Neuroendocrine and behavioral effects of maternal exposure to oral bisphenol A in female mice

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Abstract

Bisphenol A (BPA) is a widespread estrogenic compound. We investigated the effects of maternal exposure to BPA at reference doses on sexual behavior and neuroendocrine functions of female offspring in C57BL/6J mice. The dams were orally exposed to vehicle alone or vehicle-containing BPA at doses equivalent to the no observed adverse effect level (5 mg/kg body weight per day) and tolerable daily intake (TDI, 0.05 mg/kg body weight per day) level from gestational day 15 until weaning. Developmental exposure to BPA increased the lordosis quotient in naive females exposed to the TDI dose only. BPA exposure had no effect on olfactory preference, ability to express masculine behaviors or number of calbindin-positive cells, a sexually dimorphic population of the preoptic area. BPA at both doses selectively increased kisspeptin cell number in the preoptic periventricular nucleus of the rostral periventricular area of the third ventricle in adult females. It did not affect the number of GNRH-positive cells or percentage of kisspeptin appositions on GNRH neurons in the preoptic area. These changes were associated with higher levels of estradiol (E2) at the TDI dose while levels of LH, estrus cyclicity, ovarian and uterine weights, and fertility remained unaffected. Delay in the time of vaginal opening was observed during the postnatal period at TDI dose, without any alteration in body growth. This shows that developmental exposure to BPA at reference doses did not masculinize and defeminize the neural circuitry underlying sexual behavior in female mice. The TDI dose specifically exacerbated responses normally induced by ovarian E2, through estrogen receptor α, during the postnatal/prepubertal period.

Key Words
- bisphenol A
- female mice
- nervous system
- reproduction
- behaviors
- kisspeptin

Introduction

Bisphenol A (BPA) is one of the most abundant endocrine disrupters. It is used in epoxy resins and a wide variety of polycarbonate containers for the storage of foods and beverages. Environmental and health concerns about the extensive use of polycarbonate plastic have been raised, in particular, concerning BPA leaching from plastic.
Most individuals in industrialized nations are exposed to BPA contamination (Calafat et al. 2005, Vandenberg et al. 2007). BPA remains widely used in industry despite some countries recently banning the use of polycarbonate in the production of baby bottles. Known for its estrogenic activity, BPA binds to estrogen receptors (ERs) with a lower affinity than that of estradiol (E2) (Dodd & Lawson 1936) and acts as a partial agonist (Delfosse et al. 2012).

The sexual differentiation of reproductive behavior and neuroendocrine function in rodents occurs during the perinatal (late gestational and early neonatal) and postnatal periods. This process is tightly regulated by gonadal hormones and is therefore highly sensitive to hormonal changes or exposure to exogenous factors exhibiting hormone-mimetic activities.

In males, testosterone liberated from fetal and neonatal testes permanently potentiates male (masculinization) and inhibits female (defeminization) behavioral and neuroanatomical characteristics (Phoenix et al. 1959, Morris et al. 2004). The organizational effects of testosterone result in the adult expression of male-typical behaviors (such as preference for a receptive female, mounts and thrusts) and an inability to adopt the female posture (lordosis), which is triggered by male mounts. Testosterone also acts perinatally to suppress the neuroanatomical changes required for sex steroids to induce the ovulatory surge of luteinizing hormone (LH) during adulthood (Corbier 1985, Homma et al. 2009). Sex steroids regulate gonadotropin release through an inhibitory or stimulatory feedback effect. Both male and female rodents respond to negative feedback, but only females respond to positive feedback. Testosterone effects are mainly induced by E2, which is derived from neural aromatization of testosterone by aromatase cytochrome P450.

In females, the ovaries are initially inactive, and selective binding of α-fetoprotein to maternal- and male sibling-derived E2 protects the perinatal brain from its masculinizing effects (Bakker et al. 2006). Significant levels of E2 are liberated from the ovaries around postnatal day 7. Ovarian liberation of E2 between postnatal days 15 and 25 is important for a full expression of female sexual behavior during adulthood (Bakker et al. 2002, Brock et al. 2011), suggesting that this prepubertal period is also an important time window for the differentiation of female reproductive behaviors.

