Neonatal exposure to bisphenol A modifies the abundance of estrogen receptor α transcripts with alternative 5'-untranslated regions in the female rat preoptic area

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Abstract

The xenoestrogen bisphenol A (BPA) is commonly ingested by humans. We examined the effects of neonatal exposure to low versus high doses of BPA over the control of estrogen receptor α (ER α) expression in the preoptic area (POA) of prepubertal female rats. Pups received s.c. injections every 48 h of BPA (high dose, 20 mg/kg and low dose, 0.05 mg/kg) or diethylstilbestrol (DES, 0.02 mg/kg) from postnatal day (PND) 1 to PND7 and were killed at PND8 or PND21. Relative expression of ER α transcripts containing alternative 5'-untranslated regions OS, ON, O, OT, and E1 in POA were evaluated by RT-PCR. Methylation status of ER α promoters was determined by bisulfited DNA restriction analysis and ER α protein by immunohistochemistry. In PND8, the high dose of BPA and DES diminished total ER α mRNA levels,

mediated by the decreased expression of ER α -O and ER α -OT variants. In contrast, the low dose of BPA augmented total ER α mRNA by increasing the expression of the ER α -E1 variant. In PND21, both BPA doses increased total ER α mRNA by means of the augmented expression of ER α -O and ER α -OT variants. In PND21, the methylation status of the ER α promoters and the circulating levels of estradiol were similar in all experimental groups. At PND8 and PND21, DES and the high dose of BPA decreased, while the low dose of BPA increased ER α protein in the POA. These findings show that neonatal BPA exposure alters the abundance of hypothalamic ER α transcript variants and protein in a dose-dependent manner.

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Introduction

Bisphenol A (BPA), one of the most common environmental endocrine disruptors with estrogenic properties, is widely employed in the manufacture of plastics used in food packaging, dental composites, and sealants (Olea *et al.* 1996, Suzuki *et al.* 2000). BPA is not only widespread in the environment but also commonly ingested by humans. A recent study detected measurable BPA levels in 95% of the urinary samples analyzed from people in the United States (Calafat *et al.* 2005). A significant exposure of human fetuses has been reported due to BPA accumulation in maternal blood and in the amniotic fluid (Takahashi & Oishi 2000, Ikezuki *et al.* 2002, Schonfelder *et al.* 2002). Although actual BPA lowest observed adverse effects level (LOAEL) established by the National Toxicology Program's report (US Environmental Protection Agency 1993) is 50 mg/kg per day, there are several reports indicating that perinatal exposure to BPA in doses below LOAEL affects reproductive parameters in adult animals, such as neuroendocrine axis development (Khurana *et al.* 2000, Ramos *et al.* 2003), mammary gland and prostate morphology (Ramos *et al.* 2001, Durando *et al.*

2006), estrous cyclicity patterns (Rubin *et al.* 2001), and sexual behavior (Carr *et al.* 2003, Kubo *et al.* 2003, Negishi *et al.* 2004, Porrini *et al.* 2005, Fujimoto *et al.* 2006). This apparent controversy could be explained by the fact that BPA shows an inverted-U-shaped dose–response curve (Nishizawa *et al.* 2005, Wetherill *et al.* 2005, Zsarnovszky *et al.* 2005), where much of the effects observed with low-dose treatments are absent in traditional high-dose toxicological studies.

During perinatal brain development, estrogens are crucial for sexual differentiation of the central nervous system (Arnold & Gorski 1984). In mammals, α -fetoprotein protects the embryonic female brain from estrogenic action (Bakker *et al.* 2006) by strongly binding circulating estrogens. In contrast, the perinatal male brain is exposed to high levels of estrogen, synthesized locally through the aromatization of testicular testosterone (Weisz & Ward 1980, Corbier *et al.* 1992). These sex differences in hormone synthesis and exposure during this critical period result in the development of distinct male and female neuroendocrine circuits characterized by a differential expression of hypothalamic estrogen receptors (ERs; Simerly *et al.* 1990, Karolczak & Beyer 1998). In particular, the hypothalamic preoptic area (POA) contains

the anteroventral periventricular nucleus (AvPv) which is critical in the regulation of the phasic secretion of luteinizing hormone-releasing hormone in adult female rats (Polston & Simerly 2006, Wintermantel *et al.* 2006).

Akin to endogenous testosterone, the synthetic estrogen diethylstilbestrol (DES) and BPA show a very weak binding affinity to α -fetoprotein (Savu *et al.* 1979, Milligan *et al.* 1998, Nagel *et al.* 1999) and are therefore potential disruptors of hypothalamus development.

