Perinatal exposure to low-dose bisphenol A affects the neuroendocrine stress response in rats

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Abstract

Bisphenol A (BPA) is an estrogen-mimicking endocrine disruptor. Early-life exposures to low doses of BPA exert long-lasting effects on animals' reproductive and brain physiology. However, little is known about the effects of BPA on the stress–response system. Given the interaction of sex and stress hormones, we examined the effect of a low perinatal BPA exposure on the function of the hypothalamic–pituitary–adrenal (HPA) axis at rest and upon application of acute stress. Throughout pregnancy and lactation rats received daily 40 μg BPA/kg body weight orally via cornflakes. We studied the effect of this low but chronic exposure to BPA in the male and female offspring at puberty. BPA exposure led to abnormal adrenal histology including reduced zona reticularis especially in male offspring, hyperplasia of zona fasciculata in both sexes, and increased adrenal weight in female offspring. BPA-treated females had increased basal corticosterone and reduced hypothalamic glucocorticoid receptors (GR) levels. Stressed BPA-exposed females exhibited anxiety-like behavioral coping, a less rigorous corticosterone response, and did not downregulate GR in the hypothalamus, compared with control females. BPA-exposed males exhibited a heightened corticosterone stress response compared with females; they also displayed increased pro-opiomelanocortin mRNA levels and retained the prestress levels of pituitary corticotropin-releasing hormone-receptor 1, compared with control males. We found that perinatal chronic exposure to a low dose of BPA perturbs the basal and stress-induced activity of the HPA axis in a sexually dimorphic manner at adolescence. Exposure to BPA might contribute to increased susceptibility to stress-related disorders in later life.

Key Words
- ▶ adrenal cortex
- ▶ bisphenol A
- ▶ glucocorticoid receptor
- ▶ HPA axis
- ▶ rat

Introduction

The hypothalamus–pituitary–adrenal (HPA) axis plays a pivotal role in the neuroendocrine stress response. Glucocorticoids (corticosterone in rodents, cortisol in humans), synthesized mainly in the zona fasciculata of the adrenal cortex, are massively released into the circulation shortly after stress, in response to adrenocorticotropin

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(ACTH) that is rapidly secreted by pituitary corticotrophs upon stimulation by the hypothalamic-corticotropin-releasing hormone (CRH; Carrasco & Van de Kar 2003). CRH-receptor 1 (CRHR1) in the pituitary is also considered to be indispensable for the release of glucocorticoids from the adrenal cortex (Müller *et al.* 2001). The released glucocorticoids initially potentiate the stress response and subsequently terminate the HPA axis activation by exerting negative feedback at the levels of the pituitary, the hypothalamus, and in some extra-hypothalamic brain structures. Glucocorticoid receptors (GR), the classical nuclear receptors for corticosteroids, mainly mediate the negative feedback toward termination of the stress response and return to homeostasis (Joëls *et al.* 2012).

Deviations from normal HPA function have been linked to psychopathologies including affective, neuro-degenerative, and cognitive disorders (de Kloet *et al.* 2005a). A substantial number of these pathologies may originate during development. The prenatal and early-postnatal periods constitute the most critical time windows for an aberrant HPA programming by maternally derived or environmental factors (de Kloet *et al.* 2005b, Szyf *et al.* 2005, Weinstock 2008). Gonadal steroids also exert organizational effects on the HPA axis that establish sex differences in the axis components in adulthood (Patchev *et al.* 1995, Seale *et al.* 2005). The effects of phytoestrogens and environmental estrogens (xenoestrogens) on the HPA axis have not been studied extensively. Early-life exposure of rats to the soy isoflavone genistein (Ohno *et al.* 2003) or to nonylphenol (Chang *et al.* 2012) reportedly modified adrenal steroidogenesis and cortico-sterone secretion in animals' later life.

Bisphenol A (BPA) is a selective modulator of estrogen receptors. It is utilized for the production of plastics and epoxy resins, used in lining food cans and for some dental materials, as well as for many other everyday life products and therefore is widely present in our environment. BPA has been detected in more than 90% of the human tissues samples examined and there is increased concern regarding its higher bioaccumulation in fetal and infant tissues (Calafat *et al.* 2008). The dose of 50 mg BPA/kg per day has been determined to be the lowest-observable-adverse-effect level (LOAEL) dose and the reference dose of 50 µg BPA/kg body weight as the safe daily exposure for humans (Vandenberg *et al.* 2009). However, the safety of this dose has been strongly disputed due to the increasing number of animal studies reporting adverse effects upon low exposures (Welshons *et al.* 2006). Importantly, developmental exposures have been reported to exert programming effects in the rodent brain, including alterations in sexually dimorphic brain areas (Kubo *et al.* 2003, Funabashi *et al.* 2004), behavior (Farabollini *et al.* 1999, Kubo *et al.* 2001, Kawai *et al.* 2003, Rubin *et al.* 2006), cognitive skills (Carr *et al.* 2003, Miyagawa *et al.* 2007, Xu *et al.* 2007), and levels of estrogen receptors (Khurana *et al.* 2000). Published human data are also associating neurobehavioral problems in children with maternal levels of BPA during pregnancy (Braun *et al.* 2009, 2011, Perera *et al.* 2012).

Sex hormones interact with the stress response system (Handa *et al.* 1994, Viau & Meaney 1996, Viau 2002). First indications that glucocorticoids and their actions in the brain are sensitive to programming effects of BPA have been derived from our previous work (Poimenova *et al.* 2010), showing alterations in circulating corticosterone and its hippocampal receptors, along with impaired spatial recognition memory of exposed rats. Considering that this cognitive task is a kind of mild stress, the altered neuroendocrine responses of BPA litters after the task was suggestive of a BPA effect on stress response. The aim of this study was to further examine the potential effects of chronic perinatal BPA exposure on the HPA axis response under basal conditions and following acute stress. Specific objectives included investigation of BPA-induced effects on behavioral coping and on all compartments of the HPA axis of exposed rats at mid-puberty, namely, on adrenal morphology and corticosterone secretion, on hypothalamic-GR protein levels, as well as on pituitary *Crhr1* and pro-opiomelanocortin (*Pomc*) mRNA levels.

