

Bisphenol A enhances kisspeptin neurons in anteroventral periventricular nucleus of female mice

Xiaoli Wang^{1,2}, Fei Chang², Yinyang Bai², Fang Chen², Jun Zhang³ and Ling Chen^{1,2}

¹State Key Laboratory of Reproductive Medicine and ²Department of Physiology, Nanjing Medical University, Hanzhong Road 140, Nanjing 210029, China

³MOE-Shanghai Key Laboratory of Children's Environmental Health, Xinhua Hospital, Shanghai Jiao Tong University School of Medicine, Shanghai 200092, China

Correspondence should be addressed to L Chen

Email
lingchen@njmu.edu.cn

Abstract

Bisphenol-A (BPA), an environmental estrogen, adversely affects female reproductive health. However, the underlying mechanisms remain largely unknown. We found that oral administration (p.o.) of BPA (20 µg/kg) to adult female mice at proestrus, but not at estrus or diestrus, significantly increased the levels of plasma E₂, LH and FSH, and *Gnrh* mRNA within 6 h. The administration of BPA at proestrus, but not at diestrus, could elevate the levels of *Kiss1* mRNA and kisspeptin protein in anteroventral periventricular nucleus (AVPV) within 6 h. In contrast, the level of *Kiss1* mRNA in arcuate nucleus (ARC) was hardly altered by BPA administration. In addition, at proestrus, a single injection (i.c.v.) of BPA dose-dependently enhanced the AVPV-kisspeptin expression within 6 h, this was sensitive to E₂ depletion by ovariectomy and an estrogen receptor α (ER α) antagonist. Similarly, the injection of BPA (i.c.v.) at proestrus could elevate the levels of plasma E₂, LH, and *Gnrh* mRNA within 6 h in a dose-dependent manner, which was blocked by antagonists of GPR54 or ER α . Injection of BPA (i.c.v.) at proestrus failed to alter the timing and peak concentration of LH-surge generation. In ovariectomized mice, the application of E₂ induced a dose-dependent increase in the AVPV-*Kiss1* mRNA level, indicating 'E₂-induced positive feedback', which was enhanced by BPA injection (i.c.v.). The levels of *Er α* (*Esr1*) and *Er β* (*Esr2*) mRNAs in AVPV and ARC did not differ significantly between vehicle- and BPA-treated groups. This study provides *in vivo* evidence that exposure of adult female mice to a low dose of BPA disrupts the hypothalamic–pituitary–gonadal reproductive endocrine system through enhancing AVPV-kisspeptin expression and release.

Key Words

- ▶ bisphenol-A (BPA)
- ▶ kisspeptin neurons
- ▶ estrogen (E₂)
- ▶ estrogen receptor (ER)
- ▶ hypothalamic–pituitary–gonadal (HPG) axis

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Introduction

The environmental estrogenic chemical bisphenol-A (BPA) is an industrial monomer used in the production of polycarbonates and epoxy resins. There is considerable

potential for human exposure to this compound from the lining of food cans, plasticware, and dental sealants. Studies in humans have also determined that BPA can be

measured in serum, urine, amniotic fluid, follicular fluid, placental tissue, and umbilical cord blood (Ikezuki *et al.* 2002). Estimates of oral exposure to BPA are 90–930 mg during the first hour following administration of dental sealant (Markey *et al.* 2002) and up to 6.3 mg/day from food cans (Brotons *et al.* 1995). Although many women are exposed to BPA worldwide, the reproductive health risks and complications of BPA exposure during adulthood have not been evaluated. Recently, the increased incidence of BPA exposure in humans has been associated with reproductive diseases including recurrent miscarriage (Sugiura-Ogasawara *et al.* 2005), premature delivery (Cantonwine *et al.* 2010), and polycystic ovary syndrome (Kandaraki *et al.* 2011). In animal experiments, BPA exposure has been shown to alter estrous cyclicity (Rubin *et al.* 2001, Lee *et al.* 2013), impair reproductive capacity (Cabaton *et al.* 2011), and disturb hormonal levels (Rubin *et al.* 2001, Fernandez *et al.* 2009). However, the underlying mechanisms remain largely unknown.

In the female, the regular estrous cycle (EC) is controlled by the pulse mode and surge mode of gonadotrophin-releasing hormone (GNRH)/luteinizing hormone (LH) release, which is regulated by the feedback action of estradiol (E₂) secreted from the ovarian mature follicles (Adachi *et al.* 2007). However, GNRH neurons do not express estrogen receptor α (ER α (*ESR1*)), they accept E₂ signaling largely from ER α -expressing neurons elsewhere within the hypothalamus, such as the kisspeptin neurons in the arcuate nucleus (ARC) and anteroventral periventricular nucleus (AVPV) (Kinoshita *et al.* 2005). The kisspeptin neurons are known to send projections to the preoptic area (POA) in close proximity to GNRH neurons. Approximately, 90% of GNRH neurons express G protein-coupled receptor 54 (GPR54) which is intensely activated by kisspeptin (Pinilla *et al.* 2012). The kisspeptin binding to GPR54 enhances the release of GNRH, presumably through a Ca²⁺-mediated process (Strock & Diverse-Pierluissi 2004, Stathatos *et al.* 2005). The GNRH antagonist acyline blocks the ability of kisspeptin to stimulate gonadotrophin release in the rat, mouse, and primate (Gottsch *et al.* 2004). The response of the *Kiss1* gene to E₂ is opposite for the ARC and AVPV. E₂ decreases *Kiss1* mRNA in ARC, termed 'E₂-induced negative feedback', whereas increases *Kiss1* mRNA in AVPV, termed 'E₂-induced positive feedback' (Dungan *et al.* 2006). Many pieces of evidence indicate that kisspeptin neurons in AVPV, as a target of E₂ positive feedback, are considered to regulate GNRH/LH-surge generation (Ohkura *et al.* 2009). The ARC kisspeptin neuronal population, as a target of E₂ negative feedback, is considered to be involved in the

regulation of GNRH pulse generation (Ohkura *et al.* 2009). Administration of exogenous kisspeptin to the brain is able to enhance GNRH expression and release, while GPR54 antagonists block the response of GNRH neurons to exogenous kisspeptin in female rats. Given the estrogenicity of BPA, it can presumably disturb or mimic the action of E₂ to affect the E₂-induced feedback regulation in kisspeptin neurons, which leads to abnormal EC and reproductive endocrine signaling.

BPA mimics the actions of endogenous E₂ by binding to membrane or nuclear ERs (Washington *et al.* 2001). By its competitive action to endogenous E₂ (Fang *et al.* 2000), BPA has been reported to disturb the hypothalamic-pituitary-gonadal (HPG) endocrine system, resulting in the impairment of reproductive function (Markey *et al.* 2002). However, BPA is considered to be a very weak environmental estrogen, because its affinity for ERs is 10 000- to 100 000-fold weaker than that of estradiol (Okada *et al.* 2008). Results from recent studies have revealed that BPA can stimulate cellular responses at very low concentrations via rapid induction of calcium uptake and nongenomic cell signaling (Quesada *et al.* 2002, Wozniak *et al.* 2005) involving membrane-associated forms of the ERs (Wetherill *et al.* 2007), in addition to the effects initiated by binding of BPA to the classical nuclear or genomic ERs (Welshons *et al.* 2006).