For many genes, it has been demonstrated that untranslated regions (UTRs) can differentially determine protein expression by influencing mRNA stability (Sharp & Bechhofer 2005, Wang *et al.* 2005) and translational efficiency (Gauss *et al.* 2006). These UTRs can determine tissue-specific expression through the regulatory action of multiple *cis*-acting motifs (Contractor *et al.* 2004). Some of the more frequent regulatory *cis*-acting motifs that are present close to the transcription start sites are the repetitive CG-rich elements called CpG islands. These regions act as molecular switches that activate or deactivate transcription initiation through differential methylation mechanisms (Sasaki *et al.* 2003, Champagne *et al.* 2006). In the rat, it has been described that a system of untranslated first exons is associated with the promoter selection for ER α transcription initiation, and to date five active promoters called OS, ON, O, OT, and E1 (Koike *et al.* 1987, Hirata *et al.* 1996a,b, Donaghue *et al.* 1999, Osada *et al.* 2001) have been identified. Little is known about the precise function of each ER α promoter; however, it has been reported that a mechanism of promoter selection is involved in the complex stage- and region-specific regulation of ER α gene expression in many organs, including the rat brain (Kato *et al.* 1998, Hamada *et al.* 2005).

Since ER α mRNA transcription and maturation could be differentially regulated by estrogens (Varayoud *et al.* 2005), it is very interesting to know whether high and low doses of xenoestrogens can modify the differential usage of ER α gene promoters in the rat hypothalamus. Using rats exposed early in life, we examined the effects of DES and BPA over the control of ER α transcription and translation in the hypothalamic POA of prepubertal female rats. Furthermore, we studied the relative abundance of ER α transcripts with alternative 5'UTR exons and the methylation status of ER α gene promoters in prepubertal female rats in response to xenoestrogen exposure.

Material and Methods

Animals and experimental design

Pups were obtained from timed-pregnant Wistar rats housed under a controlled environment ($22 \pm 2^\circ\text{C}$; lights on from 0600 to 2000 h) with free access to pellet laboratory chow (Cooperación, Buenos Aires, Argentina) and tap water supplied from glass bottles. All rats were handled in accordance with the principles and procedures outlined in

the Guide for the Care and Use of Laboratory Animals issued by the US National Academy of Sciences.

DES and BPA were purchased from Sigma–Aldrich. At least eight, and up to ten, timed-pregnant dams were used to collect offspring for each treatment (see below). At delivery (postnatal day 0, PND0), pups were sexed according to anogenital distance and cross-fostered, distributing pups of each litter between different mothers. These actions allowed us to minimize the use of siblings to avoid potential litter effects. The cross-fostered litters were adjusted to ten pups (five females and five males whenever possible) and assigned to one out of four experimental groups: corn oil vehicle-treated pups (control females and control males), DES (female pups injected with DES 0.02 mg/kg), BPA05 (female pups injected with a low dose of BPA 0.05 mg/kg), and BPA20 (female pups injected with a high dose of BPA 20 mg/kg). All pups received s.c. injections every 48 h from PND1 to PND7, with either 40 μl corn oil alone or 40 μl corn oil containing the appropriate amount of DES or BPA. Control males were used as a reference of natural masculinization of the POA and were treated as another experimental group. Based on the US Environmental Protection Agency (US EPA) estimate, the LOAEL for oral exposure to BPA in rats is 50 mg/kg per day (US Environmental Protection Agency 1993). Experts from the National Toxicology Program's subpanel suggested a cutoff dose of 5 mg/kg per day for low-dose effects, regardless of the administration route, the duration of exposure or the age/life stage at which exposure occurred (National Toxicology Program 2001 <http://ntp.niehs.nih.gov/ntp/htdocs/liason/LowDosePeerFinalRpt.pdf>). Taking into account the above-mentioned concepts, the high dose of BPA used in our study is four times bigger than the low-dose cutoff suggested by the US EPA experts and 2.5-fold smaller than the LOAEL; meanwhile, the low dose is 100 times smaller than the low-dose cutoff and is similar to the acceptable daily intake level established by the US Environmental Protection Agency (1993 <http://www.epa.gov/iris/subst/0536.htm>). No signs of acute or chronic toxicity were observed, and no significant differences in weight gain and anogenital distance between treated and control pups were recorded during the experiment (data not shown). Alterations in maternal care were not detected between experimental groups. Pups from a single mother were killed by decapitation on PND8 or PND21, trunk blood collected, and serum stored at -20°C until used for hormone assay. The number of animals per group at each time point evaluated was ~ 12 –14.

For immunohistochemistry, brains were microdissected and fixed by immersion in 4% paraformaldehyde for 24 h at 4 – 8°C . Fixed tissue was dehydrated in an ascending series of ethanol, cleared in xylene, and embedded in paraffin. Serial 5 μm thick frontal sections encompassing the medial POA in the coronal plane (Paxinos & Watson 2005) were mounted on 3-aminopropyl triethoxysilane (Sigma–Aldrich) coated slides and dried for 24 h at 37°C .

For nucleic acid extraction, brain tissue blocks containing the POA were quickly microdissected under a GZ6 series dissecting microscope (Leica Corp., Buffalo, NY, USA).