BPA does not bind to α-fetoprotein (Milligan et al. 1998) and could therefore act in the female brain to masculinize reproductive behaviors and functions during the perinatal period. It could also interfere with E2 effects during the prepubertal period. No studies, to our knowledge, have explored the effects of BPA exposure on the sexual behavior in female mice. The studies investigating the effects of BPA on reproductive physiology report various effects on vaginal opening, fertility, and the estrus cycle. Exposure to BPA during prenatal or postnatal life resulted in advanced- (Honma et al. 2002, Nikaido et al. 2004, Nah et al. 2011), delayed- (Nikaido et al. 2005), or unaffected- (Howdeshell et al. 1999, Tyl et al. 2008) vaginal opening. Fertility was either reduced or unaltered in females exposed to BPA (Honma et al. 2002, Tyl et al. 2008, Cabaton et al. 2011). Irregular (Honma et al. 2002, Nikaido et al. 2004) or normal estrus cycles (Tyl et al. 2008) have been described for BPA-exposed females. This diversity in effects could be related to factors including differences in mouse strains, BPA dose, and time of exposure. At the neuroanatomical level, only one study reported increased levels of Kiss1 and GnRH mRNAs after exposure to high doses of oral BPA (Xi et al. 2011). These genes code for kisspeptin and GNRH, two important neuropeptides for the central regulation of reproductive function throughout life and known targets of a variety of estrogeno-mimetic endocrine disruptors (Gore 2010, Tena-Sempere 2010, Franceschini & Desroziers 2013).

Therefore, this study aimed to assess the effects of BPA exposure on sexual differentiation of copulatory behavior and reproductive physiology, as well as to investigate the neuronal populations involved in the regulation of these responses in female mice. Indeed, documenting oral BPA exposure in mice is of great interest because transgenic mice could be used to unravel the molecular mechanisms underlying BPA effects. Female C57BL/6J strain mice, widely used in reproductive and transgenic studies, were thus studied following maternal exposure during the critical developmental periods for brain sexual differentiation. BPA was orally administered to dams at the two dose levels set by the European Food Safety Agency and US Environmental Protection Agency as protective measures: the ‘no observed adverse effect level’ (NOAEL) and the ‘100-fold lower tolerable daily intake’ (TDI) dose. This mimicked the major route of contamination for animals and humans. The levels of environmental exposure to BPA could actually be similar to the TDI dose (Vom Saal et al. 2007).

Materials and methods

Animals

C57BL/6J mice, purchased from Janvier (Le Genest, France), were group housed under a controlled photoperiod (12 h light:12 h darkness cycle – lights on at 0700 h), maintained at 22 °C, and fed with a standard
diet with free access to food and water. Estrus females were caged with males overnight, and successful mating was determined by the presence of a vaginal plug (day 1 of pregnancy). Animals were handled in accordance with the European guidelines for use of experimental animals (Decree 87-848, 86/609/ECC). Experiments were carried out accordingly, to minimize animal number and discomfort and were approved by the local Department of Animal Protection and Health.

**Treatments**

Studied females, exposed to BPA in utero and during the lactational period, were obtained from a second pregnancy due to the low survival rates of pups from primiparous mothers, who have poor maternal behavior. Survival of pups from multiparous females was higher than that of those from primiparous dams (87 vs 32%; n = 38). Second-pregnancy dams were administered, by oral gavage, sesame oil (vehicle group) or sesame oil containing BPA (Sigma–Aldrich) at 5 mg/kg body weight per day (BPA–NOAEL group) or BPA at 0.050 mg/kg body weight per day (BPA–TDI group). Treatments were carried out each day from gestational day 15 until postnatal day 21 (Picot et al. 2014). After weaning, female offspring were allowed to grow until analyses. Analyses were carried out on adult females (from 8 weeks of age), with the exception of body weight measurements and vaginal opening evaluation, which started earlier.

**Reproductive physiology**

Females from the three treatment groups were weighed weekly from postnatal day 15 until 90 days of age. They were examined daily for evidence of vaginal opening from postnatal day 25.

Vaginal smears flushed with physiological saline were taken daily from adult females for 3 weeks and colored with hematoxylin–eosin to identify the estrus cycle phase under a microscope.

Females were killed at the diestrus stage for measurement of E2 and LH and morphometric analysis of the genital tract, in order to avoid variability in these parameters due to the increasing levels of E2 in the proestrus/estrus phase. Blood samples were collected and the sera were extracted as described previously (Raskin et al. 2009). The circulating levels of E2 and LH were measured by RIA (ultra-sensitive estradiol kit; Beckman Coulter, Villepinte, France) and immunoassay (ultrasensitive Elisa kit; Endocrine Technologies, Newark, CA, USA) respectively.

