

Delayed neuroendocrine sexual maturation in female rats after a very low dose of Bisphenol A through altered GABAergic neurotransmission and opposing effects of a high dose

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Rat sexual maturation is preceded by a reduction of the interpulse interval (IPI) of gonadotropin-releasing hormone (GnRH) neurosecretion. This work aims at studying disruption of that neuroendocrine event in females after early exposure to a very low dose of Bisphenol A (BPA), a ubiquitous endocrine disrupting chemical.

Female rats were exposed to vehicle or BPA 25 ng/kg.day, 25 μg/kg.day, or 5 mg/kg.day from postnatal day (PND) 1 to 5 or 15. Exposure to 25 ng/kg.day of BPA for 5 or 15 days was followed by a delay in developmental reduction of GnRH IPI studied *ex vivo* on PND 20. After 15 days of exposure to that low dose of BPA, vaginal opening tended to be delayed. In contrast, exposure to BPA 5 mg/kg.day for 15 days resulted in a premature reduction in GnRH IPI and a trend toward early vaginal opening. RNAseq analysis on PND20 indicated that exposure to BPA resulted in opposing dose effects on the mRNA expression of hypothalamic genes involved in GABA_A neurotransmission. The study of GnRH secretion *in vitro* in the presence of GABA_A receptor agonist/antagonist confirmed an increased or a reduced GABAergic tone after *in vivo* exposure to the very low or the high dose of BPA, respectively.

Overall, we show for the first time that neonatal exposure to BPA leads to opposing dose-dependent effects on the neuroendocrine control of puberty in the female rat. A very low and environmentally relevant dose of BPA delays neuroendocrine maturation related to puberty through increased inhibitory GABAergic neurotransmission.

Bisphenol A (BPA), a most ubiquitous Endocrine Disrupting Chemical (EDC), is used in the production of polycarbonate plastics and epoxy resins (1). In a recent review of thirteen studies addressing the issue of BPA effects on female rodent sexual maturation (2), exposure during the first two weeks after birth was associated with either normal (3–6) or early vaginal opening (4, 6–8) depending on the dose of BPA but without consistent dose-response effect. None among those studies found puberty to be delayed. A neuroendocrine mechanism was sug-

gested in some studies (7, 9) and invalidated in others (4). In the 13 studies summarized in the above-mentioned review, BPA was used in the μg/kg.day or mg/kg.day body weight ranges of daily exposure (2). So far, pubertal timing had never been studied after BPA administration in the ng/kg.day range which is consistent with current human exposure (0.01 to 1 μg/kg.day) (10). In humans, equivocal data have been obtained. BPA excretion was not associated with changes in pubertal timing in several studies (11–13). Increased urinary BPA levels were observed ei-

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Abbreviations:

ther in precocious puberty (14) or in relation with late menarche (15). The purpose of the present study was to further elucidate the impact of the dose of BPA on female puberty and the underlying neuroendocrine mechanism with emphasis on rat exposure to low environmentally relevant doses during the early postnatal window. The mechanistic issue was addressed using an *ex vivo* paradigm of pulsatile GnRH secretion from hypothalamic explants (16) that was shown to be affected by other EDCs in previous studies (17, 18).

The effects of EDCs on female puberty can result either from peripheral effects at the level of estrogen sensitive tissues eg, breast, uterus and vagina or from an activation of the hypothalamic-pituitary-ovarian axis (19, 20). The question arises as to whether early life exposure to BPA can affect the neuroendocrine control of puberty. During perinatal life, a critical window for organization of the central control of the reproductive axis, the female neuroendocrine system is particularly sensitive to sex steroids. In previous studies, we have shown that a single estradiol administration caused central sexual precocity and disrupted the central mechanism of ovulation (21). Also, early postnatal exposure of female rats to the estrogenic insecticide dichlorodiphenyltrichloroethane (DDT) resulted in early neuroendocrine maturation with precocious vaginal opening (VO) (17). Neonatal exposure to DES for 5 days was also associated with early puberty following a dose of 10 $\mu\text{g}/\text{kg}\cdot\text{day}$ and, unexpectedly, with a delayed puberty preceded by a delayed maturation of GnRH secretion after 1 $\mu\text{g}/\text{kg}\cdot\text{day}$ of DES (18).

Since we used exposure to DES for 5 days during the neonatal programming window in our previous study, a similar window of exposure was used initially for BPA. In such conditions, effects on GnRH secretion were observed after exposure to 25 $\text{ng}/\text{kg}\cdot\text{day}$ and 5 $\text{mg}/\text{kg}\cdot\text{day}$ whereas no effect was seen using 25 $\mu\text{g}/\text{kg}\cdot\text{day}$. None of the three doses was associated with differences in pubertal timing. Therefore, we studied the effects of a longer exposure (15 days) to the lowest and the highest doses in further experiments. For the first time in investigating puberty, we used a very low dose of BPA of (25 $\text{ng}/\text{kg}\cdot\text{day}$), which is an equivalent low dose of environmental exposure in humans (10). The delaying effects of this low dose on developmental changes in GnRH secretion and puberty were opposed to the acceleration seen after exposure to 5 $\text{mg}/\text{kg}\cdot\text{day}$. This contrast provided the rationale for RNAseq study to delineate possible opposing changes in mRNA expression of some hypothalamic genes involved in the maturational control of GnRH secretion. It appeared that the most significantly affected genes were related to GABA neurotransmission, a finding confirmed using quantitative PCR (qPCR). The involvement was interesting given the

developmental reduction in inhibitory GABA control of GnRH secretion that was substantiated in the primate in the period preceding the onset of puberty (22, 23) and also shown to occur in the rat using our hypothalamic explant paradigm (24, 25).

