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Exposure of neonatal female rats to bisphenol A disrupts hypothalamic LHRH pre-mRNA processing and estrogen receptor alpha expression in nuclei controlling estrous cyclicity

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ABSTRACT

This study examines the effects of neonatal exposure to the endocrine disruptor bisphenol A (BPA) on the neural network that controls estrous cyclicity. From postnatal day 1 (PND1) to PND7, female pups were injected with vehicle (control) or BPA (BPA.05: 0.05 mg/kg-d, BPA20: 20 mg/kg-d). At PND100 BPA.05-females showed alterations in estrous cyclicity and BPA20-females were incapable of producing an estradiol-induced LH surge. By real-time PCR we determined that hypothalamic expression of mature LH-releasing hormone (LHRH) mRNA was increased in BPA.05 and decreased in BPA20-females. Furthermore, unprocessed intron A-containing LHRH RNA was decreased in the cytoplasm of hypothalamic cells of both groups. Immunohistochemistry revealed that estrogen receptor alpha protein was up-regulated in anteroventral periventricular and down-regulated in arcuate nucleus of both groups. Our results show that BPA permanently disrupts hypothalamic LHRH pre-mRNA processing and steroid receptors expression in nuclei that control estrous cyclicity in adult rats.

1. Introduction

Bisphenol A (BPA) is a monomer widely employed in the manufacture of polycarbonate plastics and resins. Recent reports note that humans are significantly exposed to this compound during critical stages of tissue organization and development [1–4]. Although the actual lowest-observed-adverse-effects-level (LOAEL) for BPA established by the United States Environmental Protection Agency (US EPA) is 50 mg/kg-d [5], there are several reports indicating that perinatal exposure to BPA in doses below LOAEL affects reproductive health in adulthood [6–10]; clearly showing that the concept of LOAEL as currently used is inadequate, since some environmental endocrine disruptors can cause perturbations of physiological systems when exposures occur during critical developmental windows. Furthermore, there is evidence that younger animals metabolize BPA in a less efficient way [11,12], resulting in higher circulating levels of this compound and implying that oral and non-oral administration of BPA during neonatal life provide the same internal active dose [13].

During critical periods of embryonic and postnatal development, the hormonal milieu is crucial for the correct organization of neuroendocrine circuits that coordinate sex-specific physiology [14]. In the normal cycling rat, a spontaneous surge of luteinizing hormone (LH) occurs in the afternoon of the day of proestrous. This surge can also be induced by the administration of exogenous estrogen in ovariectomized rats [15]. The release of this spontaneous LH preovulatory surge is triggered by the neurosecretion of LH-releasing hormone (LHRH), also known as gonadotropin-releasing hormone (GnRH), which is dependent upon two major neuroendocrine determinants: the positive feedback actions of estrogens and a neural signal generated by the 24 h neural clock [16]. In rats, the majority of LHRH-secreting neurons are located in the preoptic area of the hypothalamus [17]. Studies on LHRH gene structure have indicated that it consists of four short exons (denoted as 1, 2, 3, and 4) and three intervening introns (A, B and C) [18]. The translation start site of the LHRH gene resides in exon 2, which encodes a signal peptide, the LHRH decapeptide, and a part of the LHRH-associated peptide. Exons 3 and 4 encode the remaining part of the LHRH-associated peptide and the 3′-untranslated region [19]. In LHRH-producing neurons, all three introns are efficiently excised from the primary gene transcript, resulting in a mature LHRH mRNA [20,21]. *In vitro* studies have shown that introns B and C are easily excised from the LHRH primary transcript, but intron A is not [22,23]. A recent report revealed that the precise and efficient excision of intron A, but not B or C, serves as a key regulatory step for the post-transcriptional regulation of LHRH biosynthesis [24].