Body length (nose to base of tail) and anogenital distance were measured. The ovaries and uterine horns of each animal were removed and weighed. Ovaries were fixed in Bouin’s buffer overnight at 4 °C, washed with ethanol, paraffin-embedded, and sliced into 5 μm sections to carry out histological analyses. These sections were mounted and deparaffinized, stained with hematoxylin–eosin, covered with a coverslip, and qualitatively examined.

To evaluate the capacity of females to produce offspring, females from the three treatment groups were individually housed with age-matched males for 4 months and examined regularly for the presence of litters. Litter size and number of litters were recorded.

**Behavioral analyses**

Tests were conducted under red light illumination for 2 h after lights off and videotaped for analysis.

**Lordosis**

Females were ovariectomized under general anesthesia and received Silastic implants containing 50 μg of estradiol benzoate (Sigma–Aldrich) in 30 μl sesame oil (Raskin et al. 2009, 2012). Four to five hours before the test, females were given a s.c. injection of 1 mg progesterone (Sigma–Aldrich) in 100 μl sesame oil. The animals were tested twice, with a time interval of 1 week between tests. Sexually experienced males were used as partners. Tests ended when females had received 20 mounts from males. The subjects displayed a lordosis posture when the four paws were grounded: the hind region lifted and the back region arched. We calculated the lordosis quotient (lordosis number/number of mounts) for each subject in response to male mounting.

**Olfactory preference**

Mice were placed into an enclosed Plexiglas Y-maze without any stimuli, for 5 min on two consecutive days, to allow them to adapt to the apparatus. Females were tested for mate preference on the third day by placing an anesthetized receptive female and gonadally intact male in boxes, one at the distal end of each arm. The time spent sniffing each goal box was scored over the 5-minute test. Results are expressed as a percentage of total time spent sniffing male or female cues. The maze was cleaned with 10% ethanol between trials (Keller et al. 2006).

**Mounting and thrusting**

Females were ovariectomized under general anesthesia and received Silastic implants filled with 10 mg testosterone. Tests were conducted 2 weeks after testosterone implantation.
Females of each treatment group were individually placed in a cage with fresh bedding and allowed to adapt for 2 h. A receptive female, ovariectomized and primed with E₂ and progesterone, was then introduced in the cage. We recorded the number of mounts and pelvic thrusting movements of testosterone-implanted females during 30 min (Martel & Baum 2009).

Immunohistochemistry

Females were killed at the diestrus stage and transcardial perfusion was performed with a solution of 4% paraformaldehyde (PFA) in phosphate buffer (PB). Brains were postfixified overnight in 4% PFA–PB, cryoprotected in sucrose, and stored until analyses. Brains were sliced into coronal sections of 30 μm using a cryotome (Leica CM 300). The sections were blocked for 2 h with 5% normal goat serum (Sigma–Aldrich) in PBS–Triton 0.1%, then incubated with anti-ERα antibody (1:400; Santa Cruz Biotechnology) for 48 h, monoclonal anti-calbindin antibody (1:1000; Sigma–Aldrich) for 24 h, or rabbit anti-GNRH (1:10 000; Caldani et al. 1988) and sheep anti-kisspeptin antibody (1:2000; Franceschini et al. 2013) for 72 h.

ERα and calbindin immunostaining was carried out over a 2 h period using biotinylated goat anti-rabbit (1:500) or anti-mouse (1:250) antibodies (Vector Laboratories, Les Ulis, France) respectively. Bound antibodies were visualized by 1 h incubation with the streptavidin complex reagent (Vector Laboratories), followed by color development with the chromogenic substrate 3,3-diaminobenzidine tetrahydrochloride (Sigma–Aldrich).

Kisspeptin and GNRH dual-label immunofluorescence was carried out using an Alexa Fluor 488-conjugated donkey anti-sheep secondary antibody (1:500; Life Technologies–Invitrogen) and a CY3 donkey anti-rabbit secondary antibody (1:500; Jackson ImmunoResearch, Montlucon, France) for kisspeptin and GNRH immunostaining, respectively, for 2.5 h at room temperature. After several rinses in PBS, the sections were immersed in a Hoechst stain bath (2 μg/ml in water; Invitrogen) for 2 min, rinsed in water (two baths, 5 min each), dried, mounted in Fluoromount-G (Southern Biotechnology, Birmingham, AL, USA) under a coverslip, and stored at 4°C in the dark.