Materials and Methods

Animal care and exposure

Female Wistar rats were purchased from the University of Liège. They were housed in standardized conditions (22.8°C, lights on from 6.30 am to 6.30 pm) and mated. Litters were standardized for size and sex ratio on the first postnatal day of life in order to have 10–12 pups per litter and an identical number of males and females. The day of birth was considered as postnatal day 1. Weaning occurred at 21 days of age. In order to avoid a litter effect, a maximum of two pups coming from the same dam were studied in a same group for a given experiment. In each litter, female pups were exposed either to corn oil or to one of the three BPA doses. The animals were raised in BPA-free cages (Polypropylene cages, Ref 1291H006, Tecnilab, Netherlands) and fed EDC- and phytoestrogen-free chow (V135 R/Z low phytoestrogen pellets, SSNIFF Diet, Netherlands). Water was supplied in glass bottles. Beginning on PND 1, female pups were subcutaneously injected with vehicle (corn oil) or BPA 25 $\text{ng}/\text{kg}\cdot\text{day}$ (consistent with low environmental exposure) or 5 $\text{mg}/\text{kg}\cdot\text{day}$ (10 times below LOAEL – Low observed adverse effect level) or an intermediate dose of 25 $\mu\text{g}/\text{kg}\cdot\text{day}$. BPA (Ref: 23,9658; Sigma–Aldrich, Saint Louis, USA) was dissolved in ethanol at an initial concentration of 100 mg/ml and then diluted in corn oil. Injections (0.05 ml) were given every 24 hours, between 11.00 am and 1.00 pm, from PND 1 to 5 or from PND 1 to 15. All experiments were carried out with the approval of the Belgian Ministry of Agriculture and the Ethical Committee at the University of Liège.

Experimental design

Effects of exposure to BPA from PND 1 to 5 on pubertal timing and GnRH secretion. Ninety female pups from 16 dams were exposed from PND 1 to 5 to corn oil, 25 ng , 25 μg or 5 $\text{mg}/\text{kg}\cdot\text{day}$ of BPA. Females were sacrificed on PND 15 ($n = 16$), 20 ($n = 12$), or 25 ($n = 8$) to study GnRH secretion *ex vivo* (4 animals/ BPA dose group) while 54 animals were followed for VO and estrous cyclicity ($n = 6$ –14/ BPA dose group and 21 controls).

Effects of exposure to BPA from PND 1 to 15 on pubertal timing, GnRH secretion and hypothalamic gene expression. One hundred and four female pups born from 18 dams were exposed to corn oil, 25 ng or 5 $\text{mg}/\text{kg}\cdot\text{day}$ of BPA from PND 1 to 15. Thirty-four females were followed for VO/estrous cyclicity ($n = 8$ –14/group) while 46 were sacrificed on PND 20 to study GnRH secretion *ex vivo*. Explants were incubated in control medium or in the presence of 10^{-4}M of bicuculline (B7561, Sigma) or muscimol (M1523, Sigma) (25) ($n = 4$ to 10/group). In 24 females also sacrificed on PND 20, the retromammillary hypothalamus was dissected and RNA was extracted

for RNA sequencing or real-time PCR ($n = 8$ /control and each BPA dose group). In Supplemental Table 1, the number of animals per experiment is specified.

Vaginal opening and estrous cyclicity

From PND 25, female rats were examined daily for imperforation of the vaginal membrane to determine age at VO. Thereafter, vaginal smears were taken every day in the afternoon until PND 80 as described previously (18). A regular cycle was defined by a regular sequence of diestrus 1, diestrus 2, proestrus and estrus (26).

Hypothalamic explant incubation and GnRH assay

GnRH pulse frequency was studied through calculation of the interpulse interval (IPI) using GnRH release from female rat hypothalamic explants. GnRH secretion was studied after exposure to different doses of BPA from PND 1 to 5 or 15 (Supplemental Table 1). After exposure to corn oil (control) or different doses of BPA from PND 1 to 5, 16 explants were studied on PND 15 (corn oil and BPA 25 ng, 25 μ g or 5 mg/kg.day) and 12 explants on PND 20 after exposure to the low (25 ng/kg.day) or the high (5 mg/kg.day) dose of BPA. Eight explants were also studied on PND 25 after exposure to the low dose of BPA (25 ng/kg.day) from PND 1 to 5. After a longer exposure (PND 1 to 15) to the low (25 ng/kg.day) or the high (5 mg/kg.day) dose of BPA, explants were studied on PND 20 (8 controls, 16 low dose of BPA and 22 high dose). The GnRH IPI was studied in the presence of a GABA_A receptor agonist, muscimol (10^{-4} M) and an antagonist, bicuculline (10^{-4} M). In previous in vitro studies using hypothalamic explants, a high GABAergic inhibitory tone on GnRH release was revealed by a reduction of the IPI in the presence of the antagonist bicuculline on PND 15. On PND 25, a lowered GABAergic tone was suggested by the disappearance of bicuculline effects and the occurrence of an increase in GnRH IPI caused by the agonist muscimol (24, 25). We used the same conditions here including bicuculline and muscimol concentrations (10^{-4} M), to investigate the effects of BPA on the regulation of GnRH secretion by GABAergic neurotransmission.

After decapitation, a tissue fragment including the preoptic region and the medial basal hypothalamus was rapidly dissected, as described in supplementary data. Each explant was transferred into an individual chamber in a static incubator (21). The incubation medium was collected and renewed every 7.5 minutes for a period of 4 hours. The GnRH release in the incubation medium was measured in duplicate using a radioimmunoassay (RIA) method with intra-assay and interassay coefficients of variation of 14% and 18%, respectively (27, 28). The highly specific CR11-B81 anti-GnRH antiserum (final dilution 1:80,000) was kindly provided by Dr. V.D. Ramirez (Urbana, IL) (29). The properties of this antibody are given in Table 1. Data below the limit of detection (5 pg/7.5-minute fraction) were assigned that value.