Activation of steroid receptors in specific hypothalamic regions like the anteroventral periventricular nucleus (AvPv) and the arcu-

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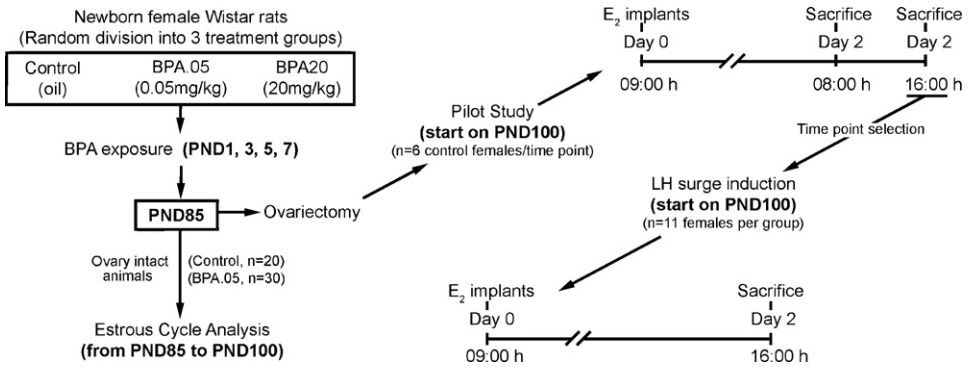

Fig. 1. Schematic representation of experimental protocol.

ate nucleus (Arc) is also necessary to achieve a normal LH surge. Estrogen receptor alpha (ERα), but not ERβ, expression in the AvPv is necessary to generate estrogen's positive feedback response in LHRH neurons [25]. Activation of progesterone receptor (PR) in the AvPv is also a required event in the estrogenic stimulation of LHRH surges [26]. Furthermore, steroid-sensitive projections from the Arc to the medial preoptic area are likely to be involved in the estrogen-mediated negative feedback control of LH secretion [27]. Moreover it has been demonstrated that ERα expression is essential to the regulation of this mechanism [28]. The mechanisms of action of ERα and PR involve a tripartite repertoire, including the receptors, their ligands, and their coregulator proteins. Two key nuclear receptor-associated cofactors are the steroid receptor coactivator 1 (SRC-1) and the repressor of estrogen receptor activity (REA). SRC-1 was the first steroid receptor coactivator to be identified and cloned; this coactivator enhances transcriptional activity of ERα and PR *in vitro* [29,30] and is expressed in a variety of hormone-responsive tissues, including the brain [31,32]. On the other hand, REA is a corepressor that specifically interacts with ERα and suppresses its transcriptional activity [33,34].

In previous reports, we demonstrated that neonatal BPA exposure alters the abundance of hypothalamic ERα transcript variants and ERα protein in prepubertal female rats [8] as well as ERα and REA protein levels in the ventromedial hypothalamic nucleus of adult female rats [10]. Here, using rats exposed to BPA early in life, we examined the effects of low and high doses of BPA on the regulation of LHRH pre-mRNA processing and the expression of ERα, PR and their cofactors (SRC-1 and REA) in hypothalamic nuclei involved in the regulation of estrous cyclicity in adult female rats.

2. Materials and methods

2.1. Chemicals

BPA and estradiol-17β were purchased from Sigma–Aldrich (St. Louis, MO, USA).

2.2. Animals and experimental design

Pups were obtained from timed-pregnant rats of an inbred Wistar-derived strain bred at the Department of Human Physiology (Santa Fe, Argentina). Animals were maintained under a controlled environment (22 ± 2 °C; lights on from 06:00 to 20:00 h) with free access to pellet laboratory chow (Cooperación, Buenos Aires, Argentina) and tap water supplied *ad libitum* in glass bottles with rubber stoppers surrounded by a steel ring. 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. The concentration of phytoestrogens in the diet was not evaluated; however, because feed intake was equivalent between control and experimental rats [35], we assumed that all animals were exposed to the same level of phytoestrogens. To minimize additional exposure to endocrine-disrupting chemicals, rats were housed in stainless steel cages with wood bedding. Clusters of timed-pregnant dams ($n = 16$) were used to collect offspring for all treatments (see below). At delivery (postnatal day 0, PND0), pups were sexed according to anogenital distance then cross-fostered by distributing pups of each litter between different mothers. These actions allowed us to minimize the use of siblings and avoid potential litter effects. To avoid a confounding effect of litter size/ratio, only litters with no less than 8 and no more than 12 pups with a sex ratio (n of females/n of males) higher than 0.7 or below 1.5 were used. Cross-fostered litters were assigned to one of three experimental groups: corn oil vehicle-treated pups (control), BPA.05 (pups injected with 0.05 mg/kg of BPA) or BPA20 (pups injected with 20 mg/kg of BPA). BPA was dissolved in charcoal-stripped corn oil (Sigma) and all pups received s.c. injections of either 40 μl of corn oil alone or 40 μl of corn oil containing the appropriate amount of BPA every 48 h from PND1 to PND7. Doses of BPA were selected using previously described criteria [8,10]. In brief, the low dose of BPA used is 100 times lower than the low-dose cutoff and is similar to the acceptable daily intake level established by the US EPA [5,36]. The high dose of BPA used herein is four times bigger than the low-dose classification cutoff suggested by NTP experts [36] and 2.5-fold lower than the US EPA LOAEL [5]. As previously reported [8,10], no signs of acute or chronic toxicity were recorded among pups and no alterations in maternal behavior were observed among foster mothers (data not shown). Pups were weaned at PND21, and females were housed (4–5 rats per cage) and given commercial pellets and tap water *ad libitum*.