The POA fragment was encompassed by the anterior portion of the anterior commissure, the beginning of the ascending optical tracts, dorsally by the ventral portion of the anterior commissure, and laterally by a virtual line that is projected from the internal capsule to the external edge of the optical tracts (Paxinos & Watson 2005). After removal, tissue samples were immediately frozen in liquid nitrogen and stored at -80°C until used for RNA or DNA analysis.

RT and real-time quantitative PCR analysis

An optimized PCR protocol was employed to analyze the relative expression levels of total ER α mRNA and ER α transcripts containing the untranslated first exons OS, ON, O, OT, and E1 (see Fig. 1A and Table 1). Four pools of two

PND8 POA brain blocks ($n=4$) or four to six individual PND21 blocks ($n=4-6$) of each experimental group were homogenized in TRIzol reagent and total RNA was extracted following the manufacturer's protocol (Invitrogen). The concentration of total RNA was assessed by A_{260} and the sample was stored at -80°C until needed. Equal quantities ($4\text{ }\mu\text{g}$) of total RNA were reverse-transcribed for 90 min at 42°C using 200 pmol random hexamer primers (Promega), 100 nmol deoxy-NTPs, and 300 U Moloney Murine Leukemia Virus reverse transcriptase (MMLV-RT, Promega) in a final volume of $30\text{ }\mu\text{l}$ of $1\times$ MMLV-RT buffer. A sample without reverse transcriptase was included to detect genomic DNA contamination. Each reverse-transcribed product was diluted with RNase free water to a final volume of $60\text{ }\mu\text{l}$ and further amplified in triplicate using the Real-Time DNA

Figure 1 Outline of ER α promoter-specific real-time PCR and combined bisulfite restriction analysis (COBRA) procedures. (A) Genomic organization of the promoter region of the rat *ERα* gene. Relative positions and orientations of promoter-specific real-time PCR primers are indicated by black arrows. The common region to all the alternative 5'UTRs of ER α is indicated by a solid grey box. (B) In the COBRA procedure, bisulfite converts nonmethylated cytosines (C) to uracil leaving 5-methylcytosine (5^mC) unaltered. Predicted changes in restriction enzyme sites following reaction of genomic DNA with bisulfite are illustrated. (C) Positions and orientations of PCR primers used for COBRA are indicated by black arrows. CpG islands are depicted by lollipop signs, *Bst* UI, and *Taq* I restriction sites are indicated by white and grey arrows respectively.

Table 1 Primers and PCR products for real-time RT-PCR experiments

	Primer sequence (5′–3′)	Product size (bp)	Genbank accession no.
Gene			
<i>ERα</i>	Forward: AATTCTGACAATCGACGCCAG Reverse: GTGCTTCAACATTCTCCCTCCTC	345	NM_012689
<i>18sRNA</i>	Forward: CAACTTTCGATGGTAGTCGC Reverse: CGCTATTGGAGCTGGAATTAC	285	M11188
<i>ERα-OS</i>	Forward: CCGAAAACACAAGGCTCCATGCT	336	NW_047550
<i>ERα-ON</i>	Forward: CTGGGGCATCTCCTTCAATATG	304	NW_047550
<i>ERα-O</i>	Forward: GACTTCTACAAACCCATGGA	272	NM_012689
<i>ERα-OT</i>	Forward: CAGCAGGTTTGCGATGTCTAA	275	X98236
<i>ERα-E1</i>	Forward: TTAACTCGGGCTCTACTC	247	X98236
<i>Exon 1</i>	Reverse: GGGCTTGCTGTTGTCCACGTAC	–	NM_012689

Engine Opticon System (Bio-Rad Laboratories) and SYBR Green I dye (Cambrex Corp., East Rutherford, NJ, USA). Primer sequences used for amplification of *ERα*, *18sRNA*, and *ERα* promoters cDNA's are shown in Table 1.

For amplifications, 5 µl cDNA were combined with a mixture containing 2.5 U Platinum *Taq*-DNA polymerase (Invitrogen), 2 mM MgCl₂ (Invitrogen), 0.2 mM of each of the four dNTPs (Promega), 1 µl of 10×SYBR Green I, and 10 pmol of each primer (Invitrogen) in a final volume of 25 µl of 1×PCR buffer. Product purity was confirmed by dissociation curves and random agarose gel electrophoresis. Controls containing no template DNA were included in all assays, yielding no consistent amplification. The cycle threshold (*C_T*) for each sample was calculated using the Opticon Monitor Analysis Software (MJ Research, Bio-Rad Laboratories) with an automatic fluorescence threshold setting. Calculation of relative expression levels of each target was conducted based on the 2^{−ΔΔCT} method (Higuchi *et al.* 1993). The efficiency of PCRs was assessed for each target by amplification of serial dilutions (over five orders of magnitude) of cDNA fragments of the transcripts under analysis. No significant differences in *C_T* values were observed for *18sRNA* between the different experimental groups. All PCR products were cloned using the TA cloning kit (Invitrogen) and specificity was confirmed by DNA sequencing (data not shown).