The objectives of this study were to dissect the influence of BPA on the kisspeptin-GPR54 and HPG reproductive endocrine axis that is well known to be critical for control of ovulation and estrus cycle. Human exposures are most likely to be through the oral route, thus adult female mice were treated with a single oral administration (p.o.) of BPA (20 μ g/kg) at proestrus, estrus, or diestrus respectively. To determine whether BPA acts directly in AVPV- and ARC-kisspeptin neurons, we carried out a single intracerebroventricular (i.c.v.) injection of BPA in proestrus or diestrus female mice. Within 6 h after administration BPA (p.o.) or (i.c.v.), AVPV- and ARC-kisspeptin expression; GPR54-mediated GNRH expression; secretion of LH, FSH, and E₂; generation of LH-surge were examined to evaluate the acute effects of BPA on kisspeptin neurons and HPG reproductive endocrine signaling. Finally, we examined the influence of BPA on E₂-enhanced AVPV-kisspeptin expression and the involvement of ER α and ER β to explore the possible targets and molecular mechanisms underlying the actions of BPA in the kisspeptin-GPR54 mediated HPG axis. Our results indicate that the exposure of adult female mice to a low dose of BPA stimulates AVPV-kisspeptin neurons to disrupt the HPG reproductive endocrine axis.

Materials and methods

Animals

Studies were carried out according to the protocols for animal use (Institute of Laboratory Animal Resources 1996). The use of animals was approved by Animal Care and Ethical Committee of Nanjing Medical University. All efforts were made to minimize animal suffering and to reduce the number of animals used. Twelve-week-old female ICR mice (Oriental Bio Service, Inc., Nanjing, China), weighing $\sim 30 \pm 2$ g, were used at the beginning of this study. The animals were housed in stainless steel cages with wood bedding to minimize additional exposure to endocrine-disrupting chemicals (temperature 23 ± 2 °C, humidity $55 \pm 5\%$, and 12 h light:12 h darkness cycle) in Animal Research Center of Nanjing University. They had free access to food and water before and after all procedures.

Examination of ECs

EC was daily examined by vaginal lavage with 0.9% saline at 0800–0900 h. The fluid was spotted thinly on a microscope slide, and the dried slides were stained with toluidine blue. The EC stage was determined by microscopic examination. We examined vaginal cytology for a total of 15 days. According to the types of vaginal epithelium cells (leukocyte cells, nucleated cells, and cornified cells), diestrus (D), estrus (E), and proestrus (P) were determined in all mice, as described by Goldman *et al.* (2007).

Exposure to BPA

Oral administration (p.o.) of BPA After 3–4 regular ECs had been observed, the mice were treated with BPA at 20 $\mu\text{g}/\text{kg}$ body weight per day. BPA (>99% purity; Sigma–Aldrich, Inc.) was dissolved in 200 μl corn oil. Control mice were treated with corn oil at the same volume. The dose was chosen on the basis of a recent report (Lee *et al.* 2013) that the oral administration of BPA at 1 or 100 $\mu\text{g}/\text{kg}$ observably affects the duration of estrus phase and the levels of reproductive hormones in adult female Sprague–Dawley rats. The lowest observed adverse effect level (LOAEL) for BPA established by the U.S. Environmental Protection Agency (EPA) is 50 mg/kg body weight per day, and the U.S. EPA reference dose (and the U.S. Food and Drug Administration acceptable daily intake) is 50 $\mu\text{g}/\text{kg}$ body weight per day (U.S. EPA 1993).

i.c.v. injection of BPA BPA was dissolved in DMSO and then in 0.9% saline to a final concentration of 0.5% DMSO. To determine the direct action of BPA in kisspeptin neurons, BPA (0, 0.02, 0.2, 2.0, 20.0, and 200.0 nM/3 μl) was injected into the right lateral ventricle (anteroposterior + 0.2 mm, lateral + 0.8 mm, dorsoventral 2.5 mm) using a stepper-motorized microsyringe (Stoelting, Wood Dale, IL, USA) with a rate of 1 $\mu\text{l}/\text{min}$. These doses were chosen because treatment with BPA (100 pM–100 nM) can decrease viability of mouse granule cells (Xu *et al.* 2002). Mice that had received an infusion of the same volume of vehicle served as the control group.

Ovariectomy

Ovaries were removed from the mice anesthetized by the intraperitoneal (i.p.) injection of chloral hydrate (400 mg/kg). Briefly, the anesthetized animal's ventral surface was shaved and cleaned and the ovaries were dissected out through midline incisions in the skin and abdominal musculature. After removal of ovary, the muscle was sutured and the skin closed with sterile wound clips.

Treatment with drugs

Treatment with E₂ i) E₂-treated ovariectomized (OVX)-mice received a subcutaneous implant at the time of ovariectomy of a Silastic brand (Dow Corning, Midland, MI, USA) capsule containing 0.625 μg E₂ (in sesame oil, Sigma–Aldrich Corp.) as described by Christian *et al.* (2005). This E₂-treatment paradigm results in constant physiological levels of E₂ in the circulation beginning on day 2. ii) On the seventh day after ovariectomy, the mice were given the subcutaneous (s.c.) injection of E₂ dissolved in sesame oil at various doses of 0, 0.1, 5, 20, 100, 200, and 400 $\mu\text{g}/\text{kg}$ for 2 consecutive days to examine the E₂-induced AVPV-kisspeptin expression.

Treatment with receptor antagonists The ER α antagonist 1,3-bis (4-hydroxyphenyl)-4-methyl-5-[4-(2-piperidinyloxy)phenol]-1H-pyrazole dihydrochloride (MPP), the ER β antagonist 4-[2-phenyl-5,7-bis(trifluoromethyl)pyrazolo(1,5-a)pyrimidin-3-yl]phenol (PHTPP, Tocris Cookson Ltd., Avonmouth, UK), and the GPR54 antagonist peptide 234 (p234, Sigma–Aldrich Corp.) were dissolved in ethanol, and then in 0.9% saline (final ethanol concentration was <0.5%). MPP (50 nmol/mouse; Harrington *et al.* 2003), PHTPP (50 nmol/mouse; Harrington *et al.* 2003, Compton *et al.* 2004), and p234 (5 nmol/mouse; Roseweir *et al.* 2009) were injected

(i.c.v., 3 μ l/mouse) at 30 min before injection of BPA using a stepper-motorized microsyringe at a rate of 0.2 μ l/min. The drugs were prepared freshly on the day of experiment. Control mice were given an equal volume of vehicle.

Measurement of plasma hormones

Blood samples were taken by jugular venipuncture under anesthetized conditions with chloral hydrate (400 mg/kg, i.p.) in 1530–1600 h at diestrus (D), proestrus (P) and estrus (E) respectively. Plasma (total 300 μ l per mouse) was separated by centrifugation at 4 °C and stored at –80 °C until assay. Hormonal levels were measured using a RIA kit provided by the National Hormone and Peptide Program (Baltimore, MD, USA). The intra- and inter-assay coefficients of variation are 5.5 and 8.9% for LH, 4.3 and 10.3% for FSH, and 6 and 5.8% for E₂ respectively. The lowest detectable levels were 0.2 ng/ml for LH, 0.39 ng/ml for FSH, and 2 pg/ml for E₂ respectively.