2.3. Vaginal cytology

Previous studies reported that female pups exposed to doses ranging from 1.2 mg/kg BPA [37] to 62.5 mg/kg BPA [38] exhibit altered estrous cycles during adulthood. To assess whether the low dose of BPA used in the present study (0.05 mg/kg of BPA) alters estrous cyclicity, vaginal smears were taken daily from control and BPA.05 females starting at PND85 for two weeks (at least 20 animals per group, Fig. 1).

2.4. Pilot study of estradiol-17β induction of LH surge in ovariectomized rats

To test the effectiveness of the LH surge induction model used in this work, a set of control females ($n = 12$) underwent bilateral ovariectomy (OVX) at PND85 under light ether anesthesia followed by 14 days of recovery. Segments of Silastic tub-

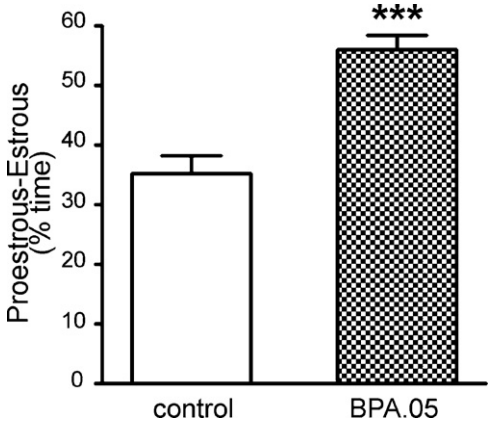

Fig. 2. Estrous cyclicity in females neonatally exposed to a low dose of BPA (0.05 mg/kg-d). Stages of estrous cycle were determined by daily examination of vaginal cytology and differences observed are represented as the percent of time that animals spent in the stages of proestrous and estrous. Each value is the mean ± S.E.M. of at least 20 animals per treatment group (***$P < 0.001$ *vs.* control females).

