. 2004), respectively, in anatomically matched sections identified using the Mouse
266 Brain Atlas (Paxinos and Franklin 2001). ER α - and PR-immunoreactive cells were analyzed in the
267 medial amygdala within an area of 0.56 mm² (plate 46), in the bed nucleus of stria terminalis within
268 an area of 0.70 mm² (plate 30), in the ventromedial hypothalamus within an area of 0.12 mm² (plate
269 46), in the medial preoptic area within an area of 0.80 mm² (plate 30), in the arcuate nucleus within
270 an area of 0.20 mm² (plate 46) and in the posteromedial cortical amygdala within an area of 0.56
271 mm² (plate 46).

272

273 ***Statistics***

274 Data were expressed as means \pm S.E.M., except for those reporting the percentage of females
275 exhibiting lordosis behavior, and analyzed by GraphPad prism 6 (GraphPad Software, San Diego,
276 USA).

277 Normality tests (Kolmogorov-Smirnov and Shapiro-Wilks tests) were performed. Two-way
278 ANOVA was used to analyze the main effect of exposure and stimulus on the number of entries for
279 olfactory preference. The Kruskal-Wallis test was used to analyze the effect of exposure on the
280 estrous cycle and stage durations, body weight, lordosis and rejection quotients, the number of
281 syllables (short, upward, one-jump), the duration of syllables (short, upward, downward,
282 modulated, mixed, one-jump, and frequency-jump), the number of ER α -immunoreactive cells in
283 the medial amygdala and arcuate nucleus, and the number of PR-immunoreactive cells in the medial
284 amygdala and bed nucleus of stria terminalis. Dunn's post hoc tests were used to determine group
285 differences. Student's one sample t-test with 0 as the theoretical value was used to analyze the
286 discrimination index for olfactory preference. Partner preference was analyzed by the Student's
287 paired t-test or the Wilcoxon test. One-way ANOVA was used to analyze the effect of exposure on
288 the remaining data. Bonferroni's post hoc tests were used to determine group differences. P values
289 of < 0.05 were considered to be significant.

290

291 **Results**

292 **Effects of adult female mice exposure to DEHP alone or in mixture on the estrous cycle**

293 Adult female mice were exposed orally for 6 weeks, and analyses of the estrous cycle were started
294 while maintaining the treatment for a further 7 weeks. Figure 1A shows five consecutive estrous
295 cycles represented for one female from each treatment group. Females exposed to DEHP alone or in
296 phthalate mixture had cycles with longer durations compared to the control female. Quantitative
297 analyses showed an effect of treatment on the mean cycle duration ($p = 0.0001$), with significant
298 longer durations for DEHP-5 (+33%; $p = 0.0003$), DEHP-50 (+35%; $p = 0.0001$) and Mix groups
299 (+20%; $p = 0.027$) than control females (Figure 1B). Detailed analyses of the duration of each stage
300 showed different effects depending on the stage of the estrous cycle. An effect of treatment was
301 observed on the proestrus stage ($p = 0.0001$), with a shorter duration in the DEHP-5 (-12%; $p =$
302 0.0021), DEHP-50 (-11%; $p = 0.0016$) and Mix groups (-16%; $p = 0.0001$) compared to control
303 females (Figure 1C). There was also an effect of treatment on the estrus ($p = 0.0001$) and metestrus
304 durations ($p = 0.0001$), whereas the diestrus stage remained unaffected ($p = 0.77$). In particular, a
305 longer duration of the estrus stage was noticed for the three treatment groups (+64% for the DEHP-
306 5 group, +38% for the DEHP-50 group, and +52% for the Mix group compared to the control
307 group). Similarly, the metestrus stage was longer for the DEHP-5 (+87%) and DEHP-50 groups
308 (+97%) in comparison to control females.

309 Monitoring of body weight during the whole period of treatment and estrous cycle analyses showed
310 no significant differences between the treatment groups (Figure S2A). After estrous cycle analyses,
311 body and uterine weights were measured at the metestrus stage. There was no effect of treatment on
312 body weight ($p = 0.48$; Figure 1D), but an effect on uterine weight was detected ($p = 0.04$), with a
313 mean increase of 25% compared to the control group (Figure 1E). Post hoc analyses did not show
314 significant differences between the treatment groups.

315

316 **Effects of adult mice exposure to DEHP alone or in mixture on lordosis behavior and** 317 **olfactory preference**

318 To determine the behavioral effects of DEHP alone or in a phthalate mixture at comparable
319 hormonal levels, all the following analyses were performed on ovariectomized females, which
320 received implants containing similar estradiol levels. Females were administered progesterone 4-5 h
321 before each behavioral test to induce their receptivity.

322 Lordosis behavior was first analyzed in naive (Test 1) and sexually experienced-females (Test 2), in
323 response to mounts of sexually experienced males as presented in Figure 2A. The percentage of
324 females showing at least a lordosis posture, was not statistically different between the four
325 treatment groups for Tests 1 and 2, although a tendency to decrease was observed in females
326 exposed to DEHP alone or in phthalate mixture (Figure 2B). The quantification of the lordosis
327 quotient showed no effect of treatment on Test 1 ($p = 0.21$), but a significant effect on Test 2 ($p =$
328 0.001) (Figure 2C). Post hoc analyses showed a lower quotient of the DEHP-5, DEHP-50 and Mix
329 groups in females, in comparison to the control group (-55% , $p = 0.0045$; -56% , $p = 0.0049$; -54% p
330 $= 0.0062$ respectively). This was mainly due to the fact that behavior in Test 2 was improved in
331 control females ($+147\%$ versus Test 1), while it remained low in the three other treated groups. The
332 rejection quotient in Test 2 was also affected by treatment ($p = 0.0201$), but not in Test 1 ($p =$
333 0.0931), with a significant higher quotient in the DEHP-50 group ($+271\%$, $p = 0.0281$ versus the
334 control group) (Figure 2D).

335 Female sexual behavior is activated by olfactory cues emitted by the male partner. We tested the
336 ability of females to discriminate between male and female odors in preference tests using
337 gonadally intact males versus sexually receptive females (Figure 2E). In this Y-maze paradigm, the
338 total time spent sniffing the stimuli was equivalent for females from the four exposure groups ($p =$
339 0.53) (Figure 2F). Two-way ANOVA of the number of entries into each arm showed no effect of
340 stimulus ($F_{(1,45)} = 1.75$, $p = 0.19$) or treatment ($F_{(3,45)} = 2.06$, $p = 0.12$) (Figure 2G). In contrast,
341 there was an effect of treatment on the olfactory discrimination index ($p = 0.0289$) (Figure 2H).

342 Females exposed to DEHP-50 or to the mix showed no preference for males over females, while a
343 preference was observed for the control and DEHP-5 groups.

344

345 **Exposure to DEHP alone or in mixture lowered female ability to attract male partners**

346 In the three-chamber test, a sexually experienced male was presented with two awake females, one
347 from the control group and the other from the group treated with DEHP alone or in mixture. Each
348 female was placed in one of two opposite compartments, separated by a neutral one (Figure 3A).
349 The number of entries into each compartment was similar for the three experimental conditions
350 (control versus DEHP-5, control versus DEHP-50, and control versus Mix) (Figure 3B). An
351 analysis of the percentage of time spent investigating each female showed that males spent more
352 time investigating control females than DEHP-5, DEHP-50 or Mix groups ($p = 0.0006$, $p = 0.0015$,
353 and $p = 0.046$, respectively) (Figure 3C).

354 To understand why females treated with DEHP alone or in phthalate mixture were less attractive
355 than their control littermates, the same females were anesthetized to avoid any social interaction,
356 and analyzed in a Y-maze paradigm. Males were again given the choice between two groups of
357 females (Figure 3D). No differences were observed in the number of entries into the stimulus arms
358 (Figure 3E). In contrast, there were differences in the percentage of time spent investigating each
359 female (Figure 3F), with males spending less time sniffing DEHP-5, DEHP-50 and Mix groups than
360 control females ($p = 0.005$, $p = 0.0011$ and $p = 0.019$; respectively).