Analysis and quantification of immunohistochemical studies

Kisspeptin cell numbers within each of the three subdivisions of the rostral periventricular area of the third ventricle (RP3V) were analyzed by counting the number of kisspeptin immunoreactive cell bodies on anatomically matched sections identified using the Mouse Brain Atlas of Paxinos & Franklin (2001) as a guide to define the anteroventral periventricular nucleus (plates 28–29; two sections per animal) and the preoptic periventricular nucleus, divided into the rostral region (plate 30; four sections per animal) and caudal region (plates 31–32, four sections per animal) (Clarkson & Herbison 2006). The quantification of GNRH immunoreactive cell bodies was carried out on four anatomically matched section selected at the level of the rostral preoptic area. In addition, each GNRH neuron was evaluated for close apposition to a kisspeptin fiber using a confocal microscope (Zeiss, LSM 700). The kisspeptin fiber was considered to be in close apposition if it was directly adjacent to the GNRH neuron cell body and/or the proximal dendrite was in the same plane of focus. Anatomically matched sections were also selected at the level of the middle arcuate nucleus for the quantification of kisspeptin fiber density (plate 45; one section per animal) by voxel counts on a set of ten serial image planes (z step distance = 1 μm) as described by Losa et al. (2011).

To assess calbindin and ERα immunoreactivity, sections were scanned using a high-resolution Nanoszoomer Hamamatsu scanner (Hamamatsu Corporation). The Mouse Brain Atlas was also used to define the sexually dimorphic cluster of calbindin immunoreactive neurons located in the medial preoptic area (plate 31; one section per animal) and ERα immunoreactive neurons in the medial preoptic area (plate 29), the ventromedial and arcuate nuclei (plate 44; one section per animal). They were compared across animal groups by tallying the number of labeled cells in each region.

Statistical analyses

Data were expressed as means ± s.e.m. Two-way ANOVA was used to analyze the main effects of treatment and experience, with Tukey tests for post-hoc comparisons. One-way ANOVA or Student’s t-tests were used for unpaired data. A χ²-test was used to analyze the percentage of females displaying the different behaviors studied. P values < 0.05 were considered to be significant.

Results

Developmental exposure to BPA at the TDI dose delayed the time of vaginal opening and increased levels of circulating E₂

The average age of vaginal opening was significantly later in the female group exposed to BPA at the TDI dose (BPA–TDI)
than in females exposed to vehicle or BPA at the NOAEL dose (BPA–NOAEL) (31.1 ± 0.4 vs 29.4 ± 0.3 for vehicle and 29.6 ± 0.3 for BPA–NOAEL; \( F_{(2, 140)} = 7.48, P < 0.05 \)). The growth curves for body weight between 15 and 90 days of age were, however, similar between vehicle and BPA-exposed groups (Fig. 1A). Body length (85.43 ± 0.42, 85.30 ± 0.44, and 84.60 ± 0.69 mm for vehicle, BPA–TDI, and BPA–NOAEL groups respectively) and anogenital distance (5.61 ± 0.10, 5.81 ± 0.10, and 5.76 ± 0.12% of body length for vehicle, BPA–TDI, and BPA–NOAEL groups respectively) were also similar for the three treatment groups.

Adult females had a similar average length of estrus cycle (Fig. 1B) and total length of each estrus cycle stage (Fig. 1C), regardless of BPA dose. Ovarian and uterine weights at the diestrus stage were also unchanged by BPA at TDI or NOAEL dose are expressed as means ± S.E.M. (n = 19–24 females per treatment group). (D) Representative histology of the ovary in females exposed to vehicle or BPA at TDI or NOAEL dose. Primary follicles (black arrows), atretic follicles (#), and corpora lutea ($) were observed in each treatment group. Scale bar = 100 μm.
exposure (Table 1). Histological analysis of ovary sections showed the presence of follicles, at different stages of folliculogenesis, and corpora lutea, indicating that ovulation occurred for all treatment groups (Fig. 1D). The levels of circulating E_2_ were significantly increased in the BPA–TDI group by comparison with females exposed to the vehicle (Table 1). Measurement of circulating levels of LH showed no significant differences in the means between the three treatment groups (Table 1), although slightly increased levels (2–2.86 ng/ml) were found in four of the ten females exposed developmentally to the BPA–TDI dose.

Analysis of female fertility showed that females exposed to vehicle or BPA during development were able to produce offspring as a result of mating tests lasting for 4 months. There was no significant difference in the number of litters (3.67±0.33, 3.67±0.67, and 3±0.00 for vehicle, BPA–TDI, and BPA–NOAEL groups respectively) and litter size (7.19±0.96, 8.20±0.32, and 6.89±0.44 for vehicle, BPA–TDI, and BPA–NOAEL groups respectively).