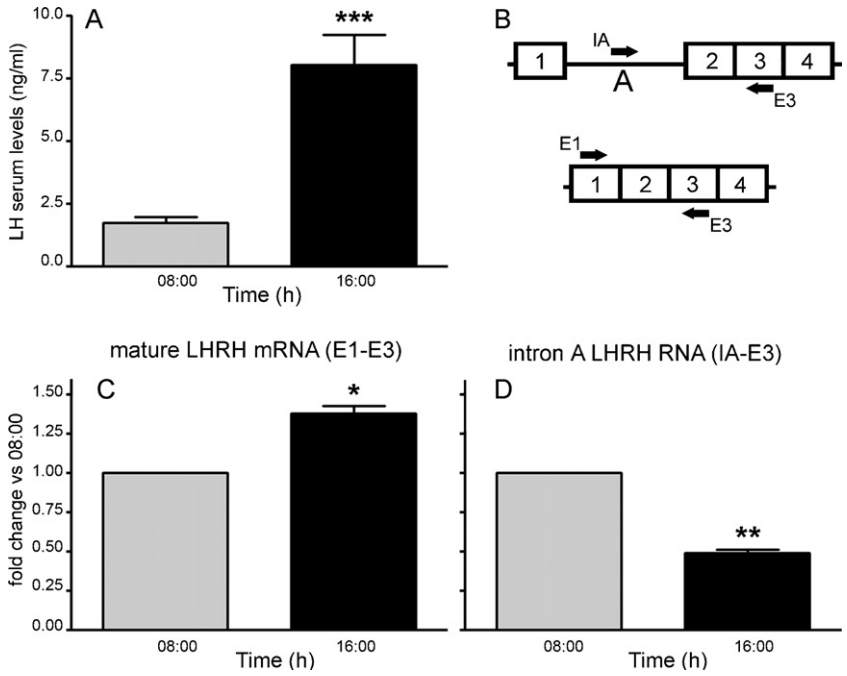

Fig. 3. LH surge timing and temporal dependence of LHRH pre-mRNA processing. Samples were obtained at 08:00 h and 16:00 h on day 2 (see Section 2) to test the effectiveness of the estrogenic model to induce an LH surge (A). LH levels were measured by RIA and each value is the mean ± S.E.M. of six animals per time point (***$P < 0.001$ *vs.* 08:00 h). To evaluate LHRH pre-mRNA processing, primers were designed (B) to amplify mature LHRH mRNA (primers E1–E3) and unprocessed intron A-containing LHRH RNA (primers IA–E3). Real-time RT-PCR assays were performed to study the temporal patterns of mature LHRH mRNA (C) and unprocessed (D) LHRH RNA cytoplasmic expression. Values were calculated by the Pfaffl method (40) and are given as mean ± S.E.M. of six animals per time point (*$P < 0.05$; **$P < 0.01$ *vs.* 08:00 h).

ing (2 cm long; inside diameter, 1.57 mm; outside diameter, 3.18 mm; Dow Corning Corp., Midland, MI) were filled with a solution of corn oil containing estradiol-17β dissolved at a concentration of 1 mg/ml and tubing ends were then sealed with NuSil Silicone adhesive (NuSil Technology LLC, Carpinteria, CA). Prior to use, all Silastic implants were incubated for 24 h in a host animal to avoid the initial large pulse of estradiol-17β release. At PND100, OVX animals were lightly anesthetized with ether and two Silastic capsules were inserted under the skin through a small dorsal midline incision, and then pushed toward the flanks. Implantation of capsules containing estradiol-17β was carried out at 09:00–10:00 h (designated day 0). At 08:00 h and 16:00 h of day 2, animals were sacrificed by decapitation ($n = 6$ per time point, Fig. 1), serum was collected and stored at −20 °C until used for LH radioimmunoassay, and hypothalamic tissue blocks were quickly dissected, frozen in liquid nitrogen and stored at −80 °C until used for RT-PCR (see below). According to our results, this experimental design induced an LH surge at 16:00 h on day 2 (Fig. 3A, see Section 3). Therefore, this time point was selected to study the effects of neonatal BPA exposure on the regulation of LHRH pre-mRNA processing in the hypothalamus and the expression of ERα, PR and their cofactors, SRC-1 and REA, in the AvPv and Arc nuclei.

2.5. Estradiol-17β induced LH surge in ovariectomized rats neonatally exposed to BPA

According to the results of the pilot experience, successive experimental animals were ovariectomized in PND85, implanted with capsules containing estradiol-17β in PND100 (day 0) and sacrificed by decapitation at 16:00 h on day 2 (Fig. 1). Trunk blood was collected and serum stored at −20 °C until used for LH radioimmunoassay. For nucleic acid extraction ($n = 5$ per group), tissue blocks containing hypothalamic tissue were quickly dissected under a GZ6 series dissecting microscope (Leica Corp., Buffalo, NY, USA) immediately after brains were removed. The hypothalamus was dissected out, bounded rostrally by the rostral edge of the optic chiasm, caudally by the caudal edge of the mammillary bodies, dorsally by the ventral portion of the anterior commissure, and laterally by a virtual line that projects from the internal capsule to the external edge of the optical tracts [39]. After dissection, tissue samples were immediately frozen in liquid nitrogen and stored at −80 °C until further use. For immunohistochemistry ($n = 6$ per group), brains were dissected and fixed by immersion in 4% paraformaldehyde for 24 h at 4–8 °C. Fixed tissue was dehydrated in ascending series of ethanol, cleared in xylene, and embedded in paraffin. In each processed brain, AvPv and Arc were sliced in a microtome in series (at least four) of six consecutive 5 μm thick frontal sections in the coronal plane. Series were separated by 20 μm from each other and matched across animals using anatomical landmarks [39] (the height of the third ventricle and the opening of the anterior commissure). All sections were mounted on 3-aminopropyl triethoxysilane (Sigma–Aldrich)-coated slides and dried for 24 h at 37 °C.