361 These results strongly suggested that the differences observed in the investigation by males were
362 probably related to the olfactory cues emitted by exposed females. To confirm this hypothesis, we
363 performed another Y-maze test where the stimuli consisted in urine collected from mice of the four
364 treatment groups (Figure 3G). Data illustrated in Figures 3H-I show comparable numbers of entries
365 into the arms, but less time spent in investigation for DEHP-5, DEHP-50 and Mix groups compared
366 to the control group ($p = 0.0049$, $p = 0.0066$ and $p = 0.015$, respectively).

367

368 **Effects of exposure to DEHP alone or in mixture on the emission of male ultrasonic**
369 **vocalizations**

370 We compared the ability of control versus DEHP- or Mix-exposed females to stimulate the
371 emission of courtship ultrasonic vocalizations by males. Recordings during the 4-min of interaction
372 between the sexual partners showed an effect of treatment on the total number of syllables ($p =$
373 0.0023), with a significant lower number in DEHP-50 and Mix groups versus the control group ($-$
374 27% , $p = 0.047$; -41% , $p = 0.0013$, respectively) (Figure 4A). Detailed analyses of each syllable
375 shows an effect of treatment on the total number of short ($p = 0.039$), downward ($p = 0.022$),
376 complex ($p = 0.041$), mixed ($p = 0.023$) and frequency-jump ($p = 0.005$) syllables (Figure 4B-D).
377 An effect of treatment was also observed on the total duration of syllables ($p = 0.031$), with a lower
378 duration in the Mix group (-51% versus the control group, $p = 0.028$) (Figure 4E). Significant
379 treatment effects were also seen on the total duration of downward ($p = 0.028$), complex ($p =$
380 0.022), and mixed ($p = 0.034$) syllables (Figure 4F-H).

381

382 **Effects of female mice exposure to DEHP alone or in mixture on general behavior and**
383 **parameters**

384 Locomotor activity was measured in a circular corridor (Figure S3). An effect of treatment ($p =$
385 0.016) was observed on cumulative activity during the 120-min test. Post hoc analyses showed a
386 lower activity (-32%) versus the control group for the Mix group ($p = 0.0097$).

387 The body weight of females was monitored during the whole period of treatment and behavioral
388 analyses (Figure S2B); no significant effects of treatment were observed. Furthermore, the absolute
389 and relative weights of uterus, an estrogen-dependent tissue, were not different between the
390 treatment groups (Table S1), confirming the comparable hormonal levels administered to
391 ovariectomized female mice.

392

393 **Effects of exposure to DEHP alone or in mixture on the neural circuitry that underlies female** 394 **sexual behavior**

395 Females exposed to DEHP alone or in an environmental mixture exhibited several impaired
396 components of sexual behavior, under normalized estrogen and progesterone levels. We asked
397 whether these behavioral alterations were due to a direct effect of phthalates on the neural signaling
398 pathways for these hormones. For this, we analyzed ER α - and PR-immunoreactivity in the neural
399 structures underlying this behavior.

400 A specific nuclear ER α signal was detected in neurons of the medial and posteromedial cortical
401 amygdala, bed nucleus of stria terminalis and ventromedial hypothalamus corresponding to the
402 principal facilitatory system (Figure 5A-C, Figure S4A-left). Quantitative analyses showed no
403 significant effect of treatment on the number of ER α -immunoreactive neurons (Figure 5D-F, Figure
404 S4B-left), or mean fluorescence density in these brain areas (Figure 5G-I, Figure S4B-right).

405 A specific nuclear PR-immunoreactivity was also observed in all these cerebral structures except
406 the posteromedial cortical amygdala where the signal was hardly detected (Figure 6A-C, Figure
407 S4A-right). Quantitative analyses showed a significant effect of treatment on the number of PR-
408 immunoreactive neurons in the medial amygdala ($p < 0.0001$), bed nucleus of stria terminalis ($p =$
409 0.0096) and ventromedial hypothalamus ($p = 0.0010$) (Figure 6D-F). Post hoc analyses showed a
410 lower number of PR-immunoreactive neurons for the three treated groups in the medial amygdala (-
411 27 to -44%), the bed nucleus of stria terminalis (-44 to -55%) and the ventromedial hypothalamus (-
412 25% to -38%). Similar results were obtained for the mean fluorescence density (Figure 6G-I).

413 Comparable immunohistochemical analyses of the inhibitory system of lordosis behavior, including
414 the medial preoptic area and arcuate nucleus, were carried out. The data obtained showed no effect
415 of treatment on the number of ER α -immunoreactive neurons or mean fluorescence density in the
416 medial preoptic and arcuate nuclei (Figures 7A, 7C and Figure S4C). In contrast, effects of
417 treatment were observed on the number of PR-immunoreactive neurons in the medial preoptic area
418 ($p = 0.0003$) and arcuate nucleus ($p = 0.0428$) (Figures 7B, 7D), with a significant lower number in

419 all treatment groups (-28 to -42% in the medial preoptic area, -20 to -41% in the arcuate nucleus,
420 compared to the control group). Similar results were observed for the mean fluorescence density
421 (Figure S4D).

422

423 **Discussion**

424 The present study shows for the first time to our knowledge that chronic exposure of adult female
425 mice to low doses of DEHP alone or in an environmental phthalate mixture impaired reproductive
426 behavior. Exposed females exhibited a lowered ability to discriminate between male and female
427 pheromones and ability to attract males and induce the emission of male courtship vocalizations.
428 They also displayed a lower lordosis quotient and inversely a higher rejection behavior, in response
429 to male mounts. These behavioral alterations were associated with a significantly lower number of
430 PR-immunoreactive neurons in the neural structures underlying female sexual behavior. In the
431 majority of analyses, the effects observed were similar between DEHP at the TDI dose (50 µg/kg/d)
432 and at a 10-fold lower dose of 5 µg/kg/d, and between DEHP alone at 5 µg/kg/d and in the phthalate
433 mixture.

434

435 In response to male mounts, female mice exposed to DEHP alone or in mixture exhibited a low
436 lordosis quotient and a higher rejection behavior after a first sexual experience. In comparison,
437 sexually experienced females of the control group showed a higher lordosis and a lower rejection
438 quotient. The analysis of general behavior showed an effect of treatment on locomotor activity
439 monitored in the circular corridor, with a significantly lower activity in the Mix group. Although
440 lower activity during mating can participate in the lowered sexual behavior of females from the Mix
441 group in particular, it is not sufficient to explain the altered mating behavior of the other treatment
442 groups. The altered lordosis behavior can be at least partly due to the lower olfactory preference
443 since exposed females were unable to discriminate between male and female pheromonal cues, in
444 particular for the DEHP-50 and Mix groups. It is, however, interesting to note that DEHP-5 group
445 females also had a low lordosis quotient despite unchanged olfactory discrimination. Altogether,
446 these data suggest that exposure to DEHP alone or in phthalate mixture impaired plasticity of the
447 neural areas involved in female sexual behavior processing. The observed alterations were probably

448 located at both the level of the olfactory system and chemosensory areas located downstream from
449 the olfactory system and involved in behavioral processing.

450
451 Females exposed to DEHP alone or in mixture were also less efficient than females of the
452 control group in attracting males. In the three-chamber test using awake non-anesthetized animals,
453 males preferred to investigate females of the control group. In these experimental conditions where
454 behavior was recorded during the dark phase, sexual partners interacted through the emission of
455 pheromones by the two partners and ultrasonic vocalization mainly by males. When they are
456 receptive, females emit specific pheromones that stimulate male behavior such as chemo-
457 investigation and courtship ultrasonic vocalizations (Bean 1982; Dizinno and Whitney 1977; Nyby
458 et al.1977; for review: Mhaouty-Kodja 2020). Interestingly, a similar lower chemo-investigation by
459 males was observed when anesthetized females, or their urine, were presented to chemo-
460 investigation by males. In addition, in the presence of females exposed to DEHP alone or in
461 phthalate mixture, males emitted a lower number of ultrasonic vocalizations for a shorter duration,
462 with a significant impact on 5 of the 9 categories of syllables (short, downward, complex, mixed,
463 and frequency-jump).