Materials and methods

Animals and experimental design

Female Wistar rats (10 weeks old) were used for breeding. Pregnancy was determined by vaginal smears and pregnant animals were randomly assigned into either the control or the BPA group ($n=5$ in each) and were housed individually under constant photoperiod cycle (12 h light:12 h darkness) and room temperature ($22 \pm 2 °C$), having free access to normal chow and tap water. The dams were administered BPA orally (40 µg BPA/kg body weight per day, Sigma–Aldrich Co.) or vehicle (water, 1% in ethanol) throughout pregnancy and lactation, using cornflakes as the carrier (Poimenova *et al.* 2010) in order to avoid stress to the animals. The dose was adjusted to body weight changes during pregnancy and lactation. In total, 37 offspring were born in the BPA group (14 males and 23 females) and 48 offspring (22 males and 26 females) in the control group. Litters were culled to ten pups.

Anogenital distances were measured at postnatal day 1. All pups were weaned at postnatal day 22. Puberty onset was monitored in female offspring through observation of vaginal opening. At mid-puberty (postnatal day 46), the offspring were dispersed per sex into non-stressed and stressed groups according to a split litter design. In some groups, more than one rat of the same sex and the same litter was included and this was taken into consideration for statistical analyses (litter effect). Four to six animals per group were killed by decapitation under basal conditions, whereas the rest of the animals were subjected to 15 min of stress and their behavior was video recorded. The stressed animals were killed at 30 or 120 min from stress onset (5–9 animals/group per time point). All animals were gently handled the last 4 days before the experiment, to adapt them to the tail vein sampling. Tail-vein blood was collected before stress (basal level) and at 30 min (t30), 60 min (t60), and 120 min (t120) from stress onset. Adrenals, hypothalami, and pituitaries were collected for analysis. Vaginal smears were examined after the animals were killed to detect the phase of the estrous cycle in each female rat. Blood collection was always taking place between 0900 and 1300 h.

All animal treatments were carried out in accordance with UK legal requirements on the ethical use of animals and the protocol was approved by the Ethical Committee of the School of Medicine, National and Kapodistrian University of Athens.

Stress procedure and behavioral measures

The stressor used was acute forced swimming. The 46-day-old rats of the stressed groups were subjected to inescapable swimming for 15 min in a glass cylinder (60 cm height × 28 cm in diameter), filled with water (24 ± 1 °C) to a depth of 40 cm. The water was changed for each animal. At the end of the session, the rat was removed from the cylinder, carefully dried, and returned to its home cage. Animals' behavioral coping was camera recorded and scored through a computer program (Registration, version 1.0.0.) by two independent observers, blindly. The duration of the following behaviors was measured during the first 5 min of stress: escaping (climbing at the walls, trying to escape), swimming (active swimming without trying to escape), and floating (staying immobile and making only the movements required to keep the head above the water).

RIA

The blood samples were centrifuged at 10 000 g for 5 min. Plasma was collected and kept at −80 °C until use.

Corticosterone levels were determined by using a standard RIA kit for small rodents (MP Biomedicals LLC, New York, NY, USA; sensitivity 1.25 ng/ml, intra-assay variation 4.4%, interassay variation 6.5%).

Adrenal histomorphometry

Adrenals were weighted and fixed in neutral formalin for 24 h. Paraffin sections (5 μm) were cut and stained with hematoxylin and eosin to assess standard histology and morphometry. Central adrenal sections from 6 to 8 adrenals per group were digitized (Leica Microsystems GmbH, Wetzlar, Germany). We calculated the thickness of total cortex and that of zona glomeruloza, fasciculata, and reticularis (Image J v.1.41 software, NIH, Bethesda, MD, USA). Specifically, the length, parallel to the adrenal radius, of these areas was measured in seven different sections per adrenal and the average value per zone, expressed as a percentage of the cortex, was used in final analysis of zone thickness. The cellular density in the outer and the inner parts of the zona fasciculata was determined by measuring the number of cell nuclei (Image J v.1.41 software) in the areas of the zona fasciculata adjacent to the zona glomeruloza and reticularis respectively. The nuclei were counted in eight frames (125 × 125 microns) per adrenal, randomly selected within each sub-area. Measurements were conducted by two independent observers, blindly. Before morphometry, the observers were aided by a pathologist to properly define the borders between the zona fasciculata and reticularis.

Western blot

Frozen hypothalami were homogenized and the whole-cell extract was electrophoresed as previously described (Poimenova et al. 2010). Proteins were transferred to nitrocellulose membranes (Schleicher & Schuell, Dassel, Germany). According to molecular weights, different parts of the membranes were incubated overnight at 4 °C with antibodies against GR (MAb-010-050, Diagenode, Liege, Belgium; 1:50; Rao et al. 2011) or GAPDH (Chemicon, Temecula, CA USA; 1:20 000), the latter was used for normalization of protein loading. The signal was visualized by chemiluminescence (ECL kit Amersham) on Fuji Medical X-ray film. Image J v.1.41 software was used to quantify the optical densities on scanned films.

RT-qPCR

RT followed by quantitative (real time) PCR was used to detect the levels of *Crhr1* and *Pomc* mRNA in the pituitaries.