Table 1: Effects of developmental exposure to BPA on ovary and uterine weights, expressed as percentage of body weight (BW), as well as circulating levels of estradiol and LH in adult females at the diestrus stage. Values are given as means ± S.E.M. (n=10–21 females per treatment group)

<table>
<thead>
<tr>
<th>Treatments</th>
<th>Ovaries weight (% BW)</th>
<th>Uterine weight (% BW)</th>
<th>Estradiol levels (pg/ml)</th>
<th>LH levels (ng/ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Vehicle</td>
<td>0.0147±0.0005</td>
<td>0.104±0.0042</td>
<td>14.83±2.054</td>
<td>0.78±0.141</td>
</tr>
<tr>
<td>BPA–TDI</td>
<td>0.0144±0.0004</td>
<td>0.100±0.0040</td>
<td>24.09±3.082*</td>
<td>1.37±0.304</td>
</tr>
<tr>
<td>BPA–NOAEL</td>
<td>0.0127±0.0007</td>
<td>0.107±0.0076</td>
<td>17.00±2.425</td>
<td>1.09±0.302</td>
</tr>
</tbody>
</table>

*P<0.05 compared with the vehicle group.

Developmental exposure to BPA–TDI increased lordosis quotient in naive females

In rodents, mating is induced by olfactory cues. We therefore analyzed whether developmental exposure to BPA affected female sexual behavior and olfactory preference.

Lordosis behavior was measured in adult females, which were ovariectomized and primed with E_2_ and progesterone, in response to the mounting of a stud male. Lordosis behavior was displayed by similar percentages of naive (Test 1) and experienced females (Test 2) who had been exposed during development to vehicle or BPA at NOAEL or TDI dose (Fig. 2A). Two-way ANOVA showed no effect of exposure (F_{(2, 41)}=1.39, P=0.26), but a significant effect of experience (F_{(1, 41)}=34.86, P<0.0001) (Fig. 2B). There was an interaction between exposure and experience (F_{(2, 41)}=3.33, P=0.046), indicating that females exposed to BPA–TDI exhibited higher sexual behavior than both vehicle- and NOAEL–BPA-exposed females, but only when they were at their first sexual experience.

In olfactory preference tests using intact males and receptive females, two-way ANOVA showed a significant effect of stimulus (F_{(1, 29)}=9.33, P=0.005) with females displaying a preference for intact males over receptive females in all three groups (Fig. 2C). The total time devoted to chemoinvestigation did not differ significantly between groups (around 100 s).

Developmental exposure to BPA did not masculinize the female brain

We investigated if exposure to BPA, particularly during the perinatal period, masculinized the behavior and neuroanatomy of female mice.

As testosterone is also needed during adulthood to activate male sexual behavior, females exposed to BPA or vehicle during development were ovariectomized during adulthood and received implants that released testosterone before testing their ability to exhibit a masculine behavior. Similar percentages of females from each group displayed mounting and thrusting behaviors toward receptive females (Fig. 3A). The numbers of mounts and thrusts were also similar between the three treatment groups, indicating no increase in the number of events in females exposed to BPA (Fig. 3B and C).

We analyzed a cluster of calbindin-D28 immunoreactive cells located in the medial preoptic area and corresponding to the rat sexually dimorphic nucleus known to be involved in male sexual behavior. E_2_, derived from the neural aromatization of testosterone, acts perinatally to masculinize this neuronal population resulting in more cells in males than in females (Gilmore et al. 2012). Developmental exposure to BPA had no effect on this cluster in the medial preoptic area as no difference in the number of calbindin immunoreactive cells was found between vehicle and BPA-exposed females (Fig. 4A and B).
Selective increase in kisspeptin immunoreactive cells in the rostral preoptic periventricular nucleus by BPA exposure

We examined whether exposure to BPA had an effect on the kisspeptin/GNRH system. GNRH neurons receive projections from kisspeptin neurons, which are direct targets of ER signaling and major gatekeepers of pubertal onset and adult female reproduction (Franceschini & Desroziers 2013). Kisspeptin neurons are localized in two hypothalamic regions: i) the RP3V including the anteroventral periventricular and the preoptic periventricular nuclei and ii) the arcuate nucleus. The kisspeptin

Figure 2
Sexual behavior and olfactory preference in adult female mice exposed to BPA during development. (A) Percentage of adult ovariectomized and hormone-primed females exhibiting lordosis behavior in response to mounting of stud males in Tests 1 and 2. (B) Lordosis quotient measured for each female in Tests 1 and 2. Data were expressed as means±S.E.M. (n=18–21 females per treatment group). ***P<0.001 compared with Test 1. (C) Time spent chemoinvestigating male vs female stimuli expressed as percentage of total time spent in chemoinvestigation. **P<0.01 compared with male stimulus in the same treatment group.