DNA extraction and bisulfite modification

DNA was isolated from pools of three POA tissue blocks of each PND21 group using the Wizard Genomic DNA Purification Kit (Promega). DNA (2 µg) in a volume of 50 µl was denatured with NaOH for 20 min at 37 °C. Thirty microliters of 10 mM hydroquinone (Sigma–Aldrich) and 520 µl of 3 M sodium bisulfite (Sigma–Aldrich) at pH 5, both freshly prepared, were added and mixed, and samples were incubated in the dark under a mineral oil layer at 55 °C for 16 h. Modified DNA was purified using Wizard DNA purification resin according to the manufacturer (Promega) and eluted into 50 µl preheated water (65 °C). Modification was completed by NaOH treatment for 15 min at 37 °C and followed by overnight ethanol precipitation. DNA was dissolved in 20 µl DNase free water and used immediately or stored at −20 °C.

Combined bisulfite restriction analysis (COBRA)

In COBRA (Xiong & Laird 1997), restriction enzyme digestion is employed to reveal DNA methylation-dependent sequence differences (Fig. 1B) in PCR-amplified bisulfite-treated genomic DNA. Using the MethPrimer software (Li & Dahiya 2002), we identified CpG-rich fragments (CpG islands) on the stage- and region-specific active *ERα* gene promoters O and OT, and in *ERα* gene exon 1 (Fig. 1C, see Results) but not on promoter E1 or on the inactive promoters

Table 2 Primers and PCR product for combined bisulfite restriction analysis experiments

	Primer sequence (5′–3′)	Product size (bp)
Gene		
<i>ERα-O</i>	Forward: ATGTTGATTTAGTGGTGTATTGT Reverse: TCCAAAAATATTCCATAAATTTATAAAAAAT	296
<i>ERα-OT</i>	Forward: AAAAAGAAAGAAAAGAAATTAGTTTGTG Reverse: CTAATCTCCCCTATTCTTAAACATC	281
<i>Exon 1</i>	Forward: TTTTGTGTATTAGATTTAAGGGAA Reverse: AAAAAAACCCTCCAACTATTAAC	272

OS and ON. For COBRA purposes, these CpG islands were checked to contain at least one restriction site for *Bst* UI and/or *Taq* I enzymes (Fig. 1C). PCR primers were designed to encompass CpG islands and to complement the bisulfite-converted DNA (Fig. 1C, Table 2). The PCR mixture contained 2 mM MgCl₂ (Invitrogen), 0.2 mM of each of the four dNTPs (Promega), 10 pmol of each primer (Invitrogen), and bisulfite-modified DNA (1 µl) in a final volume of 25 µl of 1×PCR buffer. Reactions were hot-started at 95 °C for 10 min before the addition of 2.5 U *Taq*-DNA polymerase (Invitrogen). Amplification was carried out for 36 cycles (45 s at 95 °C, 45 s at 59 °C, and 1 min at 72 °C), followed by a final 2 min extension at 72 °C. Controls with unmodified DNA and without DNA were performed for each set of primers. To ensure complete digestion of the PCR products in the subsequent restriction digestion step, each PCR product (20 µl) was loaded onto 2% agarose gel containing ethidium bromide (Sigma–Aldrich), quickly visualized under u.v. illumination and purified using the Wizard SV Gel and clean-up System kit according to the manufacturer's protocol (Promega). Fifteen microliters of each purified PCR product was then digested separately with *Bst* UI (recognition site CGCG) and *Taq* I (recognition site TCGA). Cleavage will occur only if the CG sequence has been retained during the bisulfite conversion by a methylated cytosine residue (Fig. 1B). To ensure that the bisulfite conversion was complete, a control digest was performed with *Mse*I (recognition site TTAA, data not shown). The molecular sizes of PCR and restriction products were determined by comparison with DNA standard size markers (Cien Marker, Biodynamics, Buenos Aires, Argentina).

ERα expression detected by immunohistochemistry

A standard immunohistochemical technique (avidin–biotin–peroxidase) was used to visualize ERα immunostaining intensity and distribution following a previously described protocol (Munoz de Toro *et al.* 1998, Varayoud *et al.* 2005). Overnight incubation at 4 °C was performed by applying a diluted mouse monoclonal antibody raised to full-length recombinant human ERα (working dilution 1:200; clone 6F-11, Novocastra, Newcastle upon Tyne, UK), followed by incubation with a biotinylated secondary antibody (working dilution 1:80; Sigma–Aldrich). Negative controls were obtained by replacing the primary antibody with nonimmune mouse serum (Sigma–Aldrich). The reaction was then developed using diaminobenzidine-nickel (Sigma–Aldrich) as a chromogen substrate. Samples were dehydrated and mounted with permanent mounting medium (PMYR, Buenos Aires, Argentina).