464 Altogether, these data indicate that exposure to DEHP alone or in phthalate mixture probably
465 altered the pheromonal cues emitted by females, which then impaired attraction of the male partner
466 and stimulation of ultrasonic vocalization emission.

467 Olfactory cues from female and male mice including bodily and urinary excretions play an essential
468 role during mating. As mentioned above, female olfactory cues inform the male mouse about the
469 receptive status of the female, and induce sexual arousal of the male partner. While some
470 sociosexual pheromones such as the exocrine-gland-secreting peptide 1 (Kimoto et al. 2007) or
471 major urinary proteins (Kaur et al. 2014) have been identified in males, less is known about female
472 pheromones that are necessary during mating. Sulfated estrogens derived from metabolization of
473 estrogens during the estrus phase were characterized as potential signal molecules in the female

474 urine (Nodari et al. 2008). These sulfated estrogens emitted by ovulating females were shown to
475 activate male vomeronasal receptors and promote male courtship behavior (Haga-Yamanaka et al.
476 2014; Nodari et al. 2008). In this context, it is possible that exposure to DEHP alone or in phthalate
477 mixture altered peripheral estradiol metabolism, thereby resulting in modified excretion of sulfated
478 estrogens in the urine of receptive females. In support of this hypothesis, a recent study described
479 that exposure to a low-dose mixture of food contaminants containing DEHP altered the expression
480 of estrogen sulfotransferase, the enzyme that catalyzes the formation of sulfated estrogens (Naville
481 et al. 2013).

482

483 To determine the molecular mechanisms underlying the effects of exposure to DEHP alone or in
484 phthalate mixture on female behavior, we analyzed the whole neural circuitry involved in the
485 activation of female sexual behavior. The behavioral alterations induced by exposure to these
486 molecules could not be due to differences in the levels of estradiol and progesterone, the key
487 hormones underlying behavioral activation. Indeed, levels of these hormones were normalized in all
488 females tested, through ovariectomy and hormonal supplementation. We thus quantified the number
489 of neurons expressing ER α and PR, the two key receptors mediating estradiol- and progesterone-
490 induced regulation of sexual behavior. Interestingly, a selective effect of treatment on the number of
491 PR-expressing neurons was observed, while the number of ER α -immunoreactive neurons remained
492 unaffected. A significantly lower number of PR-expressing neurons was observed in both the
493 facilitatory (medial amygdala, bed nucleus of stria terminalis, ventromedial hypothalamus) and
494 inhibitory (medial preoptic area, arcuate nucleus) systems of lordosis behavior in females exposed
495 to DEHP alone or in mixture. These differences can fully explain modified female behavior since
496 ER α -mediated up-regulation of PR is required for the induction of female receptivity in rodents
497 (Parsons et al. 1981; for review: Mhaouty-Kodja et al. 2018).

498 Two previous studies reported effects of phthalates at high doses on PR expression in females.
499 Perinatal exposure of female rats to diisononyl phthalate (20,000 ppm) lowered PR mRNAs levels

500 in the medial preoptic area (Takagi et al. 2005). A significant increase in the nuclear levels of PR
501 was observed in epithelial breast cancer cells exposed to 10 μ M DEHP (Crobeddu et al. 2019).
502 Besides its function in female reproduction, the PR has been shown to play a key role in
503 neuroprotection and promyelination (Gonzalez et al. 2020; Schumacher et al. 2014). It would be
504 interesting to study whether and how adult exposure to environmental doses of phthalates alters PR
505 expression in other female reproductive functions and non-reproductive systems.

506 The down-regulation of neural PR and lowered behavior in females are comparable to our results
507 obtained recently in males, where adult exposure to DEHP at the same doses influenced behavior
508 and induced androgen receptor (AR) down-regulation in the neural structures underlying male
509 sexual behavior (Table S2), without effect on hormonal levels (Dombret et al. 2017). Such
510 observations indicate that DEHP alone or in an environmental phthalate mixture were able to
511 impact directly the nervous system. Another intriguing observation is that DEHP exposure seems to
512 target a close subfamily of neural steroid receptors i.e. AR in males and PR in females. Indeed,
513 according to the phylogeny of the steroid receptor gene family, AR and PR are pairs of closely
514 related sister receptors among the second steroid receptor subgroup, which also includes
515 glucocorticoid and mineralocorticoid receptors (Eick et al. 2011). The first subgroup is formed by
516 ER α and ER β (Eick et al. 2011). Whether *AR* and *PR* genes share common regulatory mechanisms
517 of expression that could be similarly disrupted by DEHP exposure needs further investigation.

518

519 Analyses of intact females showed that adult exposure to DEHP alone or in an environmental
520 phthalate mixture impaired the progression of the estrous cycle. A shorter pro-estrus phase and
521 longer estrus and metestrus phases were observed in females exposed to phthalates. Effects on the
522 estrous cycle were described for 10 days or 16 weeks of adult exposure to high doses of DEHP (0.5
523 to 2 g/kg/d), with a prolonged estrous cycle and delayed ovulation in rats and mice (Davis et al.
524 1994; Li et al. 2012). Adult exposure to DEHP at 20 μ g/kg/d for 10 days or at 200 mg/kg/d for 30
525 days also prolonged the duration of the estrus phase (Hannon et al. 2014). In comparison, our data

526 indicate that effects on the estrous cycle can be observed at lower doses of DEHP (5 µg/kg/d) alone
527 or in a phthalate mixture after 6 weeks of exposure. This alteration was associated with a higher
528 uterine weight, suggesting hormonal modifications, in agreement with previous studies showing
529 altered ovarian estrogen and progesterone levels and pituitary LH and FSH levels following
530 exposure to DEHP in female mice (Chiang et al. 2020; Li et al. 2012) or sheep (Herreros et al.
531 2013).

532 The present results showing effects at different levels of the female reproductive system (estrous
533 cycle, behavior) suggest that the impact of exposure to DEHP alone or in mixture on female
534 reproduction should be important. In comparison, our previous studies in males showed an effect of
535 DEHP exposure on reproductive behavior, but not on the integrity of the HPG axis (Dombret et al.
536 2017). Given the behavioral effects induced by adult exposure to DEHP in the two sexual partners
537 (Figure 8), one may expect major adverse effects on mating in rodents. These effects may be
538 extended to other vertebrate species where sexual reproduction is tightly regulated by sex steroid
539 receptors. In humans, despite some differences with rodents in the control of the cycle, the key
540 endocrine mechanisms important for reproduction success are the same and involve also a crosstalk
541 between the ovary, the hypothalamus and the pituitary (Viguié et al. 2018). At the behavioral level,
542 sexual function in women is under this hormonal control (Mills et al. 2019). Altogether, these
543 observations suggest that the effects induced in mice by adult exposure to low doses of phthalates
544 on cyclicity and behavior might be relevant for human reproduction. Interestingly, epidemiological
545 studies reported associations between exposure to phthalates and reproductive issues including
546 premature ovarian failure (Hliseníková et al. 2020) and low interest in sexual activity (Barrett et al.
547 2014). In this context, it is important to indicate that sexual health is included since 1975 by the
548 World Health Organization (WHO) as part of reproductive health, and it is recognized that sexual
549 dysfunction may impact not only reproduction but also mental and general health, well-being and
550 maturation (WHO 2006).

551

552 The neuroendocrine and behavioral effects induced by DEHP alone or in phthalate mixture were
553 observed following continuous exposure of adult females. In males, our recent work shows that
554 exposure arrest for two months completely reversed the modifications triggered by DEHP exposure
555 in behavior and hypothalamic AR expression (Capela and Mhaouty-Kodja 2021). Whether female
556 neural structures also present this high plasticity after arrest of exposure will be assessed in further
557 studies.