Figure 3
Mounting and thrusting behaviors displayed by adult female mice exposed to vehicle or BPA during development. (A) Percentage of adult ovariectomized females with testosterone-releasing implants exhibiting masculine behavior toward receptive females. Number of mounts (B) and thrusts (C) displayed toward receptive females. Data are expressed as means±S.E.M. (n=20–22 females per treatment group).
A population located in the RP3V contains more cells in females than in males (Clarkson & Herbison 2006, 2009).

The number of kisspeptin immunoreactive cells in the rostral preoptic periventricular nucleus was significantly higher in females exposed to BPA at TDI or NOAEL dose ($F_{(2, 15)} = 5.56$, $P < 0.05$; Fig. 5A and B). In the anteroventral periventricular and the caudal preoptic periventricular nuclei, we found no significant difference in the number of kisspeptin-immunoreactive cells between treatment groups (Fig. 5B). In the arcuate nucleus, there was no difference in the density of kisspeptin fibers between the three treatment groups (Fig. 6A).

**Figure 4**
Characterization of a cluster of calbindin immunoreactive cells in the medial preoptic area. (A) Representative immunostaining of a sexually dimorphic calbindin neuronal population in adult female mice exposed during the gestational and lactational periods to vehicle or BPA at the TDI or NOAEL dose. Scale bar = 100 μm. (B) Quantification of the number of calbindin immunoreactive cells. Data are expressed as means ± S.E.M. ($n = 4$ females per treatment group).

**Figure 5**
Characterization of kisspeptin system in adult female mice exposed to BPA during development. (A) Representative kisspeptin immunoreactivity in the rostral periventricular nucleus (rPen) in females exposed to vehicle or BPA at the TDI or NOAEL dose. Scale bar = 100 μm. (B) Quantification of the number of kisspeptin immunoreactive cells per slice in the anteroventral periventricular area (AVPV), rPen, and caudal periventricular nucleus (cPen). Data are expressed as means ± S.E.M. ($n = 6$ females per treatment group). **$P < 0.01$ compared with the vehicle group of the corresponding hypothalamic area.
the forebrain or in kisspeptin neurons induced female infertility and suppressed lordosis behavior (Ogawa et al. 1998, Wintermantel et al. 2006, Mayer et al. 2010). We investigated whether increased lordosis, kisspeptin immunoreactivity in the RP3V, and E2 levels were associated with changes in the hypothalamic expression of ERα in BPA-exposed females.

We quantified the number of ERα immunoreactive cells in the three main regions involved in the regulation of the hypothalamus–pituitary–gonad (HPG) axis and expression of female sexual behavior (the ventromedial hypothalamic nucleus, medial preoptic area, and arcuate nucleus). The three treatment groups showed no significant differences in ERα immunoreactivity (Fig. 7A and B).

**Discussion**

We investigated whether developmental exposure to BPA altered the sexual differentiation of mating behavior and brain circuits in C57BL/6j female mice. Analysis of sexual behavior showed that the lordosis behavior was not abolished and was even significantly higher at the TDI dose in naive females. Administration of testosterone to gonadectomized adult females did not trigger more mounting or thrusting behaviors in BPA-exposed females than in the vehicle group. Similarly, analysis of olfactory preference, another sexually dimorphic behavior, did not reveal any masculinizing or defeminizing effects of BPA in female mice because BPA-exposed females exhibited normal interest in chemoinvestigating males rather than females. These results contrast with known perinatal effects of E2. Administration of E2 to female neonates abolishes receptivity and elicits the male pattern of sexual behavior in adulthood (Levine & Mullins 1964). Similarly, females lacking α-fetoprotein, and thus exposed to the masculinizing effects of maternal estrogens, exhibit low lordosis behavior and increased mounting behavior during adulthood (Bakker et al. 2006).