RNA sequencing

RNAseq analysis was carried out on total RNA extracted from retrochiasmatic hypothalamus of female rats on PND 20 (control: $n = 3$; BPA 25 ng: $n = 3$; BPA 5 mg: $n = 3$). Previous studies have shown that the retrochiasmatic hypothalamus contains neuro-glial networks involved in the regulation of pulsatile GnRH secretion (24, 25, 30). The extraction was done with the RNeasy Mini kit (Qiagen, Netherlands). Library preparation and sequencing were performed at the GIGA Genomics facility (University of Liège, Belgium). RNA integrity was verified on the Bioanalyser 2100 with RNA 6000 Nano chips, RIN scores were > 8 for all samples. Illumina Truseq stranded mRNA Sample Preparation kit was used to prepare libraries from 1 μ g of total RNA. Poly-A RNAs were purified with polyT-coated magnetic beads and chemically fragmented. They were used as template for first strand synthesis in the presence of random hexamers followed by second strand synthesis. cDNA ends were adenylated at 3'OH extremities before the ligation to adaptors containing the indexes. Finally, the adapters-ligated library fragments were enriched by PCR following Illumina's protocol and purified with Ampure XP magnetic beads. Libraries were validated on Bioanalyser DNA 1000 chip and quantified by qPCR with the KAPA library quantification kit. Sequencing was performed on Illumina NextSeq500. For the data analysis, Fastq files were trimmed for adaptor sequences. The reads were aligned with Tophat 2.0.9 (<http://ccb.jhu.edu/software/tophat/index.shtml>) to the rat genome. The number of reads per sample was between 11.10^6 and 22.10^6 . The overall read alignment rate was between 77.7 to 83%. Cufflinks 2.2.0 suite (<http://cole-trapnell-lab.github.io/cufflinks/manual/>) was used to generate Fragments Per Kilobase of transcript per million fragments mapped (FPKM) values and CuffDiff was used to identify significantly differentially expressed genes between the 3 conditions of treatment (q -value, ie, corrected p value ≤ 0.05). The data are presented in log₂ (fold change) to allow a representation of an increase as a positive value and a decrease as a negative value on a single plot. More details concerning these softwares and their analytical and statistical methods are given in supplemental materials and methods (31).

Data Mining Analysis

In order to identify the pathways most affected by BPA, significant RNAseq data were uploaded on QIAGEN's Ingenuity® Pathway Analysis (IPA®, QIAGEN Redwood City, www.qiagen.com/ingenuity). Canonical pathway analysis was performed on genes with a p -value < 0.05 in order to identify the pathways most affected by BPA. More information concerning this software and the analysis are given in supplemental materials and methods.

Table 1.

Peptide/protein target	Antigen sequence (if known)	Name of Antibody	Manufacturer, catalog #, and/or name of individual providing the antibody	Species raised in; monoclonal or polyclonal	Dilution used
GnRH	pGlu-His-Trp-Gly-Leu-Arg-Pro-Gly-NH ₂	CR11-B81	Dr V.D. Ramirez (Urbana, IL, USA)	Rabbit, polyclonal	1/80 000

Real-time PCR

Quantitative PCR (qPCR) analysis was used to assess mRNA levels of GAD2 and GAT2 on PND 20 ($n = 8/\text{group}$). Total RNA was extracted from retrochiasmatic hypothalamus of female rats using the RNeasy Mini kit (Qiagen, Netherlands). Five hundred nanograms of RNA for each sample were reverse transcribed using the Transcriptor first strand cDNA synthesis kit (Roche, Germany). Real-time quantitative PCR reactions were performed using FastStart Universal SYBR Green Master (Roche) (Roche, Germany) and a LightCycler 480 system (Roche, Germany). Samples were run in triplicate. Four microliters of cDNA (previously 5-fold diluted) were added to a mix of $5 \mu\text{l}$ of Sybr green, $0.3 \mu\text{l}$ of both forward and reverse primers and $0.4 \mu\text{l}$ of nuclease-free water. Ct values were obtained from each individual amplification curve and the average Ct was calculated for each target gene in each sample. Quantification of relative gene expression was performed according to the $2^{-\Delta\Delta\text{Ct}}$ method using β -actin as housekeeping gene (32). The primers sequences and information are provided in Supplemental Table 2.

Statistics

Numerical values were expressed as mean \pm SEM. For the analysis of GnRH IPI and mRNA expression (after RTqPCR), we performed a Student's *t* test to compare two groups of the same age. For data sets containing more than two groups of the same age, a one-way ANOVA followed by a Newman–Keuls multiple test was used. To compare more than two groups at different ages, a two-way ANOVA followed by a Newman–Keuls multiple test was used. To analyze the age at VO, a Cox regression model was applied. This model is an adaptation of probit analyses commonly used to study pubertal onset in humans (33). Probit analysis is a type of regression used with binomial response variable (in this study: vaginal opening, yes/no). Differences with a *p* value lower than 0.05 were considered to be statistically significant. Statistical analyses were carried out using Statistica® software.

Results

Pubertal timing after exposure to BPA from PND 1 to 5

In agreement with our previous findings (16), maturation of GnRH secretion preceding puberty was characterized by a reduction of the GnRH IPI between PND 15 and PND 25 (Figure 1) using hypothalamic explants of control female rats *ex vivo*. Exposure to $25 \text{ ng/kg}\cdot\text{day}$ of BPA from PND 1 to 5 resulted in a significant increase of GnRH IPI on PND 20 and 25, consistent with delayed maturation. In contrast, exposure to $5 \text{ mg/kg}\cdot\text{day}$ of BPA resulted in a significant reduction of GnRH IPI on PND 15 and 20, consistent with advanced maturation. The intermediate dose of $25 \mu\text{g/kg}\cdot\text{day}$ did not affect GnRH IPI. Though the low dose of BPA caused a slight delay of age at VO (Figure 1), there was no significant difference between age at VO among the 4 groups (control, BPA $25 \text{ ng/kg}\cdot\text{day}$, BPA $25 \mu\text{g/kg}\cdot\text{day}$, BPA $5 \text{ mg/kg}\cdot\text{day}$). The BPA-exposed animals showed normal estrous cyclicity from vaginal opening until PND 80. No significant difference in average weight was observed between the control and BPA groups from PND 1 to 90.