Observation of LH-surge

At proestrus, mice were anesthetized with pentobarbital (3 mg/100 μ l, i.p.) (Clarkson *et al.* 2008) and blood (100 μ l per time) was collected at 1300–1700 h by an automatic blood sampling system (DraQ, EICOM, Kyoto, Japan) as described previously (Ishii *et al.* 2013). An equivalent volume (100 μ l) of heparinized saline (5 U/ml normal saline; CP Pharmaceuticals Ltd, Wrexham, UK) was replaced through the atria cannula after each blood collection. Plasma was separated by centrifugation and stored at –80 °C until assay.

RT-qPCR

The regions of the AVPV, ARC, and POA at proestrus or diestrus at 6 h after BPA administration (p.o.) or (i.c.v.) were collected from the frozen slices (2-mm thick) and stored at –80 °C until assay. The primer sequences of *Kiss1*, *Gnrh*, *Erf1*, *Erf2*, and *Gapdh* mRNA were designed and RT-qPCR was performed as described previously (Xi *et al.* 2011). Total RNA was extracted using Trizol reagent kit (Invitrogen) according to the manufacturer's instructions. Purified RNA with an A260:A280 ratio of 1.8–2.0 was used. RNA (1 μ g) was reverse transcribed using high-capacity cDNA of the reverse transcription kit RT (TaKaRa Biotechnology CO., Ltd, Shanghai, China) in accordance with the instructions. PCRs were conducted using a LightCycler FastStart DNA Master SYBR Green I kit and an ABI Prism 7300 Sequence Detection System (Applied Biosystems). The copy number of transcripts was

calculated in reference to the parallel amplifications of known concentrations of the respective cloned PCR fragments. Standard curves were constructed and amplification efficiencies were between 0.9 and 0.95. The relative expression of genes was determined using the 2^{– $\Delta\Delta$ Ct} method with normalization to *Gapdh* expression. On the basis of melting curve analyses, there were no primer-dimers or secondary products formed. There was only one PCR product amplified for each set of primers.

Immunohistochemistry of kisspeptin

Mice at 1530–1600 h of proestrus were anaesthetized with ketamine/xylazine and 4% paraformaldehyde was perfused intraventricularly. The brains were post-fixed overnight in 4% paraformaldehyde, and then were transferred gradually into 15 and 30% sucrose until they settled. The sections (40 μ m thick) through the AVPV (0.4 mm anterior to bregma and 0.7 mm posterior to bregma) (Mayer & Boehm 2011) were cut using a cryostat. Free-floating sections were incubated in 0.5% sodium metaperiodate for 20 min and then in 1% sodium borohydride for 20 min. They were pre-incubated with 1% normal fetal goat serum (blocking solution) for 60 min, and then incubated in rabbit anti-kisspeptin polyclonal antibody (1:1000, Millipore, Billerica, MA, USA) for 24 h at 4 °C as described previously (Penatti *et al.* 2011, Quennell *et al.* 2011). The sections were treated with biotin-conjugated goat anti-rabbit IgG (1:400; Vector Laboratories, Burlingame, CA, USA) for 2 h at 37 °C. The immunoreactivity was visualized with the standard avidin–biotin complex reaction with Ni-3, 3-diaminobenzidine (DAB, Vector Laboratories). The sections were mounted on Super-Frost Plus slides (VWR Scientific, West Chester, PA, USA), air-dried, and dehydrated in graded ethanol, and cleared with Citrosolv, after which cover slips were applied. In every experiment, incubation of sections without the primary antibodies served as negative controls for immunohistochemistry. Kisspeptin-immunoreactive (kisspeptin⁺) cells in AVPV were counted using a conventional light microscope (Olympus PD70) with a 40 \times objective. The kisspeptin⁺ cells were counted in eight sections of AVPV per brain. The values obtained from eight mice were averaged to provide each group mean value.

Western blotting analysis

The regions of the AVPV at 1530–1600 h of proestrus and the regions of the ARC at 1530–1600 h of diestrus were collected from the frozen slices (2 mm thick) of brain using

16-gauge stainless steel tubing, and then stored at -80°C until assay. The amount of protein was determined using a bicinchoninic acid (BCA) protein assay kit (Pierce, Rockford, IL, USA). Protein (50 μg) was separated by SDS-PAGE and transferred to membranes. The membranes were incubated with rabbit anti-kisspeptin polyclonal antibody (1:1000, Millipore) at 4°C overnight. After several PBS rinses, the membranes were incubated with HRP-conjugated goat anti-rabbit IgG (1:5000, Millipore) for 2 h, and then were developed using an enhanced chemiluminescence detection kit (Millipore). Western blotting bands were scanned and analyzed with image analysis software (Image J, NIH).

Statistical analysis

Data were retrieved and processed with the software Microcal Origin 6.1 (OriginLab, Northampton, MA, USA). The group data were expressed as the mean \pm s.e.m. The experiment data were evaluated by Bartlett's test to examine the equality of variances. After the homogeneity of variance was determined, statistical differences among values for individual groups were determined by *t*-test or analysis of one- or two-way ANOVA, followed by the Bonferroni *post hoc* test. Statistical analysis was performed using Stata7 software (STATA Corporation, Colledge Station, TX, USA). Differences at $P < 0.05$ were considered statistically significant.

Results

Influence of BPA administration (p.o.) on activation of HPG axis and kisspeptin neurons