558 DEHP effects were observed at low doses of 5 or 50 µg/kg/d. Except for rejection behavior of
559 experienced females, olfactory discrimination and total USV number for which a more efficient
560 effect was triggered by the DEHP dose of 50 µg/kg/d for, the other behaviors and parameters were
561 similarly affected by the two doses. The lack of dose response may be due to the fact that the two
562 doses differ only by a 10-fold factor; additional lower and higher doses are maybe needed to
563 observe such dose-response. This hypothesis is supported by our previous data obtained on males
564 and showing similar effects induced by the doses of 5 and 50 µg/kg/d for partner preference, mating
565 and neural androgen receptor protein amount while the dose of 0.5 µg/kg/d was inefficient
566 (Dombret et al. 2017). The results also suggest that DEHP at 5 µg/kg/d drives the effects in the
567 phthalate mixture. This was probably due to the fact that DEHP was the predominant phthalate in
568 the mixture with a concentration 10- to 20-fold higher than the other phthalates.

569 The majority of experimental studies addressing exposure to phthalates including DEHP have used
570 high doses of these molecules. The TDI dose of 50 µg/kg/d for DEHP was established by the EFSA
571 2005 on the basis of the ability of this molecule to reduce fetal testosterone production, while the
572 oral reference dose of 20 µg/kg/d established by the US EPA 1987 was determined for an increased
573 liver weight. A recent revision by the EFSA of 5 phthalates including DEHP maintains the TDI
574 dose at 50 µg/kg/d according to effects on fetal testosterone production (EFSA 2019). Our studies at
575 the neural level addressing adult exposure (Dombret et al. 2017; present study) and some studies
576 concerning recent evidence reporting in vivo peripheral effects at doses equivalent or below the
577 reference doses for prenatal (Abdel-Maksoud et al. 2019; Barakat et al. 2019) or adult exposure

578 (Hannon et al. 2014; Lu et al. 2019) reveal the high sensitivity of both female and male
579 reproductive systems. These data should lead to the consideration of several endpoints of male and
580 female reproduction, and maybe later to the revision of reference values established for a single
581 endpoint.

582

583 **Conclusion**

584 This study shows for the first time to our knowledge that chronic exposure of adult female mice to
585 DEHP alone or in an environmental phthalate mixture interfered with several components of sexual
586 behavior. Exposed females displayed lower lordosis behavior and olfactory preference. They also
587 showed a lower ability to attract and stimulate male behavior, may be due to modifications of
588 emitted pheromonal cues by exposed females. Together with the altered duration of estrous
589 cyclicity, this led us to suggest that under comparable experimental conditions (doses used,
590 exposure period and duration), reproduction of females was more vulnerable than that of their male
591 congeners to adult exposure to phthalates. The behavioral alterations induced and driven by DEHP
592 in the phthalate mixture were probably caused at least in part by PR down-regulation in the neural
593 structures underlying female sexual behavior. All the behavioral and neural effects observed were
594 induced by doses of DEHP equivalent to or below the TDI dose. This indicates that the nervous
595 system is highly sensitive to these compounds and should be considered in this context as a relevant
596 endpoint in risk assessment for these molecules.

597

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 765

766 **Figure Legends**

767 **Figure 1. Effects of adult female mice exposure to DEHP alone or in mixture on estrous**
768 **cyclicity. A.** Representation of five consecutive estrous cycles (**a-e**) in 4 females exposed either to
769 the vehicle (control), DEHP at 5 (DEHP-5) or 50 $\mu\text{g/kg/d}$ (DEHP-50), or to a phthalate mixture
770 (Mix). The duration of the estrous cycles (in days) are indicated. **B-C.** Mean duration of the estrous
771 cycle (**B**) and mean duration of each stage of the estrous cycle (**C**) in female mice. Data, expressed
772 as means \pm S.E.M for 15 females per treatment group. Kruskal-Wallis analysis showed a treatment
773 effect on the duration of the estrous cycle ($p = 0.0001$), proestrus, estrus and metestrus ($p = 0.0001$).
774 Post hoc analyses (* $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$ versus the control group) are indicated. **D-**
775 **E.** Body (**D**) and uterine weights (**E**) are indicated as means \pm S.E.M. Treatment effect on uterine
776 weight shown by one-way ANOVA (# $p < 0.05$). Summary data for panels B, C, D, and E can be
777 found in Table S3.

778

779 **Figure 2. Effects of adult female mice exposure to DEHP alone or in mixture on lordosis**
780 **behavior and olfactory preference. A.** Lordosis behavior was tested in naive (Test 1) and
781 experienced (Test 2) females in the presence of a sexually experienced male with a two-week
782 interval duration. **B.** Percentage of female mice ($n = 11-13$ per treatment group) showing lordosis
783 behavior in the four treatment groups exposed to the vehicle (Veh, control), DEHP at 5 or 50
784 $\mu\text{g/kg/d}$ or to a phthalate mixture (Mix). **C.** Lordosis quotient, number of female lordosis
785 posture/number of male mounts, was calculated in Tests 1 and 2 for the four treatment groups
786 (means \pm S.E.M). Kruskal-Wallis analysis showed a treatment effect of treatment for Test 2 ($p =$
787 0.001); post hoc analyses (* $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$ versus the control group) are
788 indicated. **D.** Rejection quotient, number of female rejection behavior/number of male mounts, was
789 calculated in Tests 1 and 2 for the four treatment groups. Kruskal-Wallis analysis showed a
790 treatment effect for Test 2 ($p = 0.0201$); post hoc analyses (* $p < 0.05$ versus the control group) are
791 indicated. **E-H.** Olfactory preference of females towards an anesthetized male and female was

792 measured in a Y-maze paradigm (**E**). Total time spent in chemo-investigation by female mice (**F**)
793 and number of entries into the male and female stimulus arms (**G**) are presented as means \pm S.E.M.
794 The discrimination index (**H**), time spent by exposed females in male investigation minus the time
795 spent in female investigation divided by the total time of investigation, is expressed as means \pm
796 S.E.M. One-way ANOVA showed a treatment effect on the discrimination index ($p = 0.0289$);
797 positive index for the control and DEHP-5 groups ($*p < 0.05$ and $**p < 0.01$) are indicated.
798 Summary data for panels B, C, D, F, G and H can be found in Table S4.

799

800 **Figure 3. Effects of adult female mice exposure to DEHP alone or in mixture on partner**
801 **preference. A-C.** In a three-chamber test, a sexually experienced male had choice between a
802 control female exposed to the vehicle (Veh, control) and a female exposed to DEHP alone or in
803 mixture (**A**). The number of entries of males into the chamber of control female versus the chamber
804 of female exposed to DEHP at 5 (DEHP-5) or 50 $\mu\text{g/kg/d}$ (DEHP-50), or to a phthalate mixture
805 (Mix) (**B**), and the percentage of time spent investigating each female (**C**) are represented as means
806 \pm S.E.M. Males were used for 11-13 females per treatment group. Paired t-test or Wilcoxon test are
807 indicated ($*p < 0.05$, $**p < 0.01$, $***p < 0.001$ versus the control group). **D-F.** In a Y-maze test, a
808 sexually experienced male had the choice between an anesthetized female exposed to the Veh and a
809 female exposed to DEHP alone or in mixture (**D**). The number of entries of males into the arm of
810 Veh versus the arm of DEHP- or Mix-exposed female (**E**) and the percentage of time spent
811 investigating each female (**F**) are presented as means \pm S.E.M. Paired t-test or Wilcoxon test ($*p <$
812 0.05 , $**p < 0.01$, $***p < 0.001$ versus the control group). **G-I.** In a Y-maze paradigm, a sexually
813 experienced male had the choice between urine from vehicle-exposed females and urine from
814 females exposed to DEHP alone or in mixture (**G**). The number of entries of males into the arm of
815 Veh versus the arm of DEHP or Mix-exposed urine (**H**) and the percentage of time spent
816 investigating the Veh versus DEHP or Mix-exposed female urine (**I**) are presented as means \pm

817 S.E.M. Paired t-test or Wilcoxon test (*p < 0.05, **p < 0.01, ***p < 0.001 versus the control group)
818 are indicated. Summary data for panels B, C, E, F, H and I can be found in Table S5.