2.6. RT and real-time quantitative PCR analysis

An optimized RT-PCR protocol was used to analyze cytoplasmic expression of mature LHRH mRNA and unprocessed intron A-containing LHRH RNA. Cytoplasmic RNAs were isolated in accordance with a previous report [24] with modifications. Briefly, cytoplasmic RNAs were fractionated by tissue homogenization in lysis buffer (0.3 M sucrose, 0.25% sodium deoxycholate, 10 mM Tris–HCl pH 7.4, 1.5 mM $MgCl_2$, and 0.5% Nonidet P-40) and briefly centrifuged at 3000 × *g* to exclude the nuclear fraction. The cytoplasmic (upper) phase was transferred to a new tube and RNA was extracted with TRIzol (Invitrogen, Carlsbad, CA) according to the manufacturer's protocol. The concentration of total RNA was assessed by A_{260} and RNA was stored at −80 °C until needed. Equal quantities (4 μg) of cytoplasmic RNA were reverse-transcribed into cDNA with avian myeloma virus reverse transcriptase using 200 pmol of random primers (Promega). Twenty units of ribonuclease inhibitor (RNAout; Invitrogen Argentina, Buenos Aires, Argentina) and 100 nmol of a deoxynucleotide triphosphate (dNTP) mixture were added to each reaction tube in a final volume of 30 μl of 1× avian myeloma virus-reverse transcriptase buffer. Reverse transcription was performed at 42 °C for 90 min. Reactions were terminated by heating at 97 °C for 5 min and cooling on ice, followed by dilution of the reverse-transcribed cDNA with ribonuclease-free water to a final volume of 60 μl. Six samples per time point studied in the pilot experience and five samples per experimental group were analyzed in duplicate, and in all assays a sample without reverse transcriptase was included to detect contamination by genomic DNA. Primer pairs used for amplification of mature (E1–E3, Fig. 3B) and unprocessed intron A-containing LHRH mRNA (IA–E3, Fig. 3B) were E1 forward 5′-ggaagacttcagtgtcccaga-3′, IA forward 5′-tgtgtctgcagtttctgtgg-3′, and E3 reverse 5′-agagctcctcgcagatcccta-3′; and for the ribosomal protein L19 (housekeeping gene) L19 forward 5′-agcctgtgactgtccattcc-3′, L19 reverse 5′-tggcagtacccttcctcttc-3′. cDNA levels were determined using the Real-Time DNA Engine Opticon system (Bio-Rad Laboratories, Inc., Waltham, MA) and SYBR Green I dye (Cambrex Corp., East Rutherford, NJ). For cDNA amplification, 5 μl of cDNA was combined with a mixture containing 2.5 U Taq-DNA polymerase (Invitrogen), 2 mM $MgCl_2$ (Invitrogen), 0.2 mM of each of the four dNTPs (Promega), and 10 pM of each primer (Invitrogen) in a final volume of 25 μl of 1× SYBR Green I PCR Taq buffer. After initial denaturation at 95 °C for 1 min, the reaction mixture was subjected to successive cycles of denaturation at 95 °C for 15 s, annealing at 57 °C (E1–E3, IA–E3) and 55 °C (L19) for 15 s, and extension at 72 °C for 15 s (L19), 40 s (E1–E3) or 1 min (IA–E3). To test the efficiency of the cytoplasmic RNA extraction, an amplification reaction was performed using a primer directed against intron B, IB forward 5′-aagcaaccatcacttctcca-3′, and primer E3, which yielded no consistent amplification (data not shown). Product purity was confirmed by dissociation curves, and random samples were subjected to agarose gel electrophoresis. Negative DNA template controls were included in all of the assays, and yielded no consistent amplification. Fold change was determined using

the Pfaffl method [40]. The C_t and efficiency for each sample was calculated using the Opticon Monitor analysis software (MJ Research, Waltham, MA). No significant differences in C_t values were observed for L19 between the different experimental groups.