Pubertal timing after exposure to BPA from PND 1 to 15

After a longer exposure (from PND 1 to 15), the GnRH IPI on PND 20 was significantly increased by the low dose of BPA and reduced by the high dose (Figure 2), similarly to the effects observed after a shorter exposure. As illustrated by the cumulative percentage curves in Figure 2 (panel A), exposure to the low dose of BPA tended to delay VO while the high dose of BPA tended to advance VO. The Cox regression regarding age at VO revealed a significant difference ($P = .016$) between the three groups (control,

BPA $25 \text{ ng/kg}\cdot\text{day}$, BPA $5 \text{ mg/kg}\cdot\text{day}$). Pair analysis indicated that the difference was only significant between the groups exposed to the low and high doses of BPA ($P = .0043$) and not when these exposed groups were compared to controls (Figure 2). The BPA-exposed animals exhibited normal estrous cyclicity from vaginal opening until PND 80. No significant difference in average weight was observed between the control and BPA groups from PND 1 to 90, suggesting that differences in maturation of GnRH secretion and pubertal timing did not result from differences in nutritional status.

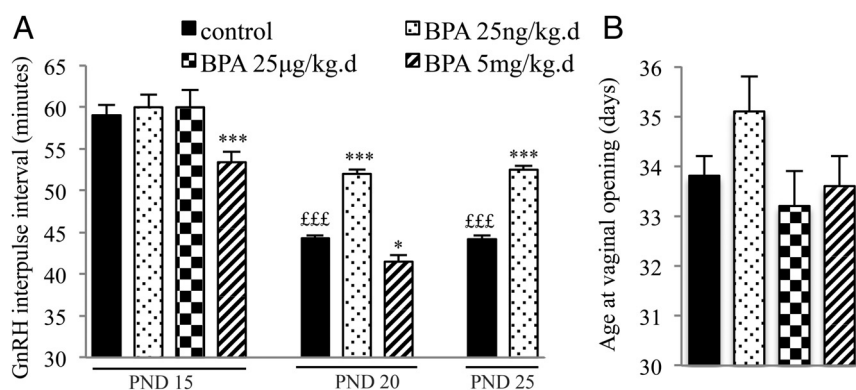


Figure 1. Effects of postnatal BPA exposure for 5 days on pulsatile GnRH secretion and female pubertal timing. A, GnRH IPI *in vitro* using hypothalamic explants obtained on PND 15, 20 or 25 from female rats after exposure from PND 1 to 5 to vehicle, $25 \text{ ng/kg}\cdot\text{day}$, $25 \mu\text{g/kg}\cdot\text{day}$ or $5 \text{ mg/kg}\cdot\text{day}$ of BPA. B, Age at vaginal opening in female rats exposed from PND 1 to 5 to vehicle, $25 \text{ ng/kg}\cdot\text{day}$, $25 \mu\text{g/kg}\cdot\text{day}$ or $5 \text{ mg/kg}\cdot\text{day}$ of BPA. Data are mean \pm SEM; * $P < .05$ and *** $P < .001$ vs control at the same age; $^{\text{f f f}}$ $P < .001$ vs control at PND 15.

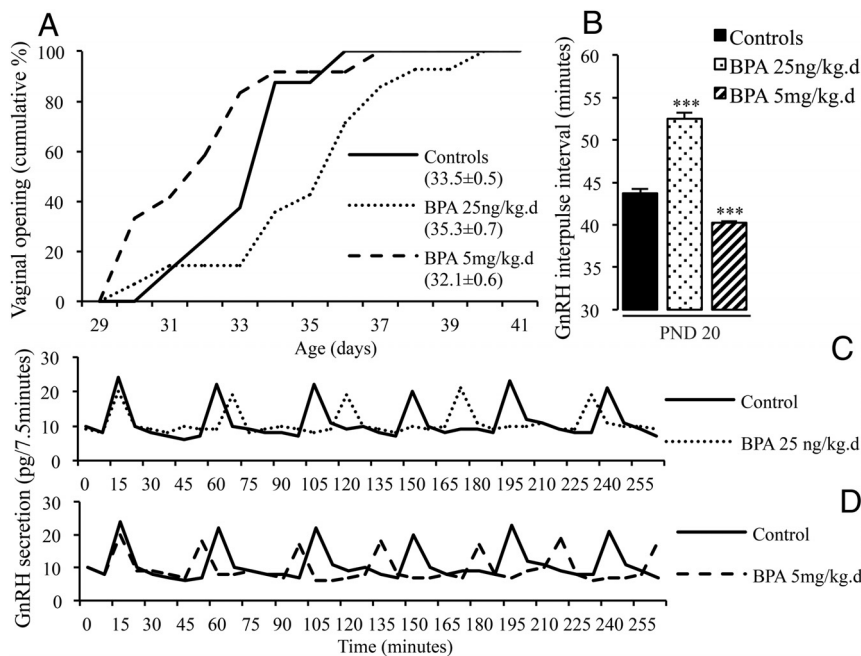


Figure 2. Effects of postnatal BPA exposure for 15 days on pulsatile GnRH secretion and female pubertal timing. A, Cumulative percentage of female rats displaying vaginal opening in relation to age in animals exposed from PND 1 to 15 to vehicle or 25 ng/kg.day or 5 mg/kg.day of BPA. Mean age at VO \pm SEM for each group is indicated in brackets. B, GnRH IPI in vitro using hypothalamic explants obtained on PND 20 from female rats after exposure from PND 1 to 15 to vehicle or 25 ng/kg.day or 5 mg/kg.day of BPA. Data are mean + SEM. *** $P < .001$, vs control group. C, and (D) Individual representative profiles of GnRH secretion after neonatal exposure to vehicle or BPA 25 ng/kg.day or 5 mg/kg.day.