Quantitation of ERα expression in the AvPv of POA by image analysis

Image analysis was performed using the Image Pro-Plus 4.1.0.1 system (Media Cybernetics, Silver Spring, MA, USA) as

previously described (Ramos *et al.* 2002). In brief, the images were recorded by a Spot Insight V3.5 color video camera, attached to an Olympus BH2 microscope (illumination, 12 V halogen lamp and 100 W, equipped with a stabilized light source), using a D-plan 10× objective (NA=0.25). The microscope was set up properly for Koehler illumination. Correction of unequal illumination (shading correction) and the calibration of the measurement system were done with a reference slide. Four brains from PND8 killed pups (*n*=4) and five brains from PND21 prepubertal animals (*n*=5) were scored in each experimental group. Typically, six or more sections of a one-in-six series were scored and averaged per animal. The images of immunostained slides were converted to a grey scale, the AvPv nucleus of POA was delimited according to Paxinos & Watson (2005). The integrated optical density (IOD) was measured as a linear combination between the average grey intensity and the relative area occupied by positive cells. This morphometric approach provides more reproducibility, since relative spatial variables are independent of oversampling errors that would be incurred in calculating the absolute volumes and total cell numbers (Gundersen *et al.* 1988, Palmer *et al.* 2000, Ramos *et al.* 2001).

Hormone assay

17β-estradiol (E₂) concentrations were determined by RIA using [2,4,6,7,16,17-³H(N)]E₂ (Perkin–Elmer Life and Analytical Sciences, Boston, MA, USA) and a specific antibody provided by Dr G D Niswender. Pools consisting of two individual PND21 serum samples were made, and five pools of each experimental group were used. The sensitivity of the assay was 12.5 pg/ml. Intra- and inter-assay coefficients of variation were 3.38 and 2.12% respectively.

Data analysis

Statistical analyses were performed using two-tailed *t* tests. Each experimental group was compared against control females only, and differences were considered significant at *P*<0.05.

Results

Effects of low and high doses of BPA on the preoptic ERα mRNA expression

In PND8, control females showed higher expression of ERα mRNA in the hypothalamic POA than control males (*P*<0.05, Fig. 2A). Females exposed to DES were defeminized and ERα mRNA expression was significantly reduced and similar to male pups (*P*<0.01, Fig. 2A). BPA treatment showed opposite and dose-dependent effects: in BPA20-treated females ERα mRNA expression levels decreased about fourfold when compared with control females (*P*<0.001). In contrast, females exposed to the low dose of BPA

Figure 2 Effect of neonatal xenoestrogen exposure on ERα mRNA expression levels in the female rat preoptic area on PND8 (A) and PND21 (B). Relative mRNA levels were measured by real-time RT-PCR and fold expression from control values were calculated for each target by equation $2^{-\Delta\Delta CT}$. Control values were assigned to a reference level of 1 and values are given as mean $\pm$ S.E.M. of at least three independent determinations (* $P < 0.05$ versus control females).

(BPA05) increased 1.4-fold their hypothalamic ERα mRNA content ($P < 0.05$, Fig. 2A).

To know if the alterations observed in PND8 were of a transient or permanent nature, we assessed ERα mRNA expression 2 weeks after the end of the postnatal treatment. On PND21 – in contrast from the PND8 findings – the hypothalamic ERα mRNA content in control females, control males, and DES-treated females was similar (Fig. 2B). Surprisingly, on PND21 in both BPA-exposed groups, ERα mRNA expression was clearly augmented ($P < 0.05$, Fig. 2B) in a dose-independent fashion. It is interesting to note that the high levels of ERα mRNA expression observed on PND8 in BPA05-treated females persisted up to PND21, increasing to just about 2.1-fold ($P < 0.05$, Fig. 2B) when compared with female controls.

BPA changes the relative abundance of ERα transcripts with alternative 5'UTRs

To determine whether total POA ERα mRNA expression changes were associated with changes in transcriptional promoter usage, relative expression levels of all exons encoding 5'UTR of the rat ERα gene were studied using a

Figure 3 Promoter usage of ERα gene on the rat preoptic area (POA). Representative ethidium bromide-stained gel electrophoresis of ERα mRNA species containing alternative 5'UTRs in PND8 and PND21 rats' POA. No transcripts from the OS and ON promoters were detected in any experimental group. Uterine cDNA from an intact adult female rat was used as a positive control of promoter-specific PCRs.

real-time PCR approach. Specific sense primers for ERα 5'-untranslated exons OS, ON, O, OT, E1 and a common antisense primer specific for ERα exon 1 were designed (Table 1, Fig. 1A). Optimal reaction conditions for all transcript variants were first evaluated in cDNA from an intact adult female uterus and in all reactions, a single band of the expected size was obtained (Fig. 3). We determined that ERα gene transcription in POA is regulated by means of promoters OT, E1, and O (Fig. 3), despite age or treatment group. Expression of transcripts containing the ON or OS exons could not be detected in POA at any time point studied.