2.7. Hormone assay

Serum levels of LH were determined by RIA using the kit provided by the NIDDK as previously described [7]. Results were expressed in terms of rat LH (RP3) reference preparations. Intra- and interassay coefficients of variation were 7.2% and 11.4%, respectively, and the lowest detectable level of LH was 0.6 ng/ml. Serum from six animals per time point studied in the pilot experience and from at least nine animals per treatment group was used to determine LH serum levels.

2.8. Antibodies

The affinity-purified antibody for ERα (clone 6F-11, 1:200 dilution) was purchased from Novocastra (Newcastle upon Tyne, UK), and the antibodies for PR (A0098, 1:200 dilution) and SRC-1 (clone 128E7, 1:3200 dilution) were from Dako Corp. (Carpinteria, CA) and Cell Signaling Technology (Boston, MA), respectively. The REA antibody was obtained and characterized in our laboratory as previously described [10]. Anti-rabbit and anti-mouse secondary antibodies (biotin conjugate, B8895/B8774, 1:200 dilution) were purchased from Sigma (St. Louis, MO).

2.9. Immunohistochemistry

A standard immunohistochemical technique (avidin-biotin-peroxidase) was used to visualize the distribution of immunostaining following a previously described protocol [8]. In brief, samples were subjected to a microwave pretreatment for antigen retrieval, followed by blocking against endogenous peroxidase activity and nonspecific binding sites. Samples were incubated with primary antibodies at 4 °C for 14–16 h, and subsequently with a biotinylated secondary antibody for 30 min. Negative controls were obtained by replacing the primary antibody with nonimmune rabbit serum (Sigma–Aldrich). The reaction was then developed using diaminobenzidine (ERα, REA, SRC-1; Sigma–Aldrich) or diaminobenzidine-nickel (PR; Sigma–Aldrich) as a chromogen substrate [41]. Samples were dehydrated and mounted with permanent mounting medium (PMyR, Buenos Aires, Argentina).

2.10. Quantification of protein expression in the AvPv and Arc nuclei 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 [42,8]. 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. A reference slide containing brain tissue sections stained in the absence of primary antibody was used to correct unequal illumination (shading correction) and to calibrate the measurement system to determine background threshold values. Within each experimental group, six brains ($n = 6$) were analyzed, and the AvPv and Arc were identified by Nissl staining of one slide per series and delimited according to Paxinos and Watson [39]. Images of immunostained slides were converted to grey scale and the percentage of immunostained area was measured. At least four sections of serial one-in-six series encompassing each nucleus studied were scored and averaged per animal for each target protein. This morphometric approach provides more reproducibility since relative spatial variables are independent of oversampling errors that would be incurred by calculating absolute volumes and total cell numbers [10,43,44,7,45].

2.11. Data analysis

All data were calculated as the mean ± S.E.M. Statistical analyses of the time-dependence of the LH surge and LHRH RNA expression as well as estrous cycles were performed using two-tailed *t*-tests. For the rest of the comparisons, one-way ANOVA analyses were performed to obtain the overall significance (testing the hypothesis that responses were not homogeneous across treatments), and differences between treatment groups were determined using the Dunnett's post test. In all cases, $P < 0.05$ was accepted as significant.

3. Results

3.1. Vaginal cytology

Vaginal smears revealed that rats from the group neonatally exposed to 0.05 mg/kg of BPA presented abnormal estrous cycles. In average, BPA.05-treated females spent significantly more time in the stages of proestrous–estrous ($P < 0.001$, Fig. 2) than control females.

3.2. LH surge timing and temporal patterns of mature and unprocessed LHRH RNA expression

To test the effectiveness of the estrogenic model used herein to induce the LH surge, a set of control females was ovariectomized on PND85 and implanted with estradiol-17β containing capsules at 09:00–10:00 h of PND100 (day 0). On day 2, trunk blood and hypothalamic tissue samples were taken at 08:00 h and 16:00 h. A significant 4.6-fold increase in LH serum levels was observed at 16:00 h ($P < 0.001$, Fig. 3A) relative to 08:00 h, which is in agreement with previous reports [15]. Moreover, LHRH pre-mRNA processing also showed a temporal shift. Using real-time PCR we observed that the levels of mature LHRH mRNA were significantly increased by about 1.4-fold at 16:00 h compared to samples obtained at 8:00 h ($P < 0.05$, Fig. 3C). In contrast, unprocessed intron A-containing LHRH RNA levels showed a significant decrease of about 2-fold ($P < 0.001$, Fig. 3D).