BPA exposure and changes in hypothalamic mRNA expression

In order to identify the hypothalamic genes possibly involved in the dose-related opposing effects of BPA on GnRH secretion and pubertal timing, a RNA sequencing study was performed using retrochiasmatic hypothalamic extracts obtained on PND 20 in female rats. After exposure to BPA 25 ng/kg.day and 5 mg/kg.day (PND 1–15), the mRNA expression of 34 and 472 genes was significantly affected, respectively (q -value < 0.05 vs controls). When comparing the two BPA doses, 1407 genes were differentially expressed. A bioinformatic analysis (Ingenuity Pathway Analysis) revealed that GABA_A receptor neurotransmission was among the systems most affected by both doses of BPA (Figure 3), based on both the number of affected genes and the statistical significance of changes in expression. The 10 canonical pathways most affected

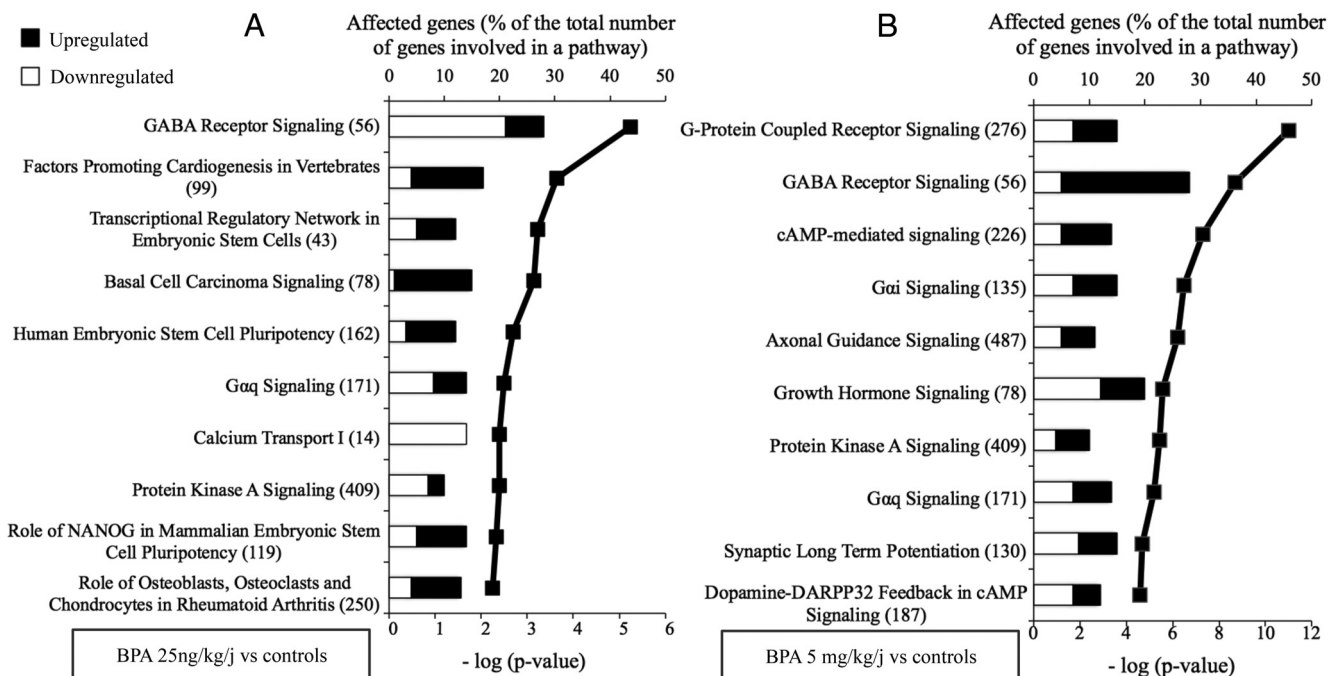


Figure 3. Hypothalamic pathways affected by 25 ng or 5 mg/kg.day of BPA. A, Top 10 canonical pathways derived from Ingenuity Pathway Analysis based on genes that are significantly down- or upregulated in the retrochiasmatic hypothalamus on PND 20 after 15 days of exposure to BPA 25 ng/kg.day. B, Top 10 canonical pathways derived from Ingenuity Pathway Analysis based on genes that are significantly down- or upregulated in retrochiasmatic hypothalamus on PND 20 after 15 days of exposure to BPA 5 mg/kg.day. The pathways are ranked based on $-\log(p\text{-value})$ in order of decreasing significance that is illustrated by the bold line. The black and the white bars refer to the percentage of upregulated and downregulated genes that is calculated relative to the total number of genes involved in each pathway (given in the brackets).

by the two doses of BPA are listed in Supplemental Tables 3 and 4. The two doses resulted in opposing changes of mRNA expression of genes involved in GABA synthesis (glutamic acid decarboxylase, GAD), reuptake (GABA transporter, GAT) and signaling (GABA_A Receptor subunits) (Table 2). Using qPCR on a larger number of samples ($n = 8$), the increase in mRNA expression of GAD2 was confirmed after 5 mg/kg.day of BPA (1.3 ± 0.07 vs 1.04 ± 0.09 in control). Likewise, the increased mRNA expression of GAT2 was confirmed after 25 ng/kg.day of BPA (1.5 ± 0.27 vs 1.09 ± 0.13 in control).