819

820 **Figure 4. Effects of adult female mice exposure to DEHP alone or in mixture on the emission**
821 **of male ultrasonic vocalizations. A.** Total number of ultrasonic vocalizations (USV) emitted by
822 sexually experienced males in the presence of females exposed to the vehicle (Veh), DEHP at 5 or
823 50 µg/kg/d, or to a phthalate mixture (Mix) during the 4-min recording. Data are expressed as
824 means ± S.E.M. Males were used for 11-13 females per treatment group. **B-D.** Total number
825 (means ± S.E.M) of syllables of the simple (**B**), complex (**C**) and frequency jump (**D**) category. A
826 significant treatment effect was found by one-way ANOVA on the number of total USVs (p =
827 0.0023), downward (p = 0.022), complex (p = 0.041), mixed (p = 0.023) and frequency-jump
828 syllables (p = 0.005), and by Kruskal-Wallis on the number of short syllables (p = 0.039). Post hoc
829 analyses (*p < 0.05, **p < 0.01 versus the control group) are indicated. **E.** Total duration (means ±
830 S.E.M) of ultrasonic vocalizations emitted by males. **F-H.** Total duration (means ± S.E.M) of
831 syllables of the simple (**F**), complex (**G**) and frequency jump (**H**) categories. A significant treatment
832 effect was found by one-way ANOVA on the duration of total USVs (p = 0.031) and complex
833 syllables (p = 0.022), by Kruskal-Wallis for the duration of downward (p = 0.028) and mixed
834 syllables (#p = 0.034); post hoc analyses (*p < 0.05 versus the control group) are indicated.
835 Summary data for panels A, B, C, D, E, F, G and H can be found in Table S6.

836

837 **Figure 5. Effects of adult exposure to DEHP alone or in mixture on neural estrogen receptor**
838 **(ER) α-immunoreactivity in the facilitatory system of female mice. A-C.** Representative nuclear
839 ERα-immunolabeling in the medial amygdala (MeA; **A**), bed nucleus of stria terminalis (BNST; **B**),
840 and ventromedial hypothalamus (VMH; **C**) of females exposed to the vehicle (Veh), DEHP at 5
841 (DEHP-5) or 50 µg/kg/d (DEHP-50), or to a phthalate mixture (Mix). Scale bar: 100 µm; OC: optic
842 chiasma, AC: anterior commissure. **D-I.** Quantitative analyses of the number of ERα-

843 immunoreactive (ir) cells (**D-F**) and mean fluorescence density (**G-I**) in the indicated brain areas.
844 Data are means \pm S.E.M. of 6 females per treatment group. Summary data for panels D, E, F, G, H
845 and I can be found in Table S7.

846
847 **Figure 6. Effects of adult female mice exposure to DEHP alone or in mixture on neural**
848 **progesterone receptor (PR)-immunoreactivity in the facilitatory system. A-C.** Representative
849 nuclear PR-immunolabeling in the medial amygdala (MeA; **A**), bed nucleus of stria terminalis
850 (BNST; **B**), and ventromedial hypothalamus (VMH; **C**) of females exposed to the vehicle (Veh,
851 control), DEHP at 5 (DEHP-5) or 50 μ g/kg/d (DEHP-50), or to a phthalate mixture (Mix). Scale
852 bar: 100 μ m; OC: optic chiasma, AC: anterior commissure. **D-I.** Quantitative analyses of the
853 number of PR-immunoreactive (ir) cells (**D-F**) and mean fluorescence density (**G-I**) in the indicated
854 brain areas. Data are means \pm S.E.M. of 6 females per treatment group. A significant treatment
855 effect was found by one-way ANOVA on cell number in the MeA ($p < 0.0001$) and VMH ($p =$
856 0.0010), and on density in the BNST ($p = 0.0003$) and VMH ($p = 0.0022$), while Kruskal-Wallis
857 analysis showed a treatment effect on cell number in the BNST ($p = 0.0096$) and density in the
858 MeA ($p = 0.0107$). Post hoc analyses (* $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$ versus the control
859 group) are indicated. Summary data for panels D, E, F, G, H and I can be found in Table S8.

860
861 **Figure 7. Effects of adult female mice exposure to DEHP alone or in mixture on neural**
862 **estrogen receptor (ER) α - and progesterone receptor (PR)-immunoreactivity in the inhibitory**
863 **system. A-B.** Representative nuclear ER α - (**A**) and PR-immunolabeling (**B**) in the medial preoptic
864 area (MPOA) and arcuate nucleus (ARC) of female mice exposed to the vehicle (Veh, control),
865 DEHP at 5 (DEHP-5) or 50 μ g/kg/d (DEHP-50), or to a phthalate mixture (Mix). Scale bar: 100
866 μ m; 3V: third ventricle. **C-D.** Quantitative analyses of the number of ER α - (**C**) and PR-
867 immunoreactive (ir) cells (**D**) in the indicated brain areas. Data are means \pm S.E.M. of 6 females per
868 treatment group. A significant treatment effect was found by one-way ANOVA on the number of

869 PR-immunoreactive cells in the MPOA ($p = 0.0003$) and ARC ($p = 0.0428$). Post hoc analyses (* p
870 < 0.05 , *** $p < 0.001$ versus the control group) are indicated. Summary data for panels C and D can
871 be found in Table S9.

872

873 **Figure 8. Proposed mechanisms for behavioral effects of adult exposure to DEHP in sexual**
874 **partners.** Chronic oral exposure of male and female mice to DEHP impacted the physiological
875 balance between the key neural sex steroid signaling pathways in the neural circuitry underlying
876 sexual behavior. A lower expression of neural androgen receptor (AR) in males and neural
877 progesterone receptor (PR) in females occurred without effects on neural ER α expression. The
878 resulting lower neural AR/ER α ratio in males might be related to a lower emission of ultrasonic
879 vocalizations (USVs) and the ability to attract female partners. In females, the lower neural PR/ER α
880 ratio was associated with a lower olfactory discrimination and expression of lordosis behavior. In
881 addition, modifications in female pheromonal cues probably due to peripheral effects of phthalates
882 were related to a lower ability of exposed females to stimulate male courtship behavior such as
883 USV emission. This suggests that combined modifications induced by DEHP exposure in both
884 sexual partners may greatly impact mating and sexual reproduction in rodents.
885 The red arrows represent the lowered sex steroid receptor ratio and male and female behaviors
886 induced by adult DEHP exposure.