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Total RNA was extracted using the Trizol reagent (Life Technologies) according to the manufacturer's instructions. RNA (1–2 μg) was reverse transcribed in a buffer containing 50 mM Tris–HCl pH 8.3, 75 mM KCl, 3 mM MgCl$_2$, 10 mM dithiothreitol, 500 μM each dNTP, 25 μg/ml oligo-dT, 2 U RNasin, and 200 U M-MLV Reverse Transcriptase (Life Technologies) according to manufacturer's instructions. Real-time PCR was performed in 96-well PCR microplates using the iQ SYBR Green Supermix, in an iQ5 thermal cycler (Bio-Rad Laboratories). The thermal cycling conditions comprised a denaturation step at 95 °C for 3 min followed by 40 cycles at 95 °C for 15 s, and 60 °C for 1 min. The comparative C_t method was used to calculate the relative gene expression by the formula ($2^{-\Delta\Delta C_t}$) for gene expression patterns and the formula ($2^{-\Delta C_t}$) for ratios of gene expression (Schmittgen & Livak 2008). Expression levels of the genes of interest were normalized to the respective levels of β-actin (*Actb*). The following primer pairs were used: rat *Crhr1*: forward: GCCGCCTACA-ATTACTTCCA, reverse: CAATGCAGACGAACATCCAC; rat *Pomc*: forward: 5′-TGTGAAGGTGTACCCCAATG-3′, reverse: 5′-TCCAGCTCCCTCTTGAACTC-3′; rat *Actb*: forward: 5′-CTAAGGCCAACCGTGAAAAG-3′, reverse: 5′-GCATACAGGGACAACACAGC-3′.

Statistical analyses

The Mann–Whitney *U* test was used for comparisons of sex ratio and vaginal opening. The effect of BPA treatment and sex on adrenals' weight and morphology as well as on stress-coping behavior was examined by using two-way ANOVA. Corticosterone data were analyzed using two-way ANOVA for repeated measures and separate two-way ANOVAs per time point. The least squares differences *post-hoc* test was used when appropriate. The effect of BPA treatment, sex, and stress on the levels of GR was analyzed by three-way ANOVA. RT-qPCR data were expressed relative to 'control conditions' (non-BPA treated or non-stressed) and were evaluated by independent samples *t*-test. Because more than one same-sex sibling contributed to some experimental groups (with $n > 5$), the birth litter was added as a covariate in the statistical analyses to correct for a possible effect on the results. Statistical significance was accepted as $P < 0.05$.

Results

Reproductive physiology

One miscarriage was observed among the BPA-exposed dams at an early stage of pregnancy. The BPA-exposed offspring comprised 38% males vs 62% females and the control offspring 45% males vs 54% females. Although the ratio of males:females in the offspring of the five BPA-treated dams ranged from 2:8 to 2:1, male birth rate (median = 2, range 1–5) and female birth rate (median = 4, range = 1–8) were not significantly different ($P = 0.310$). In comparison, the ratio of males/females in the offspring of the five vehicle-treated dams, ranged from 4/8 to 6/4 and male birth rate (median = 4, range 4–6) and female birth rate (median = 5, range = 3–8) were quite similar comparable ($P = 0.548$).

The dose of BPA used in this experiment had no significant effect on the anogenital distances of same-sex pups, as measured at postnatal day 1 (control males: 4.52 ± 0.11 mm; BPA-exposed males: 4.29 ± 0.18 mm; control females: 2.59 ± 0.08 mm; BPA-exposed females: 2.74 ± 0.09 mm; $P = 0.25$ and $P = 0.19$ respectively; *t*-test). The same was true for the time of vaginal opening (control females: median, 35 days; BPA-exposed females: median, 34 days; $P = 0.263$). More specifically, 38.5% of control and 43% of BPA-exposed females entered puberty during postnatal days 31–33. During postnatal days 34–36, 53.8% of control and the 52.2% of BPA-exposed females entered. The rest of the female animals entered puberty during postnatal days 37–38. Vaginal smears at killing of animals exhibited no difference in cyclicity between control and BPA-exposed females. The majority of females (58% of controls and 65% of BPA-treated) were at the estrous phase. The distribution in proestrous, diestrus, and metoestrus equally ranged from 17 to 10% in both groups. Ovarian morphology was similar between the two groups of females (not shown).

Histology of adrenal glands

BPA treatment significantly affected adrenal weight of mid-pubertal animals. Two-way ANOVA for treatment and sex, with litter as covariate, showed a significant effect of sex ($F_{(1,42)} = 21.89$, $P < 0.0001$) and a significant sex × treatment interaction ($F_{(1,42)} = 4.73$, $P = 0.036$) on absolute adrenal weight. *Post-hoc* analysis revealed that BPA females had heavier adrenals compared with control females ($P = 0.038$) and with BPA-treated males ($P < 0.0001$), whereas the adrenal weight difference in control animals did not reach significance ($P = 0.053$). The use of normalized adrenal/body weights showed, in addition to the aforementioned differences, a significant sex difference between control males and females (Table 1).

BPA significantly affected the histology of the adrenal cortex (Table 1 and Fig. 1). A significant effect of BPA and

Table 1 BPA effects on rat adrenals. Data are expressed as mean$\pm$s.e.m. The number of animals used in the analysis is given in parentheses