BPA exposure and GABAergic regulation of GnRH release

Based on the RNA sequencing results, we studied whether GABAergic regulation of pulsatile GnRH secretion was affected by BPA. Exposure to the low BPA dose from PND 1 to 15 resulted in a significant increase of GnRH IPI on PND 20 (51.7 ± 0.9 minutes vs 43.5 ± 0.0 minutes) while the high dose caused a significant reduction of GnRH IPI (39.4 ± 0.5 minutes), confirming the data of earlier experiments. The increase in GnRH IPI that occurred after 25 ng/kg.day of BPA likely involved increased GABAergic tone because it was no longer observed (Figure 4) when hypothalamic explants were incubated in the presence of bicuculline, a GABA_A receptor antagonist (45.8 ± 0.9 minutes vs 51.65 ± 0.9 minutes, bicuculline vs control). Moreover, muscimol did not affect the increase in GnRH IPI seen after exposure to 25 ng/kg.day of BPA. The reduction in GnRH IPI that occurred after 5 mg/kg.day of BPA (43.8 ± 1.3 minutes vs 40 ± 1.25 minutes, muscimol vs control) was no longer observed when hypothalamic explants were incubated in the presence of

muscimol, a GABA_A receptor agonist. The GABA_A receptor antagonist did not affect the reduction in GnRH IPI seen after exposure to 5 mg/kg.day of BPA (Figure 4). Taken together, these data confirm that postnatal exposure to a very low dose of BPA accounts for delayed maturation of GnRH secretion through increased GABAergic tone whereas a high dose of BPA accounts for early maturation through reduced GABAergic tone.

Discussion

We provide here the first evidence that BPA can have opposing dose-dependent effects on the neuroendocrine control of puberty in the female rat. Postnatal exposure to a high dose of BPA led to accelerated maturation of GnRH secretion and a trend toward early onset of puberty. Conversely, neonatal exposure to a very low dose of BPA led to a delay in developmental acceleration of GnRH secretion and a trend toward delayed vaginal opening. Based on these opposing effects on neuroendocrine maturation, we hypothesized that some hypothalamic genes could be differentially affected by a low vs high dose of BPA. An RNAseq analysis showed opposing variations in mRNA expression of genes related to GABA neurotransmission with confirmation using qPCR. Using GABA agonist and antagonist, a decrease or an increase of GABA inhibitory tone on pulsatile GnRH secretion was shown after exposure to a high or low dose of BPA, respectively.

As detailed in the Supplemental Table 5, a number of studies have reported that GABA neurotransmission could be affected in different brain areas after exposure to BPA during early life (34–41). GABAergic tone appeared

Table 2. Genes related to GABA neurotransmission showing significant changes in hypothalamic mRNA expression after exposure to low or high BPA doses from PND 1 to 15

Gene	FPKM Control	Down-regulated log ₂ (fold change)		p-value	Up-regulated log ₂ (fold change)		p-value
		BPA 25 ng vs. Control	BPA 5 mg vs. Control		BPA 25 ng vs. Control	BPA 5 mg vs. Control	
GAD1	65.87	-0.19		0.05 665		0.35	0.00 065
GAD2	52.05	-0.24		0.01 670		0.44	0.00 005
GAT2	5.34		-0.60	0.0006	0.39		0.00 885
GABRA2	16.69	-0.18		0.10 665		0.45	0.0015
GABRB3	17.95	-0.16		0.1294		0.63	0.00 075
GABRD	20.44		-1.09	0.00 005	0.23		0.04 135
GABRE	6.49	-0.15		0.1965		0.71	0.00 005
GABRG2	43.47	-0.04		0.73 085		0.40	0.00 075
GABRG3	3.79	-0.88		0.0003		0.61	0.00 405
GABRQ	5.67	-0.21		0.07 385		0.70	0.00 005

GAD: Glutamic Acid Decarboxylase; GAT: GABA Transporter; GABR: GABA Receptor
 FPKM: Fragments Per Kilobase Million (digital value of expression level).

to be increased after BPA exposure in some studies (34, 37) or reduced in others (36, 38, 39). In a recent study close to our conditions using whole brain extracts from animals exposed to different doses of BPA from gestational day 8 to PND 16, Cabaton et al, investigated brain composition by nuclear magnetic resonance spectroscopy on PND 21. In this non targeted metabolomics study, these authors reported that 25 $\mu\text{g}/\text{kg}\cdot\text{day}$ of BPA could reduce GABA concentrations in the brain (36). Interestingly enough they also used a very low BPA dose of 25 $\text{ng}/\text{kg}\cdot\text{day}$ and it appears that opposing effects were obtained with the low dose ie, increase in GABA concentrations (N. Cabaton and D. Zalko, personal communication). These opposing dose effects are consistent with the present study. Also, such an observation in the whole CNS could suggest that the effects reported here about a specific neuroendocrine function could be part of a much larger effect of BPA in the brain. Opposing dose effects of BPA are supported by in vitro studies of GABA-induced currents in hippocampal neurons that are inhibited or potentiated by high or low BPA concentrations, respectively (41). It has been shown that GnRH secretion can be regulated by GABA in two manners (42). Synaptic receptors generate fast (phasic) postsynaptic currents and extrasynaptic receptors generate a persistent (tonic) current controlling neuronal excitability. The latter GABA_A receptors accounting for tonic