3.3. BPA alters LHRH pre-mRNA processing

It has been demonstrated that retention of intron A in the LHRH pre-mRNA blocks the translation of downstream coding sequences [24], leading to an inefficient production of LHRH. As this mechanism is regulated by estrogenic pathways, we decided to study the effects of neonatal BPA exposure on the relative expression of mature LHRH mRNA and unprocessed intron A-containing LHRH RNA in adult female rats submitted to an estrogenic stimulation model. We observed that BPA has an inverse dose-dependent effect on the expression of mature LHRH mRNA: the levels were significantly increased in BPA.05-treated females ($P < 0.001$, Fig. 4A) and significantly reduced in BPA20-treated females ($P < 0.01$). In contrast, levels of unprocessed intron A-containing LHRH RNA were reduced about 2-fold in both BPA-exposed groups ($P < 0.01$, Fig. 4B).

3.4. Estradiol-17β induced LH surge in BPA-exposed rats

Females exposed to the low dose of BPA showed no differences in LH levels compared to control females (Fig. 4C). However, BPA20-treated females failed to produce a normal LH surge and exhibited LH levels 2-fold lower than control females ($P < 0.05$, Fig. 4C).

3.5. Neonatal BPA alters the expression of ERα and PR in the AvPv and Arc

Estrogenic stimulation of LH and LHRH surges requires activation of hypothalamic ERα and PR [25,26]. Expression of these steroid receptors was studied in neurons from the AvPv and Arc nuclei using an immunohistochemical approach. Our results showed that in the AvPv, expression of ERα was significantly increased in both BPA.05- ($P < 0.01$, Fig. 5A) and BPA20-treated groups ($P < 0.05$) compared to control females. However, ERα expression in the Arc was significantly reduced in females exposed to BPA.05 and BPA20 ($P < 0.05$, Fig. 5B). PR protein expression was significantly decreased in the AvPv of BPA.05-treated females ($P < 0.05$, Fig. 5C), but showed no changes in the Arc nucleus of either BPA-treated group (Fig. 5D).

3.6. Effects of neonatal BPA exposure on the expression of the transcriptional cofactors SRC-1 and REA in the AvPv and Arc of adult females

In the AvPv of experimental animals, SRC-1 expression was significantly decreased by about 2-fold in females exposed to the low dose of BPA ($P < 0.001$, Fig. 6A). No changes were observed in the expression of SRC-1 or REA in the Arc (Fig. 6C, D). Expression of