	Control male	BPA male	Control female	BPA female
Adrenal weight absolute (mg)	31.80 ± 2.17 (11)	28.06 ± 1.76 (7)	38.09 ± 2.18 (12)	$44.56\pm2.47^{a,b}$ (13)
Adrenal weight relative (mg/g body weight)	0.25 ± 0.01 (11)	0.22 ± 0.01 (7)	0.36 ± 0.02^{b} (12)	$0.42\pm0.02^{a,b}$ (13)
ZG thickness (percentage of cortex)	5.65 ± 0.36 (7)	4.65 ± 0.56 (6)	5.15 ± 0.32^{b} (8)	4.96 ± 0.32 (7)
ZF thickness (percentage of cortex)	88.85 ± 0.55 (7)	93.56 ± 0.70^{a} (6)	90.28 ± 0.27^{b} (8)	$92.04\pm0.43^{a,b}$ (7)
ZR thickness (percentage of cortex)	5.10 ± 0.22 (7)	2.40 ± 0.29^{a} (6)	4.45 ± 0.17^{b} (8)	2.83 ± 0.13^{a} (7)
No. of nuclei in outer ZF	60.54 ± 5.13 (5)	86.10 ± 10.96 (5)	53.97 ± 1.82 (6)	71.03 ± 3.39^{a} (6)
No. of nuclei in inner ZF	104.28 ± 4.61 (5)	$141.03\pm14.5^{a,b}$ (5)	107.05 ± 3.07 (7)	104.28 ± 6.59 (6)

ZG, zona glomeruloza; ZF, zona fasciculata; ZR, zona reticularis. Differences with $P<0.05$ were classified as significant.
[a]Significant effect of BPA.
[b]Significant effect of sex.

its interaction with sex was detected on the thickness of the zona fasciculata ($F_{(1,27)}=42.84$, $P<0.0001$ and $F_{(1,27)}=8.86$, $P=0.007$ respectively). *Post-hoc* analysis revealed that the thickness of this zone in BPA-treated males was increased, compared with control males ($P<0.0001$), control females ($P<0.0001$), and BPA-exposed females ($P=0.043$). Control females had more extended zona fasciculata than control males ($P=0.040$), whereas an opposite sex difference trend was detected between BPA-treated animals. Treatment with BPA and its interaction with sex significantly affected zona reticularis thickness ($F_{(1,26)}=108.37$, $P<0.0001$) and $F_{(1,26)}=6.80$, $P=0.016$). This zone was decreased in BPA-treated animals compared with same-sex controls ($P<0.0001$, for both). In control, but not in BPA animals, zona reticularis was more extended in males when compared with females ($P=0.032$). Furthermore, typical zona reticularis, normally comprising of a cellular network with smaller than nuclei than cells of the zona fasiculata was not detected in several regions of adrenals derived from BPA-treated males. Instead, cells from the zona fasciculata were often extended to the medulla. This was also observed in BPA-treated females, but to a lesser degree, compared with males (Fig. 1A, B, C, and D). BPA treatment increased the number of cells in the zona fasciculata, as
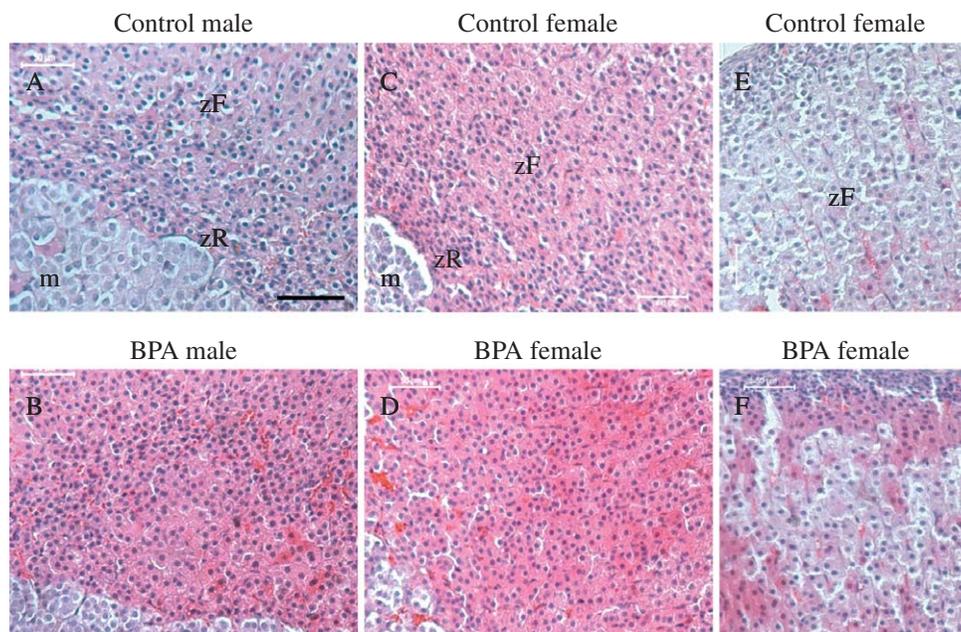

Figure 1
Representative photomicrographs from the adrenals of untreated controls and BPA-treated rat offspring depicting cortical zones. Zona reticularis (zR) is discrete in controls (A and C) but hardly distinguished from zona fasciculata (zF) in BPA-treated animals (B and D) in which large polyhedral cells from the zF extend to the medulla (m). Note the higher number of cells in the inner part of the zF in BPA-exposed males (B vs A) and in the outer part of the zF in BPA-exposed females (F vs E); hematoxylin and eosin staining. Scale bar=50 μm.

assessed from the counting of nuclei in these mononuclear cells. The number of nuclei in the inner part of the zona fasciculata was significantly affected by sex ($F_{(1,22)}=4.54$, $P=0.047$) and BPA treatment ($F_{(1,22)}=5.24$, $P=0.034$ and their interaction ($F_{(1,22)}=6.59$, $P=0.019$). BPA-exposed males had higher numbers of nuclei and hence increased cellularity (hyperplasia) in this area (Table 1 and Fig. 1A and B) compared with the control groups ($P=0.005$ for both) and with BPA-exposed females ($P=0.004$). Hyperplasia was also detected in the outer zona fasciculata due to BPA treatment ($F_{(1,21)}=11.01$, $P=0.004$) that reached significance in BPA-treated females ($P=0.025$). The same trend ($P=0.089$) toward hyperplasia was detected for BPA-treated males compared with control males (Table 1 and Fig. 1E and F).