current involve the delta subunit. Among the genes encoding the GABA_A receptor subunits, mRNA expression of Delta subunit is the only one repressed after exposure to 5 $\text{mg}/\text{kg}\cdot\text{day}$ of BPA and one among two subunit genes showing increased mRNA expression after 25 $\text{ng}/\text{kg}\cdot\text{day}$ of BPA (Table 2). This is consistent with dose-dependent dual effects of BPA on tonic GABA neurotransmission. Only a few studies addressed the neuroendocrine events that can precede or follow the changes in GABAergic tone (Supplemental Table 5). Either two separate mechanisms involving different factors or opposing variations of a common factor could explain the opposing effects of BPA on GABA neurotransmission. An insight into a possible upstream mechanism was recently provided by a study reporting that delayed neonatal maturation of a chloride cotransporter after in utero exposure to BPA contributes to activate GABA neurotransmission through reduction of intraneuronal chloride concentrations (40). The gene encoding this cotransporter (*Kcc2*) was not affected in our RNAseq study performed on PND20. Studies performed in younger animals with emphasis on epigenetic mechanisms are warranted because such mechanisms were found to account for BPA alteration of *Kcc2* expression (40) and DNA methyltransferase 1 mRNA expression was altered following BPA exposure in another study (39). In the present study, *DNMT1* mRNA expression in the female hypothalamus was found to increase significantly at PND 20 after exposure to 5 $\text{mg}/\text{kg}\cdot\text{day}$ of BPA (data not shown). Such effects consistent with involvement of epigenetic mechanisms could suggest that BPA has organizational together with activational effects. The issue is complex in the reproductive axis since some effects are likely occurring at other levels including the gonads (43, 44).

Changes in mRNA expression of the components of GABAergic neurotransmission (GAD, GAT, GABAR) can be viewed either as part of the altered GABAergic tone or as a downstream reaction to the altered GABAergic tone. In the first case, increased GAD and reduced GAT expression will be associated with increased GABAergic tone (and conversely) such as found for GAD mRNA in the basolateral amygdala (39). In the present study, the inverse observation was made since reduced GAD and increased GAT expression were associated with increased GABAergic tone (and conversely). Such findings can be consistent with effects downstream from the increased GABA tone because it was shown in neuronal culture that GABA could down-regulate GAD expression and activity (45) and up-regulate GAT activity (46). Confirmation of such an explanation would require a time-lapse study of GAD and GAT expression during and after exposure to different doses of BPA. A prerequisite to the interpretation of changes in GAD and GAT mRNA expression was to

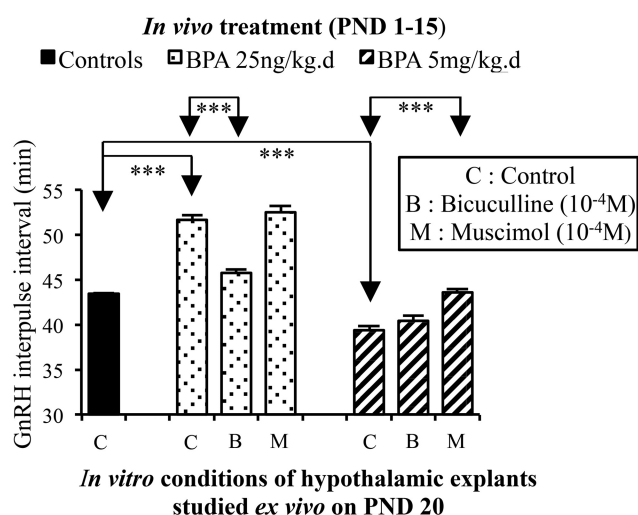


Figure 4. Effects of in vivo exposure to BPA on GABAergic regulation of GnRH secretion in vitro. GnRH IPI was calculated using secretion from hypothalamic explants obtained on PND 20 from female rats exposed in vivo to vehicle (corn oil) or 25 $\text{ng}/\text{kg}\cdot\text{day}$ or 5 $\text{mg}/\text{kg}\cdot\text{day}$ of BPA from PND 1 to 15. The in vitro conditions involve control or presence of a GABA_A receptor antagonist (bicuculline) or a GABA_A receptor agonist (muscimol) both used at 10^{-4} M concentration previously to be effective in similar conditions. A reduction in GnRH IPI by bicuculline and absent effects of muscimol indicated a high tone of GABAergic inhibition of GnRH release. Absence of bicuculline effects and increase in GnRH IPI by muscimol indicated a low tone of GABAergic inhibition of GnRH release. *** $P < .001$. Data are mean + SEM.

clarify whether the GABAergic control of GnRH secretion was increased or reduced. An answer was obtained indirectly using the GABA_AR agonist muscimol and antagonist bicuculline. Interestingly, Zhou et al (38) also found that the GABA_AR agonist was effective in conditions of BPA-induced reduction of GABAergic tone using another paradigm, whereas the antagonist was effective in control conditions. A body of literature shows that GABA is a major contributor to the prepubertal inhibition of GnRH secretion in several species (23). However, there is evidence of stimulatory GABA in adult rats and mice (47, 48) and in the fetal hypothalamus (49). Since GABA has dual effects on GnRH neurosecretion, one could hypothesize that BPA resulted in increased or reduced GnRH secretion through antagonism of inhibitory or stimulating GABA inputs. In such event however, the effects of muscimol and bicuculline would have been opposed. In addition, dual GABA effects could be determined by the developmental stage and the neuronal apparatus (GnRH neurons vs impinging regulatory neurons). Here, opposing effects were obtained in similar conditions of age and hypothalamic structure.