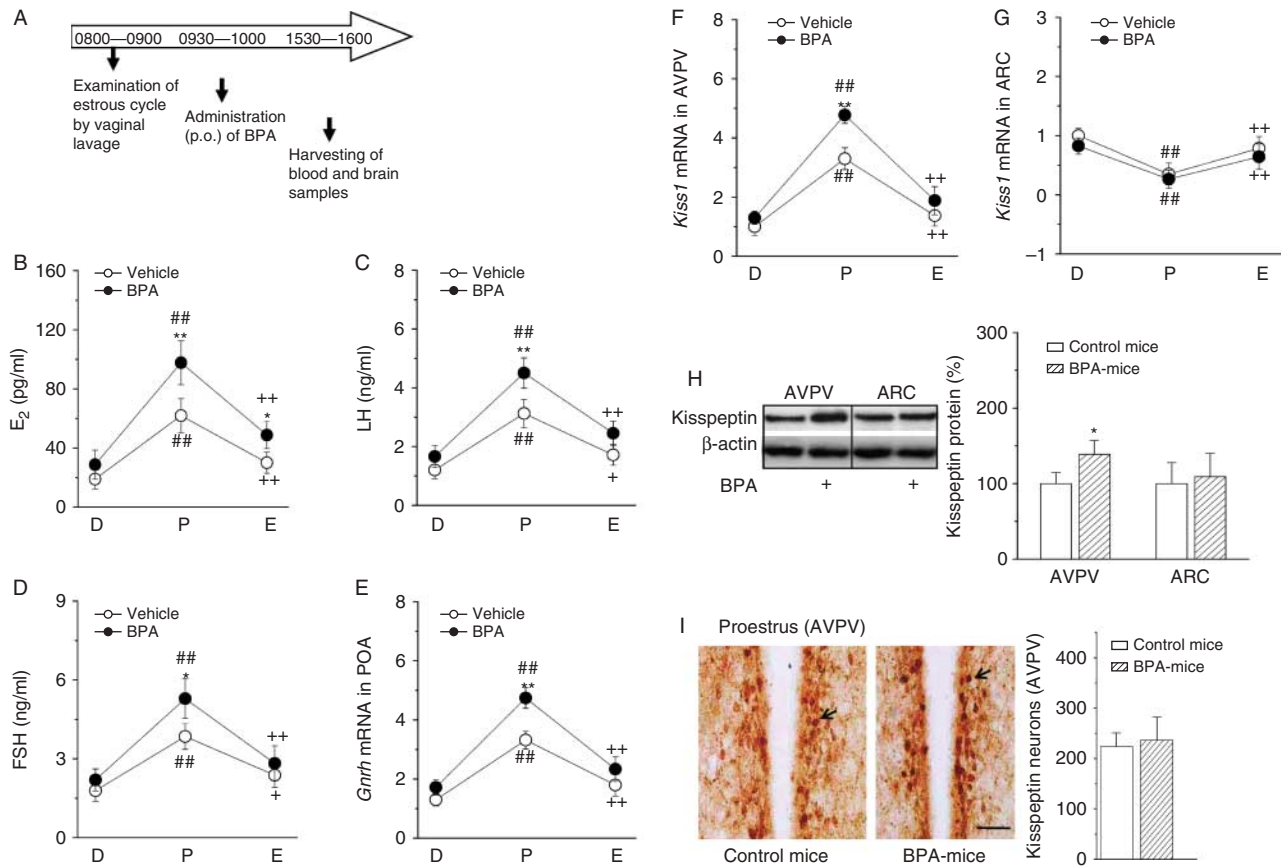
BPA was administered orally (p.o.) (20 $\mu\text{g}/\text{kg}$) at diestrus, proestrus and estrus, respectively (see the time chart for the experimental procedure; Fig. 1A). At 6 h after administration of BPA, the levels of reproductive hormones and the *Gnrh* mRNA and the kisspeptin expression were examined. Two-way ANOVA analysis showed significant effects of EC, BPA administration (BPA), and their interaction (EC \times BPA) on the levels of plasma E_2 (EC: $F_{(2,42)} = 1844.38$, $P < 0.01$; BPA: $F_{(1,42)} = 755.63$, $P < 0.01$; EC \times BPA: $F_{(2,42)} = 100.87$, $P < 0.01$; Fig. 1B), LH (EC: $F_{(2,42)} = 882.64$, $P < 0.01$; BPA: $F_{(1,42)} = 147.59$, $P < 0.01$; EC \times BPA: $F_{(2,42)} = 54.39$, $P < 0.01$; Fig. 1C), FSH (EC: $F_{(2,42)} = 462.46$, $P < 0.01$; BPA: $F_{(1,42)} = 41.95$, $P < 0.01$; EC \times BPA: $F_{(2,42)} = 31.10$, $P < 0.01$; Fig. 1D). In control mice, the level of plasma E_2 was higher at proestrus ($P < 0.01$; Fig. 1B) or estrus ($P < 0.01$) than that at diestrus. The administration of BPA further increased the levels of

plasma E_2 at proestrus ($P < 0.01$) and estrus ($P < 0.01$), but not at diestrus ($P > 0.05$), compared with controls. In contrast, the elevation of plasma LH (Fig. 1C) or FSH (Fig. 1D) was observed at proestrus in control mice, compared with estrus and diestrus ($P < 0.01$). At proestrus, the administration of BPA could elevate the levels of LH ($P < 0.01$) and FSH ($P < 0.01$), but at estrus or diestrus it could not ($P > 0.05$). Similarly, the expression of *Gnrh* was affected by EC ($F_{(2,42)} = 198.13$, $P < 0.01$; Fig. 1E), BPA administration ($F_{(1,42)} = 26.61$, $P < 0.01$), and their interaction ($F_{(2,42)} = 13.27$, $P < 0.01$). In comparison with control mice, the level of *Gnrh* mRNA in POA was markedly elevated by administration of BPA at proestrus ($P < 0.01$; Fig. 1E), but not at estrus and diestrus ($P > 0.05$).

The levels of *Kiss1* mRNA in AVPV and ARC were altered during EC (AVPV: $F_{(2,42)} = 1216.24$, $P < 0.01$; Fig. 1F, ARC: $F_{(2,42)} = 154.25$, $P < 0.01$; Fig. 1G respectively). Two-way ANOVA analysis showed the influence of BPA administration and interaction of EC \times BPA on AVPV-*Kiss1* mRNA (BPA: $F_{(1,42)} = 347.58$, $P < 0.01$; EC \times BPA: $F_{(2,42)} = 126.0$, $P < 0.01$), but not on ARC-*Kiss1* mRNA (BPA: $F_{(1,42)} = 0.64$, $P > 0.05$; EC \times BPA: $F_{(2,42)} = 1.52$, $P > 0.05$). The level of AVPV-*Kiss1* mRNA at proestrus was higher than those at estrus ($P < 0.01$; Fig. 1F) and diestrus ($P < 0.01$), and was further elevated by BPA administration ($P < 0.01$). The level of ARC-*Kiss1* mRNA at proestrus was less than those at estrus ($P < 0.01$; Fig. 1G) and diestrus ($P < 0.01$), which were hardly affected by BPA administration ($P > 0.05$). Similarly, the level of AVPV-kisspeptin protein at proestrus was increased $\sim 40\%$ by BPA administration ($P < 0.05$; Fig. 1H), whereas the administration of BPA did not alter the level of ARC-kisspeptin protein at diestrus ($P > 0.05$). In addition, at 6 h after BPA administration, the number of AVPV-kisspeptin positive (kisspeptin⁺) cells at proestrus was not altered compared with controls ($P > 0.05$, $n = 8$; Fig. 1I), whereas the AVPV-kisspeptin⁺ cells showed darker staining than that in control mice. The results indicate that exposure to low-dose BPA at proestrus is able to enhance the activation of the HPG axis and AVPV-kisspeptin neurons.

Influence of BPA administration (i.c.v.) on kisspeptin expression and E_2 involvement

To determine whether BPA acts directly on AVPV-kisspeptin neurons, a single i.c.v. injection of BPA at various doses of 0.02, 0.2, 2, 20, and 200 nM/mouse was given at proestrus and diestrus respectively (see the time chart of experimental procedure; Fig. 2A). At 6 h after BPA injection (i.c.v.), the AVPV and ARC regions were harvested

**Figure 1**

Influence of BPA administration (p.o.) on the activation of HPG axis and kisspeptin neurons. (A) Time chart of the experimental procedure. The numbers in the arrow indicate the times of experimental procedures. BPA or vehicle (control) was administered at diestrus (D), proestrus (P), or estrus (E) respectively. Blood and brain samples were harvested at 6 h after oral administration of BPA. (B, C, D and E) Each point represents the mean value of E₂, LH, and FSH levels, and *Gnrh* mRNA (expressed relative to the control value) in POA. **P*<0.05 and ***P*<0.01 vs controls. ###*P*<0.01 vs diestrus mice; +*P*<0.01 and ++*P*<0.01 vs proestrus mice (two-way ANOVA). (F) Levels of *Kiss1* mRNA in AVPV are expressed relative to the control value.