Behavioral coping

Treatment with BPA significantly modified the behavior of female rats during forced swimming stress (Fig. 2). Two-way ANOVA showed a significant effect of BPA on the duration of escaping ($F_{(1,50)}=10.17$, $P=0.003$) and floating ($F_{(1,50)}=9.41$, $P=0.004$). Further per sex analysis showed that BPA females spent more time in trying to escape and less time immobile compared with control females, whereas no significant differences were detected in male animals.

Corticosterone secretion

Repeated-measures ANOVA for the effect of sex and treatment on corticosterone measures before stress (basal level, 0 min) and at 30, 60, and 120 min post stress showed a significant effect of sampling time ($F_{(3,30)}=14.73$,
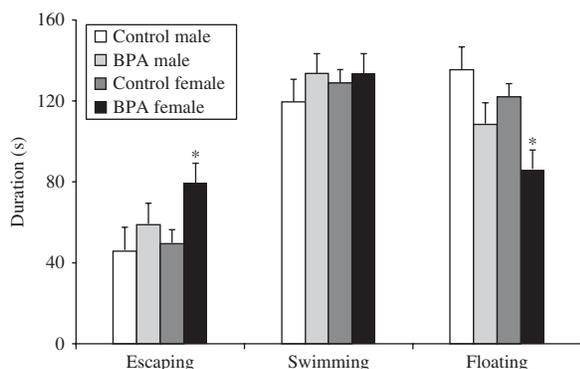

Figure 2
Behavioral coping of BPA-treated rats and vehicle-treated controls observed during the first 5 min of swimming stress. Data are mean±S.E.M. *Significant effect of BPA. Two-way ANOVA. Differences with $P<0.05$ were classified as significant.

$P<0.0001$) and its interaction with sex ($F_{(3,30)}=11.13$, $P<0.0001$). Litter was included as covariate. Control males, as well as BPA-exposed males, had significantly increased corticosterone levels at 30 and 60 min post stress compared with basal levels ($P<0.0001$ for all comparisons) and these returned to baseline levels by 120 min post stress (Table 2). Similarly, control females displayed increased corticosterone levels at 30 and 60 min post stress compared with basal levels ($P<0.0001$ and $P=0.026$ respectively) and these returned to baseline levels at 120 min post stress. Corticosterone in BPA-exposed females was significantly increased from baseline to 30 min post stress ($P<0.0001$), returned to pre stress levels at 60 min ($P=0.65$), and further decreased below baseline levels at 120 min post stress ($P<0.0001$).

Further analysis within each time point showed that BPA-exposed females had higher basal corticosterone levels than all other groups ($P<0.0001$). In the controls, no significant sex differences were detected at basal conditions. At 30 min post stress, both control and BPA-exposed females had higher corticosterone levels than males ($P<0.0001$ and $P=0.001$ respectively), whereas BPA-exposed females had lower hormone levels compared with the control females ($P=0.003$). At 60 min post stress, corticosterone levels of BPA-exposed females were significantly lower compared with both control females ($P=0.044$) and BPA-exposed males ($P=0.046$). No sex differences were observed in the control groups at 60 min post stress. At 120 min post stress, corticosterone levels were higher in BPA-exposed females compared with males ($P=0.043$).

The fold increase in corticosterone levels for each animal was calculated for the time points of 30, 60, and 120 min post stress, with respect to basal levels (Table 2). Significant sex effects were obtained at 30 min ($F_{(1,24)}=7.17$, $P=0.014$), 60 min ($F_{(1,18)}=24.09$, $P<0.0001$), and 120 min post stress ($F_{(1,18)}=8.14$, $P=0.013$). At 30 min post stress, the mean fold increase in corticosterone was significantly higher in BPA-treated males compared with females ($P=0.011$), whereas no sex difference was detected in the controls. At 60 min post stress, both male groups exhibited higher increases in corticosterone levels than females ($P=0.04$ for the controls and $P=0.001$ for BPA). At 120 min post stress, BPA-treated males still had a higher fold increase in corticosterone compared with BPA females ($P=0.007$), whereas no such difference was detected in the controls.

Hypothalamic GR levels

Three-way ANOVA, with litter as covariate, revealed significant interaction of BPA treatment, sex, and stress

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Table 2 Rat plasma corticosterone concentrations and fold increase during stress. Data represent mean±s.e.m. of plasma corticosterone concentrations of control and BPA-treated animals under basal conditions (t0) and at 30 min (t30), 60 min (t60), and 120 min (t120) from stress onset, as well as the fold increase from basal conditions at the aforementioned time points. The number of animals sampled for each group is given in parentheses

Corticosterone	Control male	BPA male	Control female	BPA female
t0 (ng/ml)	26.87±6.9 (5)	24.70±8.7 (6)	57.21±8.7 (7)	133.51±17.2[a,b] (9)
t30 (ng/ml)	587.33±35.6[c] (7)	583.63±43.3[c] (7)	1304.81±126.4[b,c] (10)	1027.8±70.8[a,b,c] (10)
t60 (ng/ml)	284.12±29.7[c] (5)	215.41±32.7[c] (4)	287.62±97.1[c] (6)	120.10±24.9[a,b] (7)
t120 (ng/ml)	33.73±5.9 (5)	23.76±3.1 (5)	65.58±21.7 (6)	42.61±6.1[b,c] (8)
t30/t0 (fold increase)	22.66±3.7 (5)	30.02±8.2 (6)	19.42±2.5 (6)	8.56±1.0[b] (8)
t60/t0 (fold increase)	9.53±2.1 (4)	8.71±2.2 (4)	2.95±1.4[b] (4)	1.00±0.2[b] (7)
t120/t0 (fold increase)	1.16±0.4 (4)	1.13±0.2 (4)	0.56±0.2 (5)	0.41±0.06[b] (6)

Significance was accepted for $P<0.05$.
[a]Significant effect of BPA.
[b]Significant effect of sex.
[c]Significant change from basal (t0).

on GR protein levels in the hypothalamus ($F_{(1,34)}=7.61$, $P=0.001$). Subsequent comparisons showed that under basal conditions BPA-treated females had reduced GR levels compared with control females ($P<0.0001$). Control females had higher GR levels than control males ($P<0.0001$), whereas no sex differences were seen in BPA-treated animals (Fig. 3). At 120 min post stress, control females had reduced GR levels ($P=0.008$) compared with prestress conditions. No significant changes from basal values were observed for control males and the BPA-treated animals following stress.