Based on RNAseq data, G-Protein-coupled receptors represent another mechanistic component that bioinformatics analysis identified as a candidate system possibly involved. This was particularly interesting given the critical stimulating role of GPR54 and its ligand kisspeptin in the neuroendocrine control of puberty and reproduction (50, 51). However, neither *Kiss1* nor *GPR54* mRNA expression appeared to be affected in the present RNAseq analysis. We reported recently that DES exposure (1 and 10 $\mu\text{g}/\text{kg}\cdot\text{day}$) reduced *KISS1* expression in the hypothalamus (18). In the present study however, exposure to BPA did not affect *KISS1* mRNA level in the mediobasal hypothalamus (data not shown). After neonatal exposure of female rats to 0.1 or 0.5 mg/kg.day of BPA for 5 days, Navarro et al (9) reported reduced *KISS1* mRNA expression on PND 30 and Patisaul et al (52) found reduced kisspeptin immunoreactivity in the arcuate nucleus after neonatal exposure to 50 mg/kg.day of BPA for 4 days. Because kisspeptin is a gatekeeper of GnRH neuron stimulation, these findings are in contrast to the early vaginal opening that was reported after neonatal or early postnatal exposure to BPA doses ranging 0.05–100 mg/kg.day (4, 7, 8). These discordant findings could suggest that BPA effects on kisspeptin are not causal in the mechanism of early sexual maturation and might be reactive to that process, such as discussed about GAT and GAD mRNA expression.

Several studies have reported an effect of BPA on pubertal onset in female rodents. Taken as a whole, those studies show that BPA effects depend markedly on the age

window and dose of exposure, with possible nonlinear dose-response relationship (2, 53). To our knowledge, our study is the first one demonstrating the impact of such a low and environmentally relevant dose of BPA on the neuroendocrine mechanism of sexual maturation. Twenty-five ng/kg.day of BPA affect the frequency of GnRH secretion and mRNAs expression of several hypothalamic genes. The effect of the very low dose of BPA on frequency of GnRH secretory pulses is robust and reproducible in our model. Moreover, it is consistent with the trend toward delayed VO that is seen subsequently. The amplitude of GnRH secretory pulses is not affected (data not shown). Using the ex vivo paradigm of hypothalamic explant, changes in amplitude of GnRH release were only apparent after in vitro stimulation with secretagogues such as glutamate or glutamate receptor agonists (21, 54). Since we aimed at changes in endogenously driven pulsatile secretion of GnRH in the present study, GnRH secretagogues have not been used.

The neuroendocrine system appears to be exquisitely sensitive to BPA endocrine disruption that may not be manifested phenotypically. When the female rats are postnatally exposed to 25 ng/kg.day for 5 or 15 days, age at puberty is not significantly different from controls but the frequency of GnRH secretion is significantly decreased. Presumably, the effects on maturation of GnRH secretion evidenced at PND 20 and 25 are not lasting enough or sufficient to affect significantly pubertal timing about ten days later. We made a similar observation in male rats exposed for 5 days after birth (2). Thus, absence of phenotypic changes in some of the previous studies does not necessarily mean absence of effect. Such high neuroendocrine sensitivity to BPA, functionally evidenced through changes in pulsatile GnRH secretion, is also consistent with a significant change in hypothalamic mRNA expression of 34 genes following exposure to that very low dose of BPA.

The study of mRNAs expression showed some limitations. Our RNAseq analysis was limited to 3 samples for each group and confirmed on 8 samples only for some of the genes involved in GABA synthesis, reuptake and signaling. However, the incubation of control or BPA-exposed hypothalamic explants with a GABA receptor agonist or antagonist robustly confirmed the effects of BPA on GABAergic neurotransmission. A short exposure of 5 or 15 days differs from the constant human exposure throughout life. However, such short exposures allow the delineation of a window of sensitivity to BPA. Also, the effects of BPA on puberty are unlikely to result from direct effects on target peripheral tissues such as the female genital tract because the exposure ended several weeks before puberty and BPA is a nonpersisting chemical. Accordingly,

the data are likely to reflect alterations of functions organized during early postnatal life.

Human exposure to BPA varies between 0.01 and 1 $\mu\text{g}/\text{kg}\cdot\text{day}$ (10). Because we have studied a very low dose of 25 $\text{ng}/\text{kg}\cdot\text{day}$, we chose subcutaneous injections in order to guarantee accuracy of the exposure. This route of administration was used as well in all the other studies with BPA exposure in early postnatal life (reviewed in (2)). While not relevant to the human route of exposure, subcutaneous injections have been shown to lead to similar serum concentration as oral administration (55). The question remains as to whether such a low level of exposure makes a difference from contamination through food, water and cages. We have attempted to control for this through housing conditions and the control animals exposed to corn oil were raised in similar conditions.

Postnatal life (5 to 15 first days after birth) has been shown to be particularly sensitive to the effects of BPA on the neuroendocrine mechanism of puberty (2). Further studies could underscore the significance of early postnatal life as a critical window by comparing the effects of a similar exposure to BPA in adulthood. Questions remain as to whether a longer and sustained exposure involving the whole prenatal life could result in effects similar to those reported here. Moreover, further studies should aim at delineation of the genetic mechanisms that take place upstream and downstream to GABA neurotransmission and account for divergent effects on pubertal timing.

In conclusion, our study shows that alteration of pubertal timing by BPA in the rat is not necessarily towards precocity. Instead, delayed maturation of GnRH secretion can be caused by environmentally relevant exposure with the mechanistic involvement of GABA neurotransmission. Taking together our data and the literature, it appears that the effects of BPA are not limited to the neuroendocrine control of the reproductive axis. BPA seems to affect GABA neurotransmission in multiple brain areas. Though rodent data do not necessarily apply as such to human health, the involvement of GABA shown in the present study points to the likelihood of detrimental effects of early life exposure to BPA in humans. GABA plays indeed a critical role in brain development and function and disorders of human behaviors including autism (56) that are possibly more frequent after early exposure to BPA (57–59). The US Environmental Protection Agency (EPA) has considered that a daily exposure lower than 50 $\mu\text{g}/\text{kg}\cdot\text{day}$ was “safe” (60). EFSA very recently proposed to set the daily tolerable intake at 4 $\mu\text{g}/\text{kg}\cdot\text{day}$ (61). This study reinforces the idea that defining a threshold for BPA effects is misleading not only because doses much below the so-called “safe” limit can interfere with the hormonal system

but also because a low and a high dose can have opposing effects.

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