The behavioral observations are supported by the results of the neuroanatomical analyses. We observed no increase in the number of calbindin immunoreactive neurons, a sexually dimorphic population highly sensitive to the masculinizing effects of perinatal E2. The number of kisspeptin cells in the RP3V, a neuronal population normally reduced by perinatal E2 (Kauffman et al. 2007, Gonzalez-Martinez et al. 2008), was even higher in BPA-exposed individuals. These data indicate that during the perinatal period, when endogenous ovarian estrogens are undetectable and exogenous estrogens have limited brain access (due to their binding to α-fetoprotein), BPA at
reference doses was unable to mimic the masculinizing and defeminizing effects of perinatal E_2 on the reproductive functions and behaviors in female mice. In accordance with this hypothesis, previous studies in rats reported unchanged (Kwon et al. 2000, Adewale et al. 2009, Monje et al. 2009, Ryan et al. 2010, Jones et al. 2011) or increased (Farabollini et al. 2002) female sexual behavior and unaltered volume of the rat sexually dimorphic nucleus following early exposure to BPA at doses equivalent or lower than the TDI dose (Takagi et al. 2004, He et al. 2012). However, as behaviors and brain circuits may exhibit different sensitivities to estrogen signaling, it is possible that our BPA exposure treatment may have masculinized/defeminized other behaviors and circuits not investigated in this study as reported for tyrosine-hydroxylase-expressing cells in the preoptic area and open-field activity in CD1 mice (Rubin et al. 2006).

At the neuroendocrine level, more RP3V kisspeptin-immunoreactive cells were observed in adult females exposed developmentally to BPA, regardless of the BPA dose. A significant increase was found in the preoptic periventricular nucleus and not in the two other RP3V subdivisions (anteroventral periventricular and caudal preoptic periventricular nuclei). The studies administering BPA at the TDI dose to neonatal rats did not detect any significant effect on kisspeptin immunoreactivity in the anteroventral periventricular nucleus (Patisaul et al. 2009, Losa-Ward et al. 2012). The observed increase in kisspeptin immunoreactive cells could be due to BPA-induced transcriptional regulation of Kiss1 because increased hypothalamic levels of Kiss mRNAs were observed following oral exposure to high doses (25–50 mg/kg) of gestational and lactational BPA in CD1 mice (Xi et al. 2011).

There is now accumulating evidence indicating the involvement of kisspeptin neurons located in the RP3V nucleus in the positive feedback exerted by E_2 to trigger the preovulatory surge of LH during proestrus, while those of the arcuate nucleus integrate the negative signal of E_2. BPA treatment had no effect on kisspeptin immunoreactive fiber density in the arcuate nucleus or on the number of GNRH neurons in the preoptic area. It is therefore possible that increased E_2 levels result, at least partly, from a change in the balance between negative- and positive-feedback responses at the hypothalamic portion of the HPG axis.

Figure 7
Quantification of the number of ER\(\alpha\) immunoreactive cells in hypothalamic areas. (A) Representative ER\(\alpha\) immunolabeling in the medial preoptic area (MPOA), ventromedial hypothalamus (VMH), and arcuate nucleus (ARC) of adult female mice exposed during development to vehicle or BPA at the TDI or NOAEL dose. Scale bar = 50 \(\mu\)m. (B) Number of ER\(\alpha\) immunoreactive cells. Values are expressed as means ± S.E.M. (\(n=3–5\) females per treatment group).
although a direct effect of BPA on follicular steroidogenesis (Xi et al. 2011) cannot be excluded.

The observations that females developmentally exposed to BPA exhibited normal estrus cyclicity, ovarian and uterine weights, and were able to ovulate and produce offspring indicate that BPA-induced alterations did not interfere with the normal functioning of the HPG axis. A normal length of estrus cycle was also described in studies using ICR and CD1 mice exposed to BPA during the postnatal period (Nikaido et al. 2005, Nah et al. 2011) or during both gestation and lactation (Tyl et al. 2008) whereas BPA exposure during the prenatal period has been shown to increase estrus cycle length (Honma et al. 2002, Nikaido et al. 2004). This indicates again that the time window of BPA exposure could result in different effects. It is, however, important to note that our fertility studies were carried out on young adult females and deleterious effects of developmental exposure to BPA on reproductive capacity may be observable at advanced ages (Cabaton et al. 2011).