Pituitary *Pomc* and *Crhr1* levels

The mRNA levels of *Crhr1* in the pituitary did not differ significantly between control and BPA-treated animals of the same sex under basal conditions (Fig. 4A). Control males showed a trend ($P=0.07$) toward reduced *Crhr1* mRNA levels at 30 min compared with non-stressed levels that reached significance at 120 min post stress ($P=0.002$). In contrast, BPA-treated males retained prestress levels of *Crhr1* mRNA at 30 and 120 min following stress, as no differences between basal and post-stress levels were detected in this group. *Crhr1* mRNA levels were found to be significantly higher at 2 h from stress onset in BPA-exposed males compared with the respective control male group ($P=0.027$). At 30 min post stress, both female groups had significantly decreased *Crhr1* mRNA levels compared with basal ($P=0.016$ and $P=0.002$ for controls and BPA respectively). This decrease in female groups was also detected at 120 min post stress ($P=0.005$ and $P=0.021$ for controls and BPA respectively). Comparisons between female animals at 30 and 120 min post stress showed no significant differences due to BPA treatment.

The mRNA levels of *Pomc* did not differ significantly between control and BPA-treated animals, independently of sex, under basal conditions and at 30 min after stress (Fig. 4B). *Pomc* levels were increased at 120 min following stress in BPA-treated males compared with the same group without stress ($P=0.007$) as well as with stressed males not treated with BPA ($P=0.01$). In contrast, BPA-exposed females following stress did not differ from BPA-exposed females not exposed to stress or from control females.

Summary of results

Continuous BPA exposure during the perinatal period led to abnormal adrenal histology including reduced zona reticularis especially in male offspring, hyperplasia of zona fasciculata in both sexes, and increased adrenal weight in female offspring. Under basal conditions, BPA-exposed females had increased plasma corticosterone and reduced GR levels in the hypothalamus compared with controls. BPA-exposed females exhibited an anxiety-like coping during swimming stress. Following stress, BPA-exposed females had a less rigorous corticosterone response and failed to display downregulation of GR in the hypothalamus, as control females did. BPA-exposed males exhibited heightened corticosterone stress response compared with females. Following stress, BPA-exposed males retained the prestress levels of *Crhr1* mRNA and increased *Pomc* mRNA levels in the pituitary compared with all other groups.

Discussion

The current study provides evidence that continuous perinatal BPA exposure at a dose similar to the environmentally
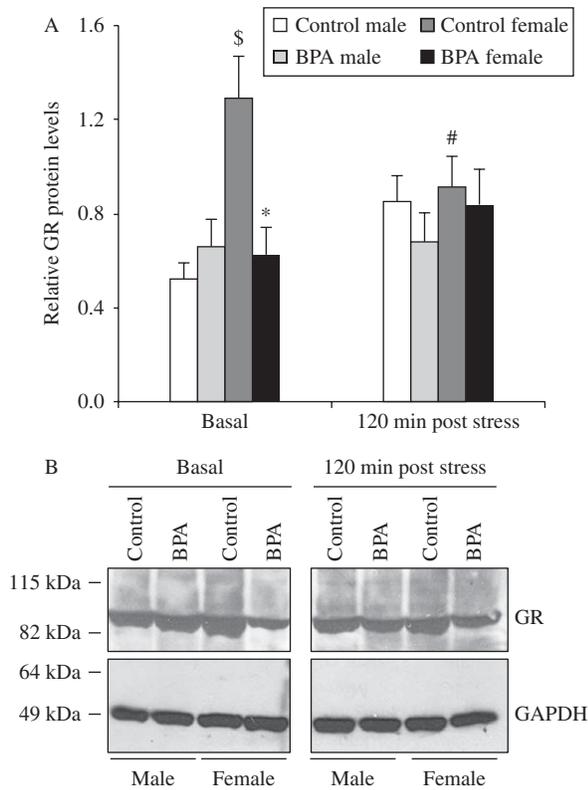
Figure 3

Effect of BPA treatment, sex, and stress on basal and 120 min post stress protein levels of glucocorticoid receptor (GR) in the hypothalamus of mid-adolescent rats. (A) Quantification of the western blot data. Relative protein levels refer to GR levels relative to GAPDH levels as calculated by densitometry of western blot analysis of total protein extract. Data represent mean ± s.e.m. *Significant effect of BPA; $significant effect of sex; #significant effect of stress. Three-way ANOVA. Differences with $P < 0.05$ were classified as significant. (B) Representative western blots.

relevant human exposure (Vandenberg et al. 2007) induces long-lasting alterations in the HPA axis of the rat. The effect on the HPA axis of pubertal offspring was apparent in both peripheral and central components of the axis and was sexually dimorphic. The BPA dose used in our study is below the reference dose of 50 μg/kg body weight per day that is the currently accepted LOAEL for humans (US Environmental Protection Agency 1988 Bisphenol A (CASRN 80-05-7). Integrated Risk Information System. Available at www.epa.gov/iris/subst/0356.htm, accessed Sept 2013). Recent pharmacokinetic studies, taking into account the differences in BPA metabolism between rodents and humans have estimated that exposures to 400 μg/kg body weight per day produce bioactive BPA concentrations in their blood, within the range of recorded human blood concentrations (Vandenberg et al. 2012).