At PND8, ERα 5'UTR variant expression in the POA showed a great sexual dimorphism, since control females showed higher levels of ERα-OT, ERα-O, and ERα-E1 transcripts than male pups ($P < 0.05$, Fig. 4A–C). DES-treated females presented diminished ERα-OT and ERα-O transcription rates when compared with female controls ($P < 0.05$, Fig. 4A and B), together with a significant 1.6-fold increase in the ERα-E1 transcript variant expression ($P < 0.05$, Fig. 4C). BPA exposure showed a dose-dependent perturbation of ERα 5'UTR variant expression. When compared with female control pups and like DES-treated rats, the high dose of BPA (BPA20) clearly diminished ERα-OT and ERα-O transcript content ($P < 0.01$, Fig. 4A and B) and augmented the ERα-E1 variant expression ($P < 0.05$, Fig. 4C). Females exposed to the low dose of BPA (BPA05) showed no changes in the ERα-OT and ERα-O transcript content and only a 1.6-fold significant rise in the ERα-E1 transcript variant ($P < 0.05$, Fig. 4A–C).

In prepubertal PND21 females, a different pattern of ERα transcript variants was detected following xenoestrogen exposure. ERα-O and ERα-OT transcripts were significantly increased in DES- ($P < 0.01$), BPA20- ($P < 0.01$), and BPA05- ($P < 0.005$, Fig. 4D and E) treated females. Regarding the

Figure 4 Effect of neonatal xenoestrogen exposure on the relative abundance of ERα transcripts with alternative 5'UTRs in the female rat preoptic area on PND8 (A–C) and PND21 (D–F). Relative mRNA levels of the stage- and region-specific promoters ERα-O (A and D), ERα-OT (B and E), and ERα-E1 (C and F) were measured by real-time RT-PCR and fold expression from control values were calculated for each target by equation $2^{-\Delta\Delta CT}$. Control values were assigned to a reference level of 1 and values are given as mean $\pm$ S.E.M. of at least three independent determinations (* $P < 0.05$ versus control females).

exon-E1 mRNA variant, a twofold decrease in BPA20- ($P < 0.01$) and BPA05- ($P < 0.05$) treated animals were found (Fig. 4F). It is important to note that at this time point, no differences between male and female controls were observed in ERα 5'UTR variant transcription rates (Fig. 4D–F).

Methylation status of ERα transcriptional promoters in prepubertal female rats

To find out if DES- and BPA-induced changes in alternative 5'UTR ERα transcript levels are being caused through a DNA methylation mechanism, we used a COBRA approach to determine the methylation status of transcriptionally active promoters in prepubertal PND21 animals. In all experimental groups, exon 1 and promoter ERα-O were completely unmethylated, while the ERα-OT promoter presented a constitutive, treatment-independent, low level of methylation in the CpG island, as evaluated by *Bst* UI restriction analysis (Fig. 5). We could not find differences in the CpG methylation status of active ERα promoters between experimental groups.

Differential ERα protein expression in the AvPv

To determine if alterations prompted by postnatal xenoestrogen exposure in ERα mRNA expression were extensive in the ERα protein, we determined the relative ERα protein levels in PND8 and PND21 rats in neurons from the AvPv

nucleus (Fig. 6A–J). On PND8, ERα protein levels were significantly reduced in the AvPv ($P < 0.005$, Fig. 6K) of male pups and in DES- and BPA20-treated females when compared with female controls. In contrast, the AvPv of females exposed to the low dose of BPA showed a clear increase in ERα protein expression ($P < 0.001$). Two weeks after exposure, ERα expression levels remained significantly

Figure 5 Determination of the methylation status of promoter's ERα-OT CpG island. (A) Representative ethidium bromide-stained gel electrophoresis of a COBRA assay with the restriction enzyme *Bst* UI; no differences could be observed in the methylation pattern of the ERα-OT region between experimental groups. (B) Map of the PCR product with the sizes of the predicted digestion products. There are two consecutive methylation sensitive *Bst* UI sites in the 281 bp PCR product.

Figure 6 Effects of neonatal xenoestrogen exposure on ER α translation in the AvPv nucleus of the POA. (A–J) Representative photomicrographs of immunohistochemical detection of ER α in the AvPv nucleus of PND8 (A–E) and PND21 (F–J) in female rats. The AvPv nucleus was delineated in accordance with Paxinos & Watson (2005). Scale bar, 300 μ m; 3V, third ventricle; ac, anterior commissure; oc, optic chiasm. (K and L) Quantification of the results obtained by immunohistochemistry in the AvPv of PND8 (K) and PND21 (L) animals. Data are expressed as IOD, which is measured as a linear combination between the average immunostained intensity and the relative area occupied by positive cells in the AvPv. Each column represents the mean $\pm$ S.E.M. of at least six semi-serial sections per animal of four (PND8) or five (PND21) animals per group (* P < 0.05 versus control females).

reduced in male controls, as well as DES- and BPA20-treated females (P < 0.001, Fig. 6L), whereas in BPA05-treated rats, ER α IOD levels were still significantly increased with respect to female controls (P < 0.05).