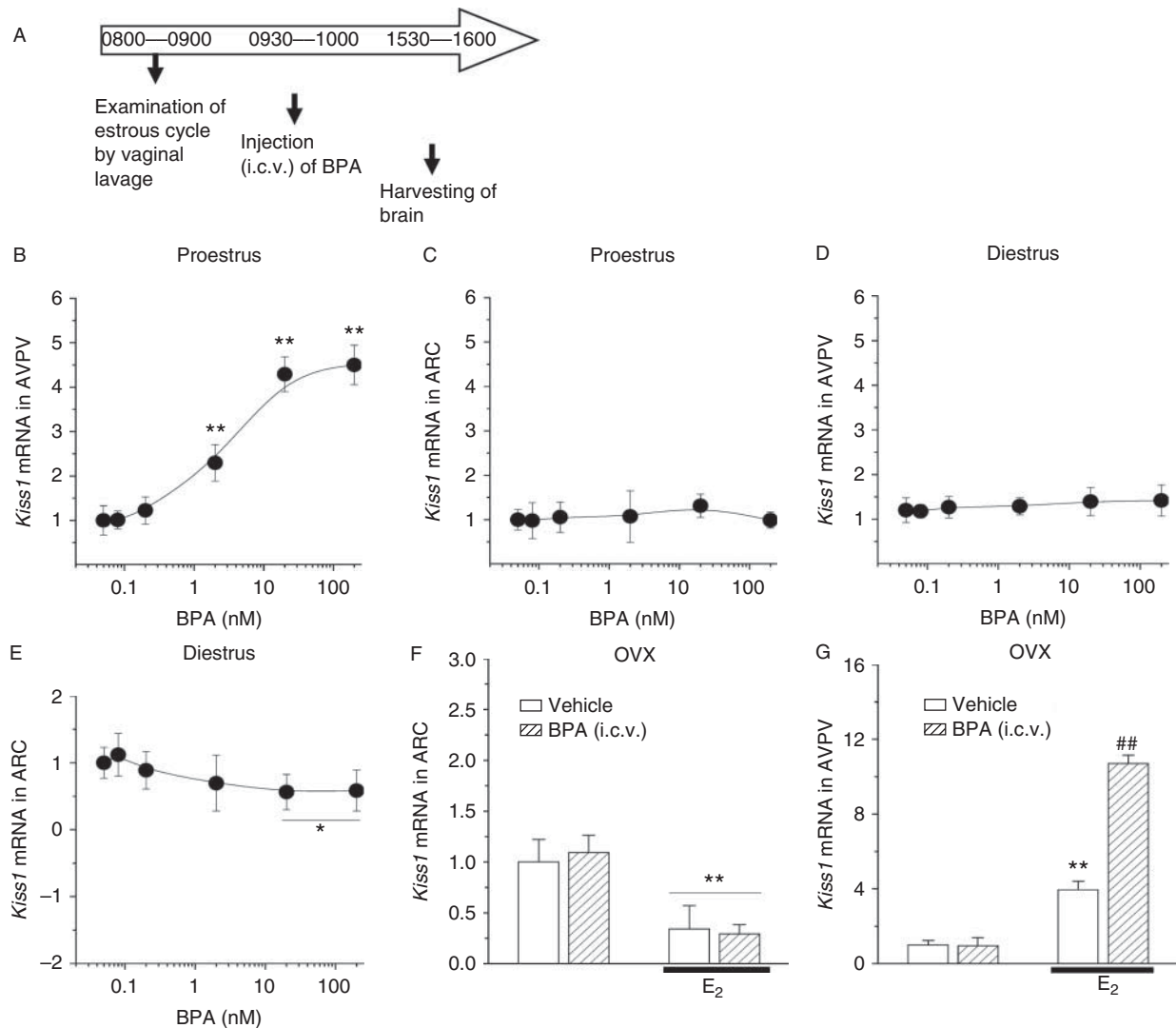
in order to measure the level of *Kiss1* mRNA. The injection of BPA at proestrus could dose-dependently increase the AVPV-*Kiss1* mRNA with EC₅₀ 2.745 nM ($F_{(5,30)}=121.0$, $P<0.01$; Fig. 2B), whereas it did not affect the level of ARC-*Kiss1* mRNA ($F_{(5,30)}=0.86$, $P>0.05$; Fig. 2C). In contrast, the injection of BPA at diestrus failed to affect the levels of AVPV-*Kiss1* mRNA ($F_{(5,30)}=0.98$, $P>0.05$; Fig. 2D). The ARC-kisspeptin expression at diestrus was slightly inhibited only at higher doses of BPA than at 20 nM ($P<0.05$, $n=6$; Fig. 2E). In addition, the application of E₂ was able to increase the level of AVPV-*Kiss1* mRNA in OVX-mice ($F_{(1,28)}=1875.53$, $P<0.01$; Fig. 2F). However, the injection of BPA (i.c.v.) could only enhance the AVPV-kisspeptin expression in OVX-mice treated with E₂

***P*<0.01 vs controls. ###*P*<0.01 vs diestrus mice; ++*P*<0.01 vs proestrus mice (two-way ANOVA). (G) Levels of *Kiss1* mRNA in ARC are expressed relative to the control value. ###*P*<0.01 vs diestrus mice; ++*P*<0.01 vs proestrus mice (two-way ANOVA). (H) Western blot analysis of kisspeptin in AVPV and ARC. **P*<0.01 vs controls (t-test). (I) Representative images of kisspeptin neurons (black arrows) in AVPV of control- and BPA-treated mice (left panels). Scale bars=100 μm. Bars indicate the number of kisspeptin-positive cells in AVPV (t-test). A full colour version of this figure available via <http://dx.doi.org/10.1530/JOE-13-0475>.

($F_{(1,28)}=552.95$, $P<0.01$), but not in OVX-mice ($P>0.05$). In contrast, the injection of BPA (i.c.v.) had no effects on the ARC-kisspeptin expression in OVX-mice or OVX-mice treated with E₂ ($F_{(1,28)}=0.066$, $P>0.05$; Fig. 2G), although treatment with E₂ alone in OVX-mice could reduce the level of ARC-*Kiss1* mRNA ($F_{(1,28)}=193.09$, $P<0.01$). The results indicate that, in the presence of high concentrations of E₂, BPA can enhance the AVPV-kisspeptin expression.

Influence of BPA injection (i.c.v.) on activation of HPG axis and GPR54 dependence

In accordance with the BPA-enhanced AVPV-kisspeptin expression at proestrus, at 6 h after BPA injection (i.c.v.),

**Figure 2**

Influence of BPA injection (i.c.v.) on kisspeptin expression and E₂ involvement. (A) Time chart of experimental procedure. The numbers in the arrow indicate the times of various experimental procedures. (B and C) At proestrus BPA-injection (0.02–200 nM/3 μl/mouse)-induced changes in AVPV- and ARC-*Kiss1* mRNA. Each point represents the mean value of AVPV- and ARC-*Kiss1* mRNA. ***P* < 0.01 vs vehicle-treated group (one-way ANOVA). (D and E) At diestrus BPA-injection (0.02–200 nM/3 μl/mouse)-