Reproductive physiology

The perinatal low-dose BPA exposure had no obvious effect on the reproductive physiology of their offspring, as deduced by observations of anogenital distance, puberty onset, ovarian histology and vaginal smears, in agreement with the literature regarding low-dose exposures (Ashby 2001, Rubin et al. 2001, Tinwell et al. 2002, Kubo et al. 2003, Yoshida et al. 2004, Patisaul & Polston 2008, Poimenova et al. 2010). However, the reduced male:female offspring ratio in BPA-treated dams, though not statistically significant in this study, may imply a male embryo selective vulnerability in utero that worth further investigation.

Adrenal histology and function

Our observations indicate for the first time, to our knowledge, that continuous low-dose exposure to BPA
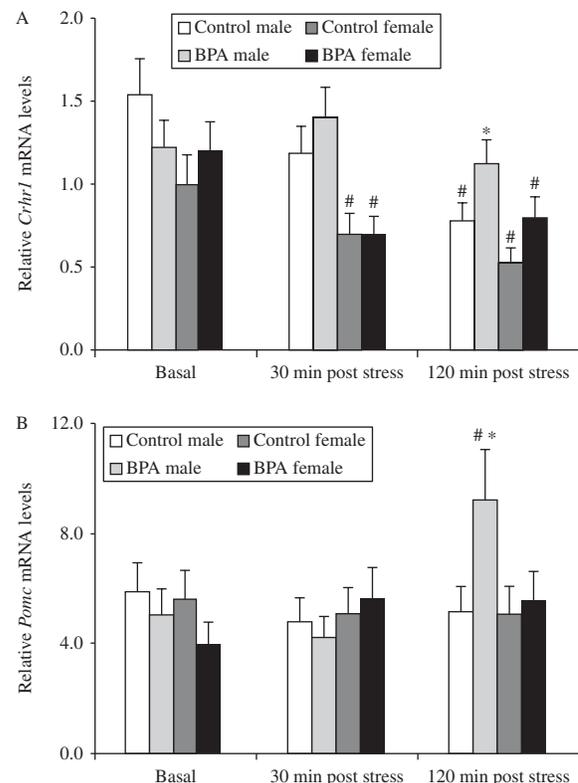

Figure 4

Effect of BPA treatment, sex, and stress on basal, 30 and 120 min post stress mRNA levels of *Crhr1* (A) and *Pomc* (B) in the pituitary of mid-adolescent rats. Relative mRNA levels refer to *Crhr1* or *Pomc* mRNA level relative to *Actb* mRNA level as calculated by RT-qPCR analyses of total mRNA. Data represent mean ± s.e.m. *Significant effect of BPA, compared with the 120 min post stress control males; #significant effect of stress compared with the non-stressed (basal) animals of the same sex and BPA treatment groups. Independent samples *t*-test. Differences with $P < 0.05$ were classified as significant.

may affect adrenal function leading to long-lasting effects on animal's physiology. Fetal adrenal cortex development is dependent on placental estrogen and the existence of both types of estrogen receptors in the fetal gland (Kaludjerovic & Ward 2012) allows for a genomic action of BPA during developmental exposures. Additional non-genomic or epigenetic BPA actions in the adrenals, already reported for other tissues (Zsarnovszky *et al.* 2005, Doshi *et al.* 2011), cannot be excluded. The few reports on the effects of xenoestrogens in the adrenal cortex upon early-life exposure support their effect on male steroidogenesis. Administration of a high dose of genistein in weanling male rats significantly altered adrenal steroidogenesis at puberty and also expanded the zona fasciculata and reticularis (Ohno *et al.* 2003). Perinatal exposure to nonylphenol increased adrenal cortex steroidogenic activity and ACTH in male animals only and basal plasma corticosterone in both sexes (Chang *et al.* 2012), in partial compliance with our corticosterone findings.

Given the importance of normal adrenal development for the feto–placental unit and perinatal survival (Ishimoto & Jaffe 2011), we cannot exclude the possibility that fetal adrenal abnormalities may have contributed to the reduced number of male offspring from BPA-exposed dams. In addition, the lack of proper cytoarchitecture and the reduced zona reticularis, the main site for extragonadal androgen production, in BPA-treated males, imply a feminizing action of BPA during adrenal gland development that warrants further investigation due to the role of this zone in adrenarche onset and related pathologies in primates (Belgorosky *et al.* 2008).

Corticosterone responses

The heavier adrenals of BPA-exposed females vs control females and the extended and hyperplastic zona fasciculata of BPA-treated rats imply alterations in the responsiveness of adrenal cortex to central stress regulators. Indeed, altered corticosterone secretion was witnessed, compared with the non-treated rats, both under basal conditions and following stress, which was sexually dimorphic. Under resting conditions, BPA-treated females had higher basal corticosterone levels than control females and BPA-treated males, in agreement with our previous findings (Poimenova *et al.* 2010), whereas no sex differences were detected between control animals. The lack of sex differences in plasma corticosterone levels of the untreated controls is in compliance with the literature for pubertal rats, reporting that sex differences in this system normally emerge after puberty (Sencar-Cupovic & Milkovic 1976, Romeo 2010). Following stress, BPA-treated females exhibited a blunted stress response at 30 min compared with control females; in contrast to BPA-treated males, which responded similarly to control males. The stress-induced corticosterone levels reported here are in close agreement with the high values reported in the literature for pubertal corticosterone responses (Bourke & Neigh 2011, Foilb *et al.* 2011). Previous studies have shown that prepubertal rats irrespective of sex have a protracted corticosterone response to stress compared with adults and that at both ages females demonstrate a higher peak and a faster return to baseline than males (Romeo *et al.* 2004*a*,*b*). Our observations for control rats are in agreement with the results of the aforementioned studies, whereas BPA-exposed females showed an even faster return than controls. Interestingly, chronic exposure of neonatal female rats to estradiol led to increased corticosterone levels and HPA axis hyperactivity under resting conditions (Patchev *et al.* 1995), suggesting similarities in programming actions of estradiol and BPA.