E₂ serum levels remain unaltered in prepubertal females postnatally exposed to BPA and DES

No differences in E₂ serum levels were observed between prepubertal PND21 rats of all experimental groups (Fig. 7).

Note that control female and control males E₂ serum levels were similar at this time point.

Discussion

To our knowledge, this is the first study showing that neonatal BPA exposure affects the relative abundance of alternative 5'UTRs ER α transcripts in the female rat POA. Furthermore, female rats exposed to the high dose of BPA downregulated the expression of total ER α mRNA, while

Figure 7 Estradiol serum levels in prepubertal animals. Blood was collected on PND21 and analyzed for 17 β -estradiol. Each value is the mean $\pm$ S.E.M. of five serum samples per treatment group.

females exposed to a dose of 400 times smaller presented the opposite effect. Two weeks after the end of the xenoestrogen exposure, the ER α mRNA hypothalamic expression remained significantly high in both BPA-treated groups, suggesting a long-term effect. Alterations caused by early postnatal BPA exposure are not restricted to ER α transcription, but are extensive in ER α translation in the sexually dimorphic AvPv nucleus of POA. When female rats were analyzed immediately after the end of the high-dose BPA exposure (PND8), an abrupt decrease in ER α protein levels was observed. Surprisingly, the low-dose BPA exposed animals showed a significant upregulation of the ER α protein, clearly showing an opposite dose-dependent effect of BPA on the transcription and translation control of the ER α gene in the female POA. Taken together, the above-mentioned findings suggest that the high dose of BPA, like DES treatment, provoke defeminization of ER α expression in the POA of female offspring, which is consistent with the estrogenic nature of BPA (Olea *et al.* 1996). Remarkably, the opposite effect was seen in the low dose-exposed females, suggesting that the estrogenic action of BPA is an emergent property that depends on the experimental context complexity. In lower complexity contexts, like *in vitro* experimental conditions, the estrogenic or antiestrogenic qualities of a substance are robustly determined; however, in *in vivo* conditions, the estrogenic or antiestrogenic action is an emergent phenomena dependent on multiple variables like doses, end-points evaluated, animal genomic background, etc. (vom Saal & Hughes 2005). Specifically, the results obtained in the high-dose BPA group on PND21 could indicate an over compensation mechanism that take place in response to the initial ER α reduction observed on PND8. In a previous work (Ramos *et al.* 2007), we found a similar over-compensation mechanism in the control of estrogen-dependent genes in the hippocampus of rat neonatally exposed to xenoestrogens. These results suggest the existence of an internal regulatory mechanism to compensate an initial

xenoestrogen-induced declination of estrogen-dependent gene transcription.

A possible mechanism involved in the xenoestrogen-mediated ER α disruption could include differential promoter usage, as evidenced by changes in the relative abundance of alternative spliced 5'UTR transcripts (Kato *et al.* 1998, Hughes 2006). Our results show that the reduction in ER α mRNA expression observed in newborn females exposed to the high dose of BPA is correlated with low levels of ER α -O and ER α -OT transcripts. Otherwise, in newborn females exposed to the lower dose of BPA, the increase observed on ER α transcription could be explained by the increment in transcripts from promoter ER α -E1 and the unaltered transcription levels from promoters ER α -O and ER α -OT. It is interesting to note that even though newborn females exposed to the high dose of BPA also presented an increment in transcripts from promoter ER α -E1, which does not seem to be enough to balance the low levels of promoters O and OT activity. On PND21-treated females, both doses of BPA induced an approximately twofold upregulation in total ER α mRNA levels, which was mediated by means of the increased expression of ER α -O and ER α -OT variants. These results strongly suggest that the ER α -OT and ER α -O promoters are very important in ER α transcription control during the early postnatal period in hypothalamic structures.

The selective promoter usage in estrogen-mediated ER α transcriptional regulation has been demonstrated in several breast cancer cell lines (Donaghue *et al.* 1999) and in rat brain (Hamada *et al.* 2005). Moreover, differential promoter activity has been shown to be a key regulator of tissue-specific ER α expression (Kato *et al.* 1998, Donaghue *et al.* 1999). Here, we suggest that the alternative promoter usage of the ER α gene is regulated by BPA exposure in a dose-sensitive manner and therefore this mechanism could be, at least in part, responsible for the different and sometimes contradictory effects observed in BPA action on gene transcription (Khurana *et al.* 2000, Kwon *et al.* 2000, Aloisi *et al.* 2001, Ramos *et al.* 2003, Patisaul *et al.* 2006). Since sexual differentiation of the hypothalamus is estrogen-dependent and mediated at least partly through ER α (Simerly *et al.* 1997), BPA deregulation of this receptor could affect the natural estrogen-directed dimorphisms of POA circuits. In a previous work performed in our laboratory, it has been demonstrated that low and high doses of estrogen can differentially regulate the splicing of the rat ER α mRNA coding region (Varayoud *et al.* 2005). Selective promoter usage and alternative mRNA splicing events are estrogen-dependent mechanisms of ER α gene variation, arising as sources of complexity in the endocrine disruptor's toxicology. In every particular experimental paradigm, it is very difficult to predict if BPA-mediated disruptions would be similar to those observed in natural or synthetic estrogen exposure experiments (vom Saal & Hughes 2005, vom Saal & Welshons 2006). The results obtained here clearly show that BPA acts as a classical estrogen (like DES), defeminizing the ER α protein expression of females' POA only in the high-dose experiment, whereas the low-dose