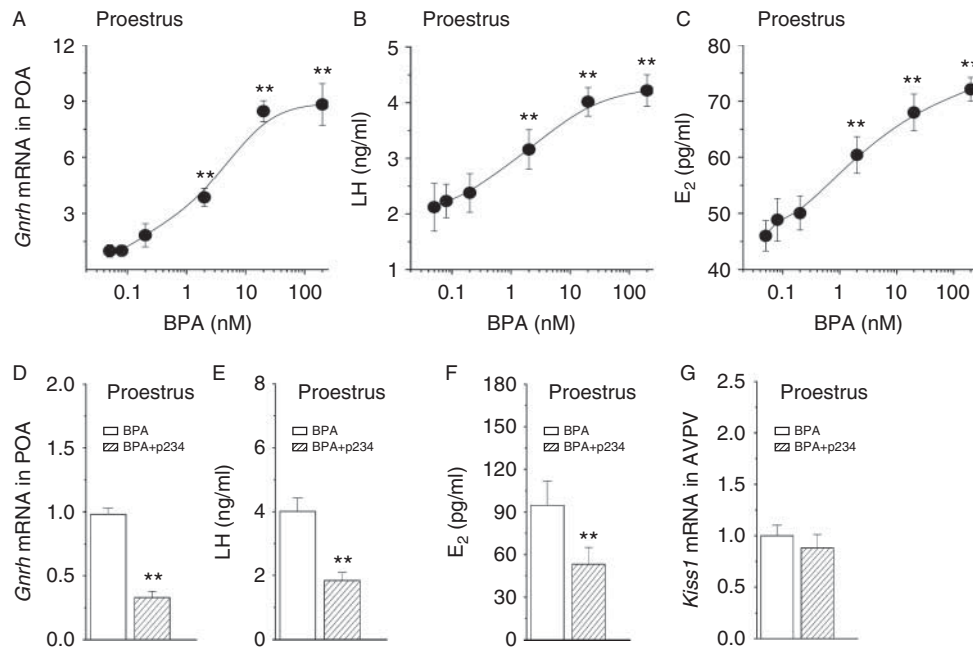
induced changes in AVPV- and ARC-*Kiss1* mRNA. **P* < 0.05 vs vehicle-treated group (one-way ANOVA). (F and G) E₂ dependency of BPA-regulated AVPV- and ARC-kisspeptin expression. Bars represent mean levels of AVPV- or ARC-*Kiss1* mRNA 6 h after BPA injection (i.c.v.) in OVX-mice treated with vehicle or E₂. ***P* < 0.01 vs OVX-mice; ##*P* < 0.01 vs E₂-treated OVX-mice (two-way ANOVA).

the levels of *Gnrh* mRNA ($F_{(5,30)} = 202.80$, $P < 0.01$; Fig. 3A), plasma LH ($F_{(5,30)} = 45.03$, $P < 0.01$; Fig. 3B), and E₂ ($F_{(5,30)} = 74.41$, $P < 0.01$; Fig. 3C) showed a dose-dependent increase. More importantly, treatment (i.c.v.) with the GPR54 blocker p234 (5 nmol/mouse) at 30 min before BPA injection could perfectly abolish the BPA-induced increase in the *Gnrh* expression ($P < 0.01$, $n = 8$; Fig. 3D) and the release of LH and E₂ ($P < 0.01$, $n = 8$; Fig. 3E and F), even though the increased AVPV-*Kiss1* mRNA was not affected ($P > 0.05$, $n = 8$; Fig. 3G). The results indicate that BPA

enhances the release of HPG hormones through stimulation of AVPV-kisspeptin neurons.

Influence of ER antagonists on BPA-stimulated AVPV-kisspeptin neurons

E₂-driven AVPV-kisspeptin biosynthesis depends on the ER α activation (Smith *et al.* 2005). To confirm targets of the BPA-stimulated AVPV-kisspeptin neurons, the ER α antagonist MPP or the ER β antagonist PHTPP was used

**Figure 3**

Influence of BPA injection (i.c.v.) at proestrus on activation of the HPG axis and GPR54 dependence. (A, B and C) Each point represents the mean level of *Gnrh* mRNA, and mean plasma levels of LH and E_2 6 h after BPA injection (0.02–200 nM/3 μ l/mouse). ** $P < 0.01$ vs vehicle-treated group (one-way ANOVA). (D, E, F and G) Effects of the GPR54 antagonist on BPA injection

(i.c.v.)-enhanced HPG axis. The GPR54 antagonist p234 was injected (i.c.v.) 30 min before BPA injection (i.c.v.). Bars show plasma mean levels of LH and E_2 , and the levels of *Gnrh* mRNA and AVPV-*Kiss1* mRNA. ** $P < 0.01$ vs BPA-treated group (*t*-test).

(i.c.v.) at 30 min before BPA injection (i.c.v.). At proestrus, the administration of MPP alone, but not PHTPP, could attenuate the level of AVPV-*Kiss1* mRNA ($F_{(2,41)} = 605.21$, $P < 0.01$; Fig. 4A). In contrast, the enhancing effects of BPA (i.c.v.) on AVPV-kisspeptin expression was abolished by treatment with MPP ($F_{(2,41)} = 282.42$, $P < 0.01$). As expected, treatment with MPP could perfectly block the BPA-induced increase in *Gnrh* mRNA and plasma LH and E_2 ($P < 0.01$, $n = 8$; Fig. 4B, C and D). At 6 h after BPA injection (i.c.v.), the levels of *Er α* (*Esr1*) and *Er β* (*Esr2*) mRNA in AVPV and ARC showed no significant differences from those for controls ($P > 0.05$, $n = 8$; Fig. 4E and F). The results indicate that BPA-stimulated AVPV-kisspeptin neurons depend on ER α activation.

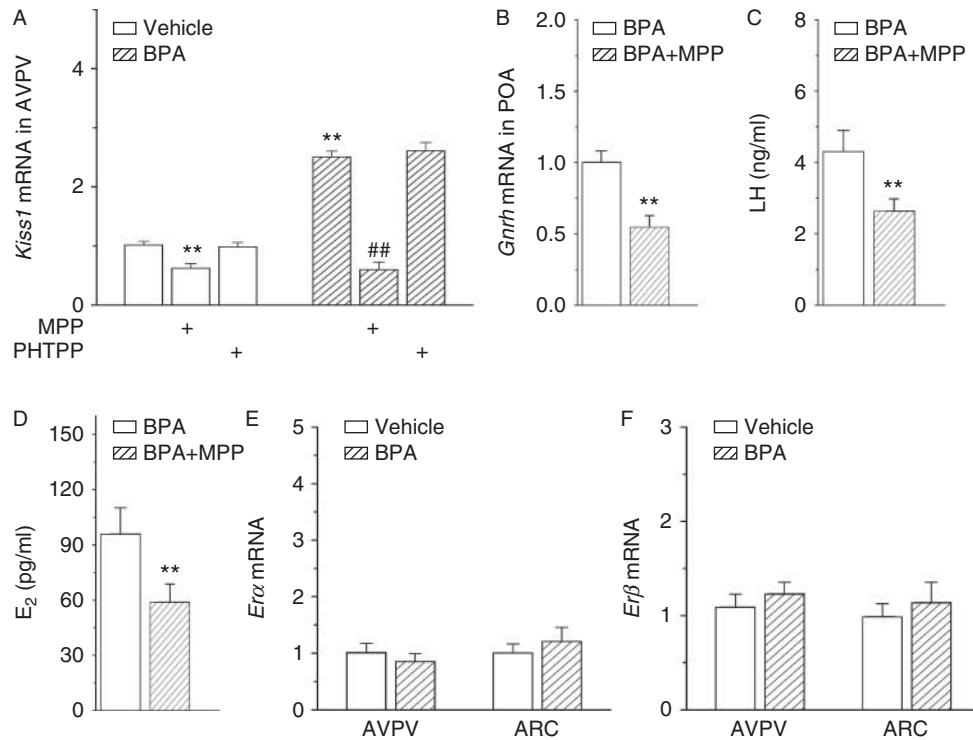
Influence of BPA injection (i.c.v.) on E_2 -enhanced AVPV-kisspeptin expression

The administration (s.c.) of E_2 (0, 0.1, 5, 20, 100, 200, and 400 μ g/kg) in OVX-mice induced a dose-dependent increase in the levels of AVPV-*Kiss1* mRNA within 6 h with a sigmoidal-shaped curve with an EC_{50} of 45.20 μ g/kg ($F_{(6,98)} = 321.23$, $P < 0.01$; Fig. 5), indicating ' E_2 -induced positive feedback'. Interestingly, the injection of BPA

(i.c.v.) could shift the curve of E_2 -increased AVPV-*Kiss1* mRNA (EC_{50} 15.25 μ g/kg, $F_{(6,98)} = 14.66$, $P < 0.01$) leftward. The results further demonstrate that BPA can enhance E_2 -induced positive feedback in AVPV-kisspeptin neurons.