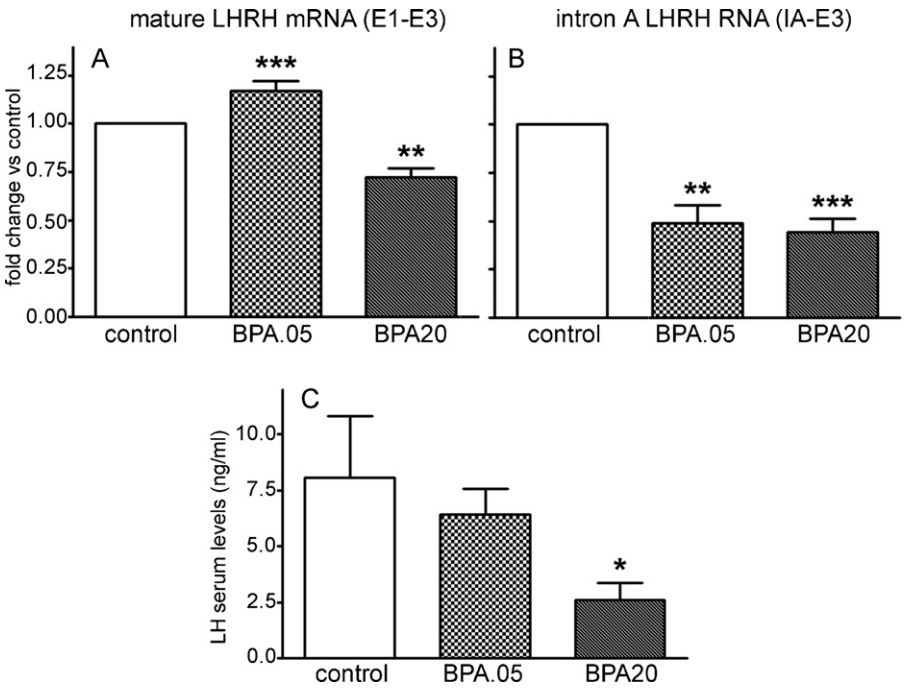

Fig. 4. Effect of neonatal BPA exposure on the hypothalamic expression of mature LHRH mRNA (A), unprocessed intron A-containing LHRH RNA (B) and estrogen implant-induced afternoon surge of LH (C). Relative cytoplasmic RNA expression was measured by real-time RT-PCR and fold change from control values was calculated by the Pfaffl method (40) and is given as the mean ± S.E.M. of five animals per treatment group (**$P < 0.01$, ***$P < 0.001$ *vs.* control females). LH serum levels were measured by RIA and each value is the mean ± S.E.M. of at least nine animals per treatment group (*$P < 0.05$ *vs.* control females).

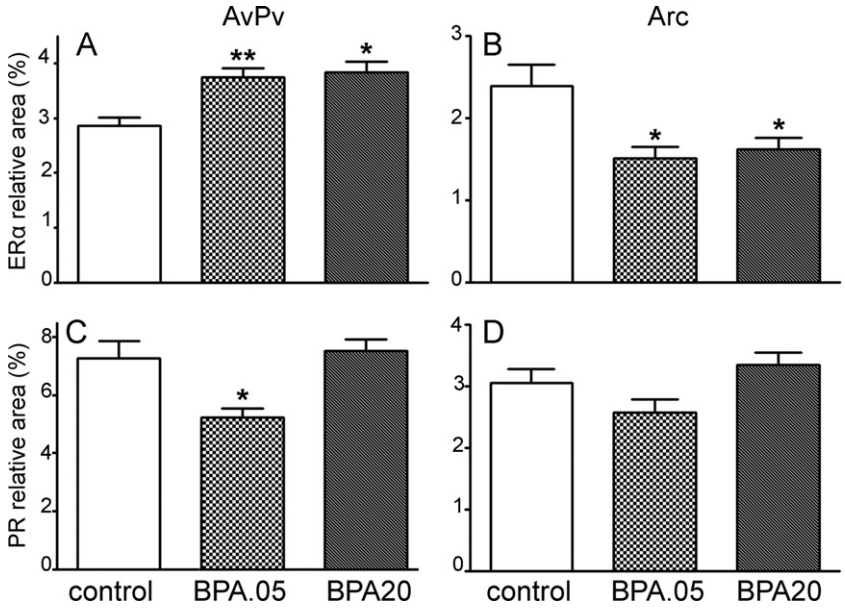

Fig. 5. Effects of neonatal xenoestrogen exposure on ERα (A, B) and PR (C, D) expression in the AvPv (A, C) and Arc (B, D) hypothalamic nuclei of adult females. AvPv and Arc were delimited according to Paxinos and Watson [39]. Data are expressed as the relative area occupied by positive cells; each column represents the mean ± S.E.M. of at least four semi-serial sections per animal of six animals per treatment group (*$P < 0.05$; **$P < 0.01$ *vs.* control females).

ERα, PR, SRC-1 and REA in the nuclei AvPv and Arc is illustrated in Figs. 7 and 8.

4. Discussion

This study demonstrates that female rats neonatally exposed to a dose of 0.05 mg/kg of BPA showed alterations in their estrous cyclicity. These results are in agreement with recent reports where abnormalities in the estrous cycle were shown using animals exposed via drinking water to 1.2 mg/kg of BPA (but not animals exposed to 0.1 mg/kg of BPA) [37] and animals postnatally exposed via subcutaneous injection to 25.0–62.5 mg/kg of BPA (but not animals injected with 2.5–6.2 mg/kg of BPA) [38]. However in both aforementioned studies doses of BPA several times higher to the low dose used herein showed no effects on estrous cyclicity. A feasible explanation for such discrepancy could be the different rat strains used [46,47]. Since disruption of mechanisms regulating spontaneous LH and LHRH surges could be implicated in the altered estrous cyclicity observed in BPA-exposed females, we decided to investigate post-transcriptional regulation of hypothalamic LHRH