887

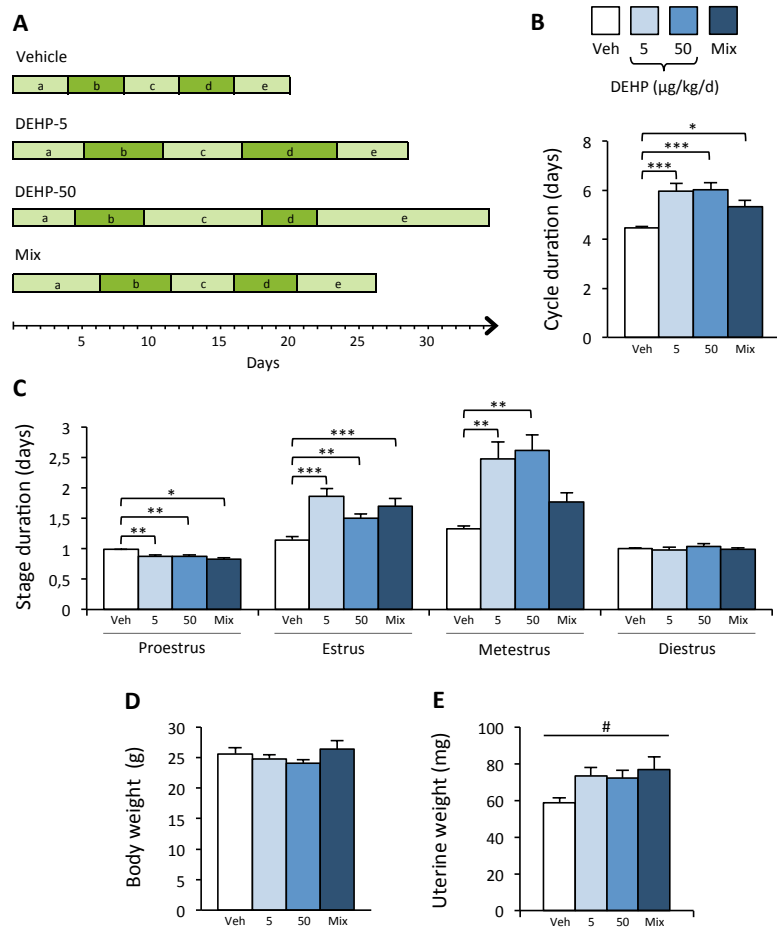


Figure 1

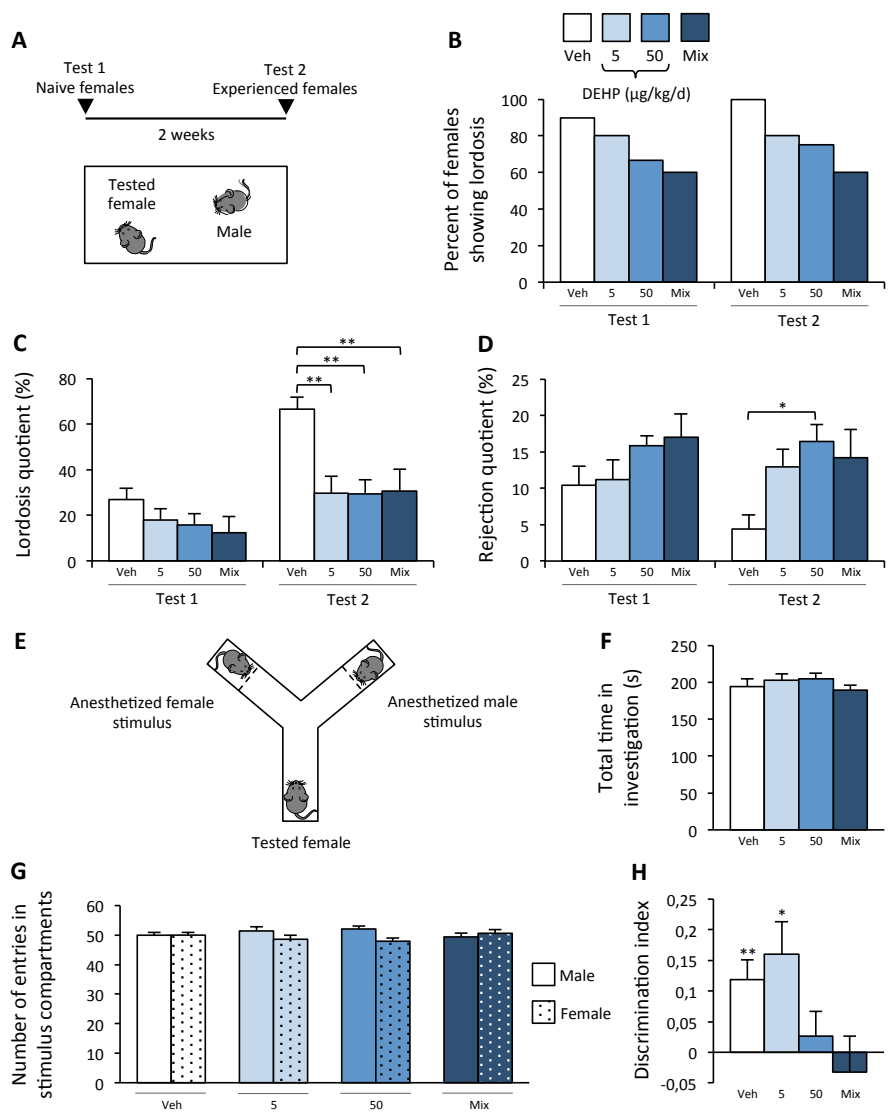


Figure 2

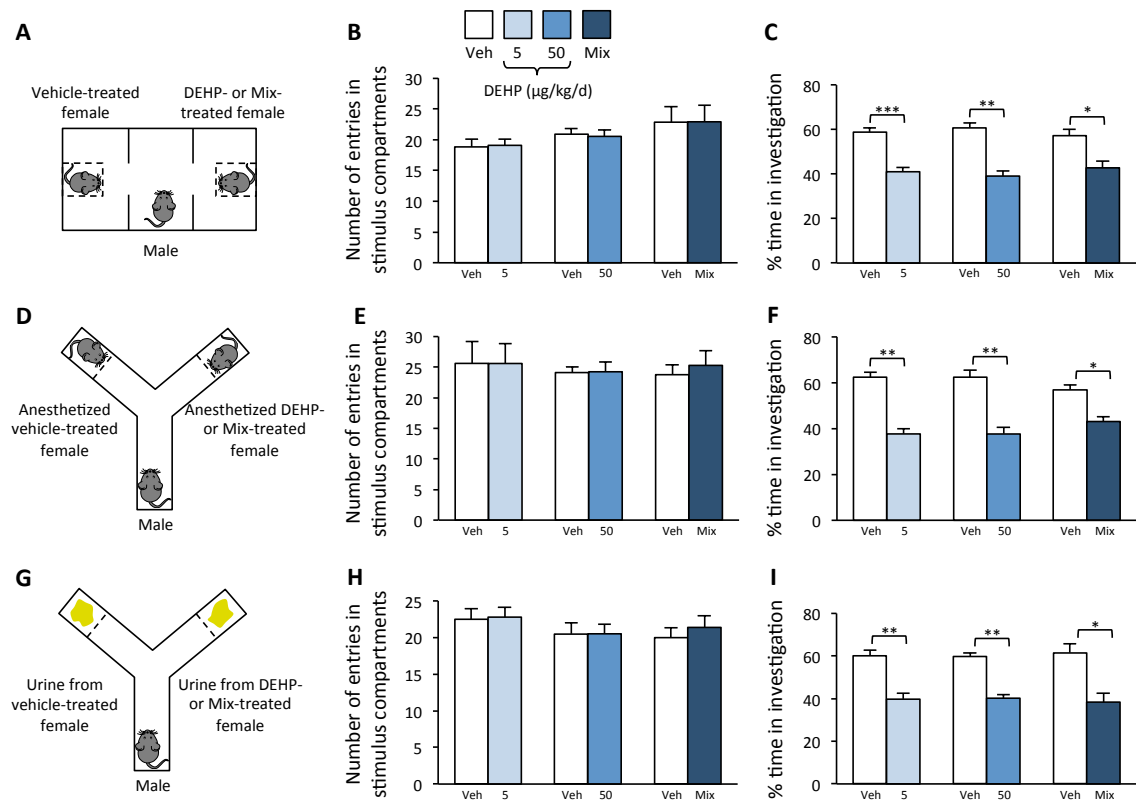


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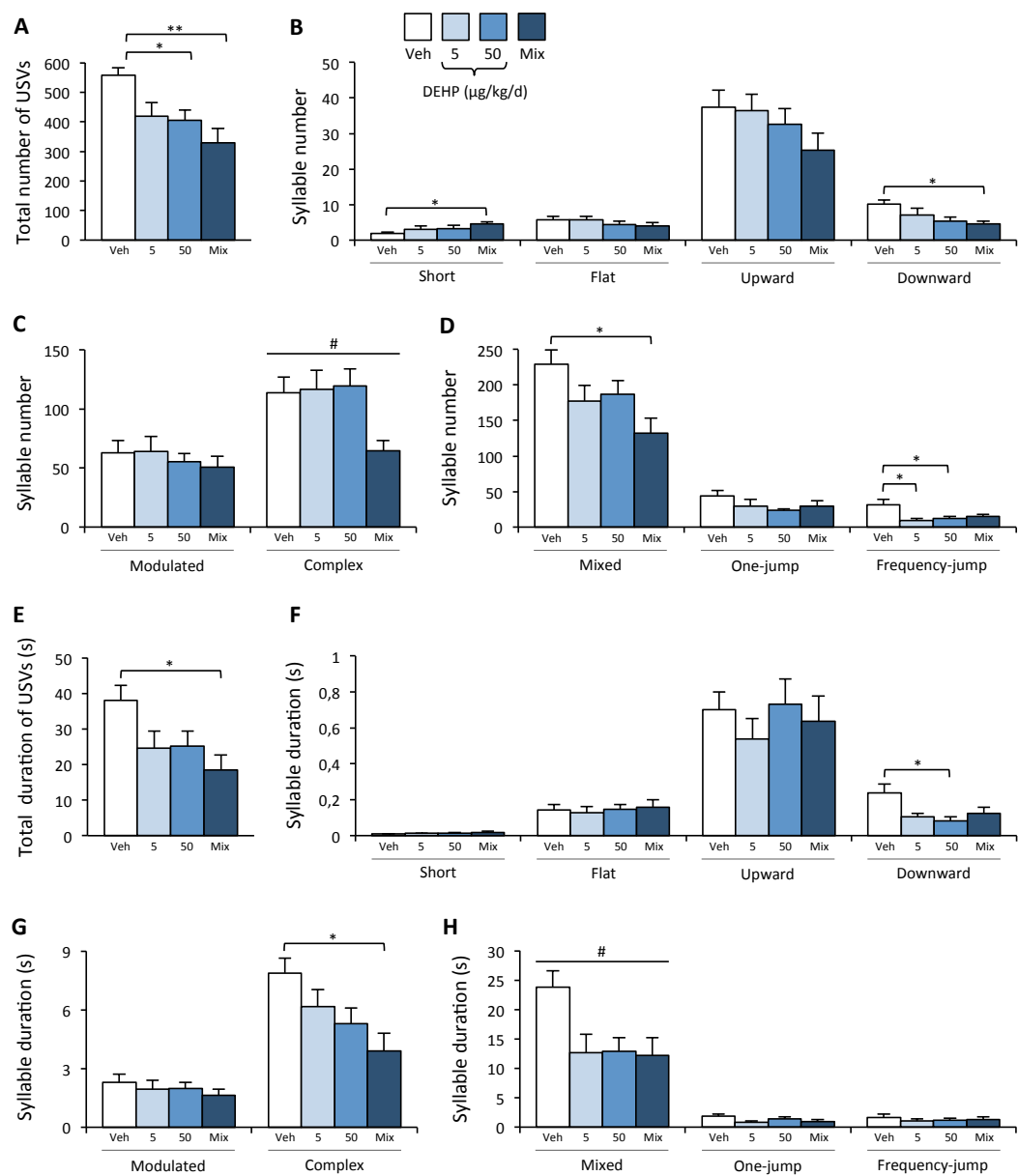