Influence of BPA-enhanced AVPV-kisspeptin on LH surge

The AVPV-kisspeptin neurons, as a target of E_2 -positive feedback, are involved in the generation of the GNRH surge. The final experiment was designed to examine whether the injection of BPA (i.c.v.) at proestrus affects the production of the LH surge (see the time chart of the experimental procedure; Fig. 6A). Repeated-measures ANOVA revealed that the basal levels of plasma LH were increased by BPA injection (i.c.v.) ($F_{(1,18)} = 74.22$, $P < 0.01$). As shown in Fig. 6B, in mice at proestrus, a distinct peak of plasma LH concentration was observed during 1600–1700 h ($n = 10$), showing an apparent LH surge. In contrast, the basal level of plasma LH at 1300–1500 h was elevated by administration of BPA ($P < 0.01$, $n = 10$), which led to a decrease in the amplitude of the LH surge, a difference between the baseline level of LH at 1300 h and the peak concentration of LH. However, the administration of BPA failed to alter the peak timing and the peak

**Figure 4**

Influence of ER antagonists on BPA-enhanced AVPV-kisspeptin expression and activity of the HPG axis. The ER α antagonist MPP or the ER β antagonist PHTPP was injected (i.c.v.) 30 min before BPA injection (i.c.v.). The blood and brain samples were harvested at 6 h after BPA injection (i.c.v.). (A) Bars show the mean levels of AVPV-Kiss1 mRNA. $^{**}P < 0.01$ vs vehicle-treated group;

$^{##}P < 0.01$ vs BPA-treated group (two-way ANOVA). (B, C and D) Bars show the level of GnRH mRNA and mean plasma levels of LH and E₂. $^{**}P < 0.01$ vs BPA-treated group (t-test). (E and F) Influence of BPA on the expression of ER α and ER β in AVPV and ARC. Bars show the mean levels of ER α and ER β mRNA at proestrus in the vehicle-treated and BPA-treated groups.

concentration of the LH surge relative to controls ($P > 0.05$, $n = 10$). The results indicate that the acute administration of BPA does not affect the generation of the LH surge.

Discussion

The present study provides, for the first time to our knowledge, *in vivo* evidence that exposure of adult female mice to a low dose of BPA enhances ER α -mediated positive regulation of AVPV-kisspeptin expression and release.

BPA stimulates AVPV-kisspeptin neurons leading to potentiation of the HPG axis

One of the principal observations in this study is that low-dose BPA can selectively stimulate AVPV-kisspeptin neurons in adult female mice. This conclusion is deduced mainly from the following observations. i) The oral administration of BPA (20 μ g/kg) could increase the level of AVPV-Kiss1 mRNA and kisspeptin protein, whereas it

hardly affected the ARC-kisspeptin expression. ii) A single injection (i.c.v.) of BPA could dose-dependently increase the levels of AVPV-Kiss1 mRNA within 6 h. iii) Either the administration of BPA (p.o.) or the injection of BPA (i.c.v.) could increase the plasma LH, FSH, and E₂ levels, and the level of GnRH mRNA within 6 h, which was sensitive to GPR54 antagonist. A recent study (Lee *et al.* 2013) has reported that in adult female rats, exposure to BPA at doses of 1 and 100 μ g/kg increases serum LH levels and LH protein content in the pituitary gland. Thus, the results indicate that the administration of BPA increases the activation of the HPG axis through enhancing AVPV-kisspeptin expression and release. iv) Consistent with the effects of BPA on ER α -mediated gene expression (Matthews *et al.* 2001), the enhancing effects of BPA on the AVPV-kisspeptin neurons and the activation of the HPG axis were blocked by the ER α antagonist. v) The injection of BPA (i.c.v.) potentiated the E₂-induced AVPV-kisspeptin expression, indicating that BPA can enhance the E₂-induced positive feedback in AVPV-kisspeptin neurons. The results from a wide variety of studies support the indication that

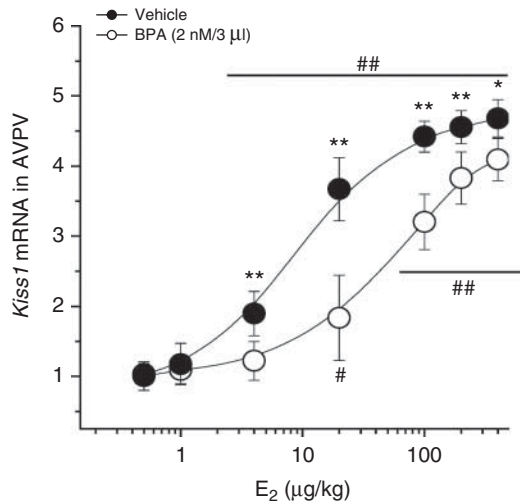


Figure 5

Influence of BPA injection (i.c.v.) on E₂-enhanced AVPV-kisspeptin expression. Open circles and filled circles represent the mean level of AVPV-*Kiss1* mRNA in OVX-mice treated with E₂ alone (0–400 µg/kg) or E₂+BPA (2 nM/3 µl/mouse), respectively. **P*<0.05 and ***P*<0.01 vs E₂-treated group; #*P*<0.01 and ##*P*<0.01 vs vehicle-treated group (two-way ANOVA).

kisspeptin stimulates gonadotrophin secretion via the activation of GNRH neurons expressing GPR54 (Kinoshita *et al.* 2005). Irwig *et al.* (2004) have demonstrated that the central administration of kisspeptin induces *Fos* expression of GNRH neurons in rats.

The level of ARC-*Kiss1* mRNA at proestrus was lower than that at diestrus and was reduced by treatment with E₂ in OVX-mice, indicating that the ARC-kisspeptin neurons in adult female mice receive E₂-induced negative feedback regulation. The molecular mechanisms by which E₂ produces opposite effects on *Kiss1* mRNA expression in AVPV and ARC are unknown. Although E₂ apparently acts through the same ERα to produce opposite results in AVPV and ARC, it seems likely that something intrinsic to the two cell types causes the opposing regulatory effects of E₂. For example, E₂ acting through ERα recruits co-activators of transcription in AVPV-kisspeptin neurons and co-repressors of transcription in ARC (Klinge *et al.* 2001, Gottsch *et al.* 2009). Interestingly, our results show that the ARC-kisspeptin expression was hardly affected by the administration of BPA, although the high dose of BPA caused a slight decline in the ARC-*Kiss1* mRNA. The ratio of ERα to ERβ in ARC-kisspeptin neurons is less than that in AVPV, because the E₂-induced decline in ARC-*Kiss1* mRNA appears greater in ERβ-knockout mice than in control mice (Smith *et al.* 2005). The BPA-stimulated AVPV-kisspeptin neurons depend on the activation of ERα

by high concentrations of E₂. ERβ can oppose ERα-mediated gene transcription (Matthews & Gustafsson 2003). Taking into account of our data, as well as results from other studies, we propose that the differences in expression of ERα and ERβ are a possible molecular basis for the selective action of BPA on AVPV-kisspeptin neurons.