effect is exactly the opposite. These events could be associated with the BPA property to modulate different promoter regions of regulatory genes (like *ERα*) in a dose-dependent manner. The molecular mechanism underlying this promoter selection is not known and is under study in our laboratory. The sequences of the *ERα*-O and *ERα*-OT promoters possess a small 5'-upstream open reading frame (5'upORF). Several regulatory roles have been assigned to these 5'upORF, such as limiting the protein expression by a translation reinitiation mechanism (Kos *et al.* 2002, Blaschke *et al.* 2003). Further research will help to elucidate if any of these mechanisms are involved in the xenoestrogen regulation of ERs in the rat hypothalamus.

Early developmental perturbations due to xenoestrogen exposure have been linked to the adult-onset of reproductive pathologies, including cancer (Munoz-de-Toro *et al.* 2005, Durando *et al.* 2006); however, the molecular basis for this imprinting event is not known. A recent report showed that neonatal estradiol or BPA exposure causes early and prolonged hypomethylation of the 5'-flanking region of the phosphodiesterase type 4 gene (*PDE4D4*) in the rat prostate gland, resulting in continued and elevated *PDE4D4* expression (Ho *et al.* 2006). Using COBRA, we could not find any differences in the methylation status of the active *ERα* promoters between xenoestrogen-treated and control groups, suggesting that DES and BPA alter the hypothalamic *ERα* gene transcription control using a methylation-independent mechanism.

The results presented here are in accordance with a recently published report where it was evidenced that exposing female rats during the first PNDs to a high dose of BPA (100 mg/kg per day) causes a significant decrease in tyrosine-hydroxylase (TH) and *ERα* double-labeled neurons in the AvPv (Patisaul *et al.* 2006). In another work, CD-1 mice treated with low doses of BPA (25 and 250 ng/kg per day) from day 8 of gestation through day 16 of lactation clearly decreased the number of TH neurons in the AVPV (Rubin *et al.* 2006). Moreover, the fact that *E*₂ serum levels in prepubertal females are not affected by postnatal BPA exposure suggests that the disruptions observed 2 weeks after the end of xenoestrogen treatment could be attributed to an organizational disruption in the transcription control of estrogen-sensitive genes like *TH* and *ERα* rather than disturbances in the endogenous estradiol.

Our findings are consistent with an inverted-U-shaped dose-response curve previously reported for BPA hormone action (Nishizawa *et al.* 2005, vom Saal & Hughes 2005, Wetherill *et al.* 2005, Zarnovszky *et al.* 2005). Alarming, the lower BPA concentration used in this study (50 µg/kg) falls in the expected human exposure range, as microgram quantities of BPA were detected in urinary samples of a reference human population (Calafat *et al.* 2005) and in early pregnancy amniotic fluid (Ikezaki *et al.* 2002, Engel *et al.* 2006). This is of particular concern to fetus health, where subtle alterations will probably remain undiscovered until the onset of sexual maturity. It was clearly shown that *in utero* exposure to

environmental doses of BPA affects the hypothalamic-pituitary axis, increasing POA *ERβ* mRNA expression and prolactin levels in male offspring as well as LH secretion and estrous cyclicity in females (Rubin *et al.* 2001, Ramos *et al.* 2003). Moreover, xenoestrogen exposition in newborns is expected to continue as BPA is released from polycarbonate baby bottles (Brede *et al.* 2003) and is also present in regular powdered infant and follow-up formulas (Kuo & Ding 2004).

Our results show that BPA exposure during the early postnatal period alters the abundance of hypothalamic *ERα* transcript variants and protein in a dose-dependent manner. Based on present data and previous reports, we could suggest that xenoestrogen exposure during critical periods of development disrupt hypothalamic gene expression, interfering with the normal maturation of sexually dimorphic areas of the forebrain. Actually, we are evaluating the consequences of the xenoestrogen-induced *ERα* disruption on the gonadotropin-releasing hormone neurocircuitry.

In summary, a comprehensive analysis is needed to evaluate the potential hazards to humans and wildlife from exposure to BPA at doses below the prior LOAEL dose of 50 mg/kg per day.

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