Figure 4

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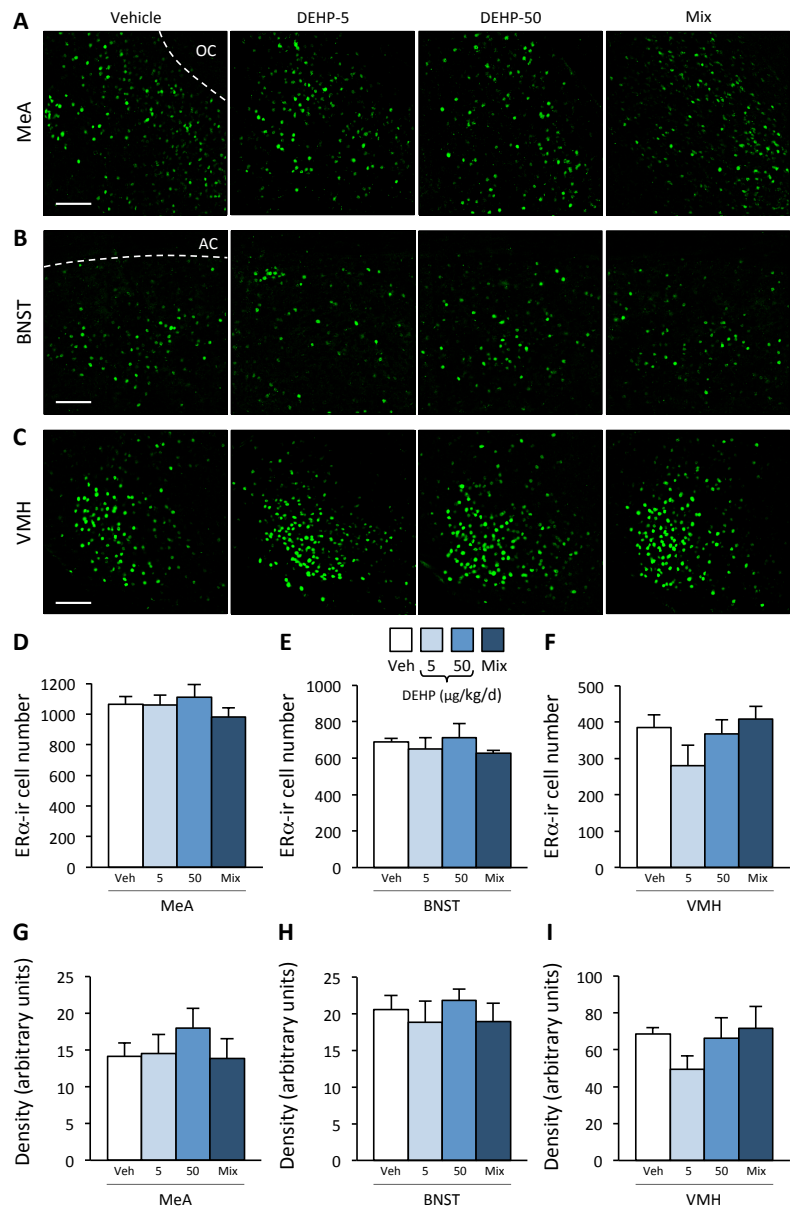


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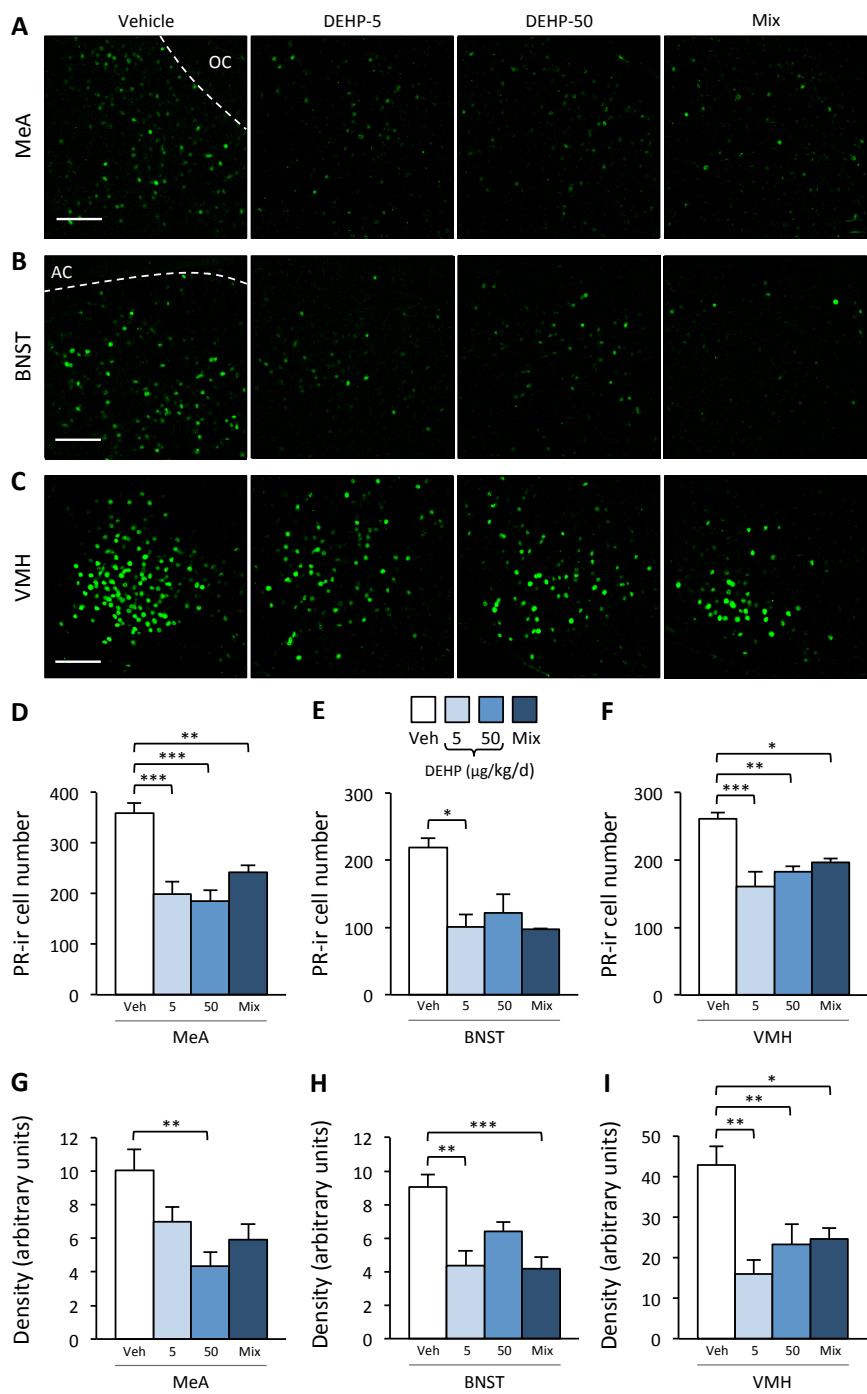