Possible molecular mechanisms of BPA-enhanced activity of AVPV-kisspeptin neurons

The next question we should address may be the molecular mechanism(s) of the BPA effects on E₂-induced positive feedback in AVPV-kisspeptin neurons. Generally, environmental and exogenous E₂ enhances the functioning of E₂ in at least four possible ways: by binding to ER to mimic the action of endogenous E₂; by modulating ER to enhance the E₂-induced responses; by increasing the expression of ER; or by modifying the synthesis, transport, metabolism, and excretion of E₂. First, the BPA-enhanced AVPV-kisspeptin expression and release only occurred

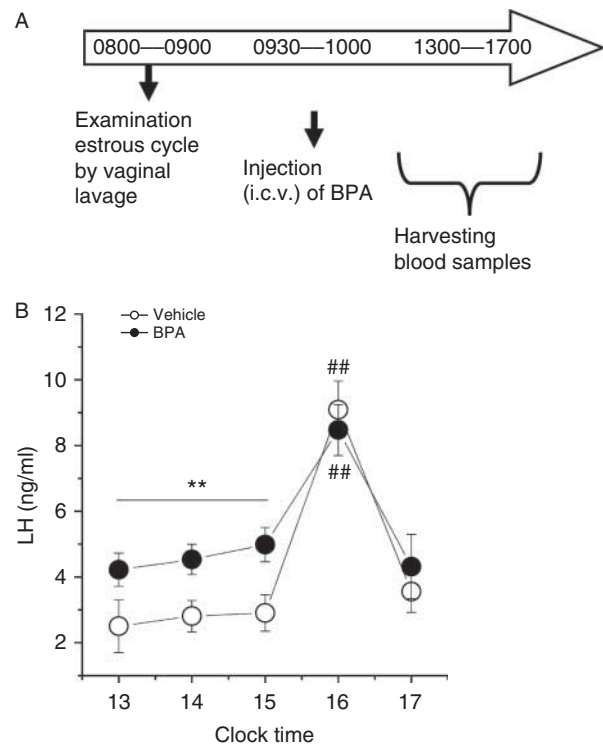


Figure 6

Influence of BPA injection (i.c.v.) on LH-surge. (A) Time chart of experimental procedure. The numbers in the arrow indicate the timing of various experimental procedures. (B) Hourly changes in plasma LH levels were measured at proestrus. ***P*<0.01 vs controls; ##*P*<0.01 vs 1300 h (two-way ANOVA).

at proestrus, but not at diestrus or in OVX-mice. BPA's binding affinity for ER α has been demonstrated to be over 1000-fold lower than that of E₂ (Gould *et al.* 1998). Therefore, it is conceivable that the BPA itself has no E₂-like effects or a very weak estrogenic activity, which is insufficient to trigger the activation of ER α . Interestingly, in the presence of E₂, a high concentration of BPA is able to exert the enhancing effect to further increase the E₂-induced AVPV-kisspeptin expression. However, the administration of BPA does not alter ER α expression in AVPV and ARC. Thus, it is highly probable that BPA, as a positive modulator of ER α activation, augments the E₂-induced response. In fact, BPA has been reported to enhance the ER α -mediated response through rapid activation of ERK1/2 signaling pathway (Gertz *et al.* 2012, Li *et al.* 2012), as well as the generation of second messengers such as cAMP, cGMP, and intracellular Ca²⁺ (Alonso-Magdalena *et al.* 2012, Belcher *et al.* 2012), or the activation of the transcription factor cAMP-responsive element-binding protein (CREB; Quesada *et al.* 2002). However, BPA has been reported to have a direct adverse effect on the regulation of E₂ production. We observed that at estrus, the acute administration of BPA could increase E₂ levels, which was not associated with an increase in LH level and AVPV-kisspeptin neurons. An earlier study reported that the treatment of cultured theca-interstitial cells with BPA increased StAR and P450 side-chain cleavage (P450_{sc}) expression (Zhou *et al.* 2008). However, there is an apparently conflicting report describing that exposure of adult female rats to BPA for 90 days significantly decreases E₂ serum concentration through reduction of P450_{arom} and StAR protein levels (Lee *et al.* 2013). This discord may arise from the difference in treatment duration and doses of BPA. In addition, a BPA metabolite, 4-methyl-2,4-bis (4-hydroxyphenyl) pent-1-ene (MBP), has been demonstrated to have transcriptional activity at nanomolar concentrations, which is 1000-fold higher than the estrogenic activity of BPA (Baker & Chandsawangbhuwana 2012). Although our results do not support the metabolism of BPA introduced by injection (i.c.v.), further studies are needed to clarify the effects of MBP on AVPV-kisspeptin neurons.

Acute administration of BPA does not affect LH-surge generation

The expression of *Kiss1* mRNA in the AVPV tends to be the highest at 1500 h, around the time of the LH-surge peak (Adachi *et al.* 2007). At the time of the preovulatory LH-surge, the levels of FOS protein in AVPV-kisspeptin

neurons are increased in an E₂-dependent manner (Smith *et al.* 2006). The responsiveness of the AVPV-kisspeptin neurons to E₂ has been demonstrated to be critical for the generation of LH surges (Ishii *et al.* 2013), although AVPV-kisspeptin neurons can well maintain their circadian activation when lack of E₂ does not allow LH surges. It is proposed that the preovulatory LH surge is generated only when E₂-induced kisspeptin production is high enough to drive GNRH neurons (e.g., at proestrus). Local injection of a kisspeptin-specific MAB to the POA of female rats at 1300–2100 h on the day of proestrus completely blocks the preovulatory LH-surge (Kinoshita *et al.* 2005) or E₂-induced LH-surges (Gottsche *et al.* 2009). A single injection of kisspeptin can induce a large and sustained LH release (Matsui *et al.* 2004, Shahab *et al.* 2005). Interestingly, we observed that the injection of BPA (i.c.v.) could elevate the basal level of plasma LH to reduce the amplitude of the LH-surge through stimulation of the AVPV-kisspeptin neurons; however, it did not alter the timing and peak concentration of LH-surge generation. Thus, we consider that at 6 h after the administration of BPA the generation of the LH surge is not affected. A chronic high level of LH in female *Er α* -knockout mice has been reported to reduce the size of the LH surge, which was accompanied by ovarian dysfunction and subsequent infertility (Couse & Korach 1999). Therefore, further studies are needed to investigate the chronic effects of BPA administration (p.o.) on the AVPV-kisspeptin expression and the LH-surge generation.

Conclusion

The present study provides *in vivo* evidence that the acute exposure of adult female mice to low doses of BPA can stimulate AVPV-kisspeptin neurons to induce the hyperactivation of the HPG axis. Although much is to be done, our results indicate that exposure to a low dose of BPA during adulthood, by enhancing ER α -mediated positive regulation in AVPV-kisspeptin neurons can disrupt HPG reproductive endocrine signaling, which might subsequently affect the estrous cyclicity and ovarian function.

Declaration 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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