HPA axis responsiveness and behavioral phenotype

The interpretation of the adrenal, hormonal, and neuroendocrine findings of this study indicates that the HPA axis of female BPA-exposed offspring under basal conditions is hyperactive, resulting in increased circulating corticosterone that leads to a reduction in GR levels by a process termed homologous downregulation (Oakley & Cidlowski 1993), consequently limiting cellular responsiveness to glucocorticoids. The blunted span of glucocorticoid response is expected to disturb regulatory circuits involving adrenals, hypothalamus, and pituitary. The dysfunctional adrenal provides more corticosterone than needed. The moderately increased basal corticosterone levels of BPA-exposed females are probably capable of activating hypothalamic GR, resulting in constitutive translocation and blunted recycling of the receptor. This appears to have resulted in the 'atypical,' for resting conditions, autologous downregulation of GR (Oakley & Cidlowski 1993). The hyperactive HPA axis of BPA-exposed females under basal conditions is reflected in their behavioral stress response during the first 5 min of exposure to swim stress, characterized by increased anxiety-like behavior (escaping) and concomitant reduction of immobility.

In line with the results expected for animals with a dysfunctional HPA axis, BPA-exposed females following stress did not display downregulation of GR levels in response to the stress-induced corticosterone increase,

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as control females do, toward termination of axis activation (Makino *et al.* 1995). Heightened levels of glucocorticoids or impaired CRH signaling during adolescence have been proposed to modify brain plasticity and emotionality (Sapolsky 2003) resulting in increased susceptibility to stress-related psychopathologies in adulthood (Becker *et al.* 2007). Accordingly, the biological outcome of the observed HPA axis deregulation in BPA-exposed females may be a sustained inability to successfully cope with stressful events.

Compared with females, pubertal BPA-exposed males exhibited a more active HPA axis under stress, reflected in their high and extended rise in plasma corticosterone and in their higher pituitary *Crhr1* and *Pomc* levels following stress. This sex difference may relay on the potent pituitary signals of BPA-exposed males driving corticosterone secretion and the proper response of their hypertropic and extended zona fasciculata. In comparison with control males in our study, BPA-exposed males appear less mature regarding their pituitary stress responsiveness. According to Foilb *et al.* (2011), the stress-induced ACTH response is more robust and extended in prepubertal than mature male rats, reaching adult levels after postnatal day 50.

Sexual dimorphism of BPA effects on the stress axis

Perinatal BPA exposure often exerts a sex-specific effect on their offspring's physiology and behavior, including reduction or reversal of existing sex differences (Richter *et al.* 2007). In this study, sexually dimorphic effects of BPA on the HPA axis components were observed that may have contributed to sex differences in stress responsiveness. BPA acting as a weak estrogen could have modified the steroidogenic activity or sensitivity of the developing adrenal cortex to pituitary signals in a sex-specific way. Accordingly, we could detect sex-dependent, BPA-induced alterations of adrenal histology, able to alter corticosterone secretion and stress responses of the exposed animals. In addition, sex-specific alterations were detected in pituitary and hypothalamic mediators of BPA-treated animals, further supporting this hypothesis.

Circulating gonadal hormones can modulate adult HPA reactivity (Handa *et al.* 1994, Viau & Meaney 1996, Viau 2002), but they did not seem to mediate stress responses in rats younger than 50 days (Foilb *et al.* 2011). Nonetheless, to exclude the possibility that the detected BPA effects on HPA axis reflect alterations in gonadal steroids of the exposed animals, we have previously

(Poimenova *et al.* 2010) determined the circulating levels of progesterone and testosterone in mid-pubertal BPA-exposed rats treated under the same protocol. No differences were found between BPA-exposed and control animals, in compliance with previous studies (Ramos *et al.* 2003, Muñoz-de-Toro *et al.* 2005). In addition, in this study, monitoring of the estrous cycle also did not reveal differences between control and BPA-treated females that could possibly influence stress responses.

Limitations of the study

This study has limitations mainly due to the low number of births ($n = 5$ per treatment), which did not allow equilibrated sex ratios in BPA-exposed litters. This could have possibly biased maternal care, subsequently affecting some physiological outcomes (Meaney 2001). In a previous study (Della Seta *et al.* 2005), maternal care was affected in dams treated with 40 μg BPA/kg body weight per day, but this was not influenced by the sex of the pup, in contrast to the control dams. In a more recent study (Kundakovic *et al.* 2013), the maternal behavior of dams exposed to BPA during pregnancy, at a dose similar to that used in our study, did not differ from normal. Furthermore, in the same study, the modified maternal care at BPA doses lower or higher than ours did not affect the BPA-induced effects on offspring gene expression. A bigger sample would have also allowed the use of more than one BPA dose to examine the 'non-monotonic' curve of BPA actions previously reported (Vandenberg *et al.* 2007). Finally, more studies are required to examine whether the observed alterations in the pubertal HPA axis are retained or modified in the adult state, under the influence of the gonadal steroids.

In conclusion, continuous exposure of rats in early life to BPA at a dose that is lower than the currently accepted LOAEL (50 μg/kg) can modify basal and stress-induced HPA axis function at puberty in a sexually dimorphic way. This implicates BPA in aberrant HPA axis programming, potentially leading to stress-related disorders in later life.

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