Exposure to oral BPA at the TDI dose from gestational day 15 until weaning induced delayed vaginal opening. Other studies using mouse strains with various exposure routes and BPA doses have reported advanced vaginal opening or first vaginal estrus associated with BPA exposure during the prenatal period (Nikaido et al. 2004) or unaltered vaginal opening when mice were exposed to BPA from pre-breeding until weaning (Tyl et al. 2008). Although advanced vaginal opening is described as the most common effect of early exposure to endocrine disrupters, it is important to note that delayed vaginal opening was observed with varying exposure periods or BPA doses. For instance, the same dose of BPA induced delayed vaginal opening in ICR mice exposed between postnatal days 15 and 18 (Nikaido et al. 2005) and early vaginal opening in CD1 females exposed during the prenatal period (Nikaido et al. 2004). Bourguignon et al. (2013) described early vaginal opening after neonatal exposure to diethylstilbestrol (DES) at 10 μg/kg per day and delayed vaginal opening at 1 μg/kg per day, indicating that precocity could be attributed to both peripheral and central effects of DES effects while delayed vaginal opening could be more related to neuroendocrine effects. Therefore, the discrepancy in BPA effects on the age at vaginal opening could be due to differences in the time of BPA exposure and/or the dose used.

The delayed time of vaginal opening in C57BL/6J females was not due to delayed body growth. The vaginal opening might represent one of the first processes triggered by increasing levels of postnatal E2. Further studies should investigate whether delayed vaginal opening influences the age at first estrus and pubertal onset in BPA-exposed females.

BPA induced a long-term increase in lordosis behavior and kisspeptin immunoreactivity in the RP3V. Interestingly, these two parameters are known to increase around puberty in a manner dependent on the postnatal rise in E2 release from the ovaries (Clarkson et al. 2009, Brock et al. 2011). As BPA exposure lasted until postnatal day 21, it is thus likely that BPA exerted its predominant effect during the postnatal/prepubertal period to reinforce the action of endogenous E2, most probably on the ERα signaling pathway. The importance of this pathway in the regulation of female reproduction and sexual differentiation of the brain and of behaviors is demonstrated by the infertile phenotype and reduced RP3V kisspeptin neuronal population and lordosis behavior in female mice lacking aromatase or neuronal ERα (Bakker et al. 2002, Wintermantel et al. 2006). The delayed vaginal opening observed in females exposed to BPA also favors the suggestion that BPA acts during the postnatal/prepubertal period as E2 can affect this process through the postnatal action of ERα in kisspeptin cells (Mayer et al. 2010). Although we did not detect any long-term effect of BPA exposure on ERα expression in the brain, it remains possible that BPA transiently influenced ERα expression or its signaling pathway during the postnatal/prepubertal period, reinforcing the feminizing developmental effects of endogenous E2.

The majority of BPA effects described here were found at the TDI and not the 100-fold higher NOAEL dose. Except for the increased number of kisspeptin cells in the RP3V, which was induced by both doses, increased sexual behavior and circulating levels of E2 and delayed vaginal opening were all observed only at the TDI dose. This adds arguments in favor of the emerging idea in the field of endocrine disruption that more effects of BPA are observed at low but relevant doses than at high doses (Welshons et al. 2003, Jenkins et al. 2011). Such non-monotonic responses, known for endogenous hormones such as E2, follow the dynamics of hormone receptor occupancy and saturation. Specific responses for E2 are observed in response to low physiological ranges below the concentration at which the receptor-binding site is half occupied, while no or only nonspecific effects not mediated by ER are observed at high ranges. It is possible that BPA at the lower reference dose exacerbated E2 effects by acting directly through ERα or indirectly through other receptors, which in turn regulate the ERα-signaling pathway.

In conclusion, gestational and lactational exposure to BPA enhanced lordosis behavior at the TDI dose and augmented kisspeptin immunoreactivity in the RP3V.
nucleus at both the NOAEL and TDI doses in C57BL/6J female mice. BPA at the TDI dose also delayed the time of vaginal opening and increased diestrus levels of E2. The comparison with known effects of perinatal E2 indicates that BPA at reference doses, in the absence of endogenous E2, did not affect sexual brain development during the perinatal period. BPA may act, in particular at the TDI dose, during the postnatal/prepubertal period to increase the action of ovarian E2 on the ERα signaling pathway.

The role of sex steroids in sexual differentiation of the nervous system has been well documented in many mammalian species. In addition, BPA pharmacokinetics in women, female monkeys, and mice seem to be similar (Taylor et al. 2011), although the characterization of BPA bioavailability still needs to be improved in humans. It is therefore possible that BPA triggers responses similar to those described in this study in females of other mammalian species.

Declarations of interest
The authors declare that there is no conflict of interest that could be perceived as prejudicing the impartiality of the research reported.

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