Figure 6

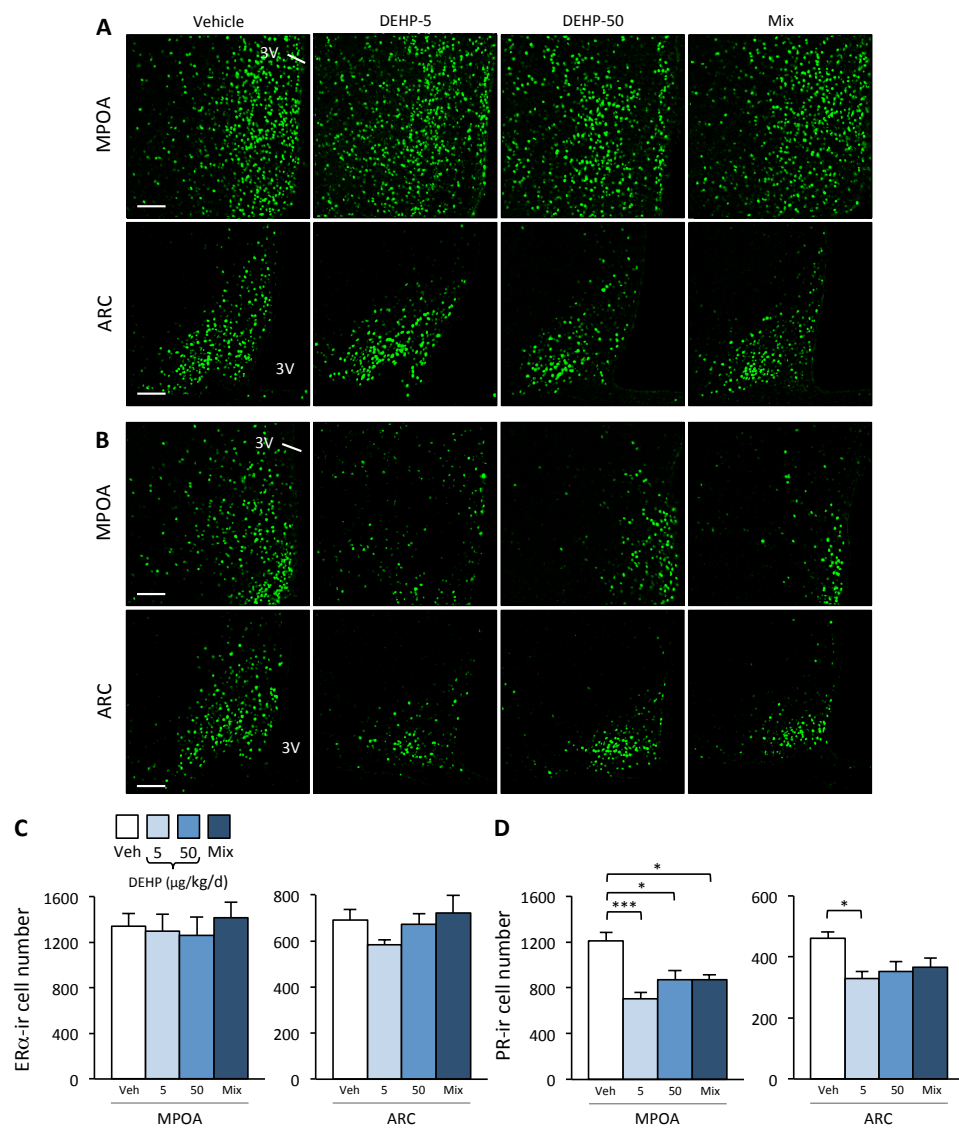


Figure 7

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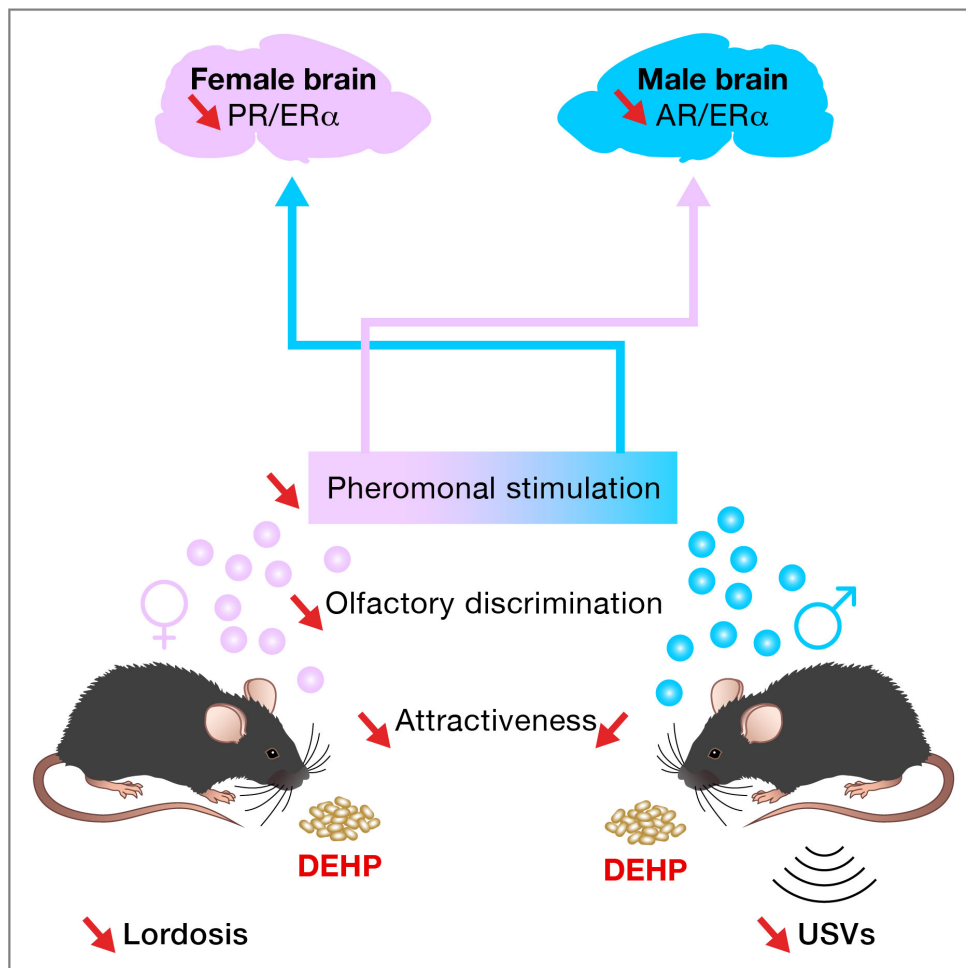


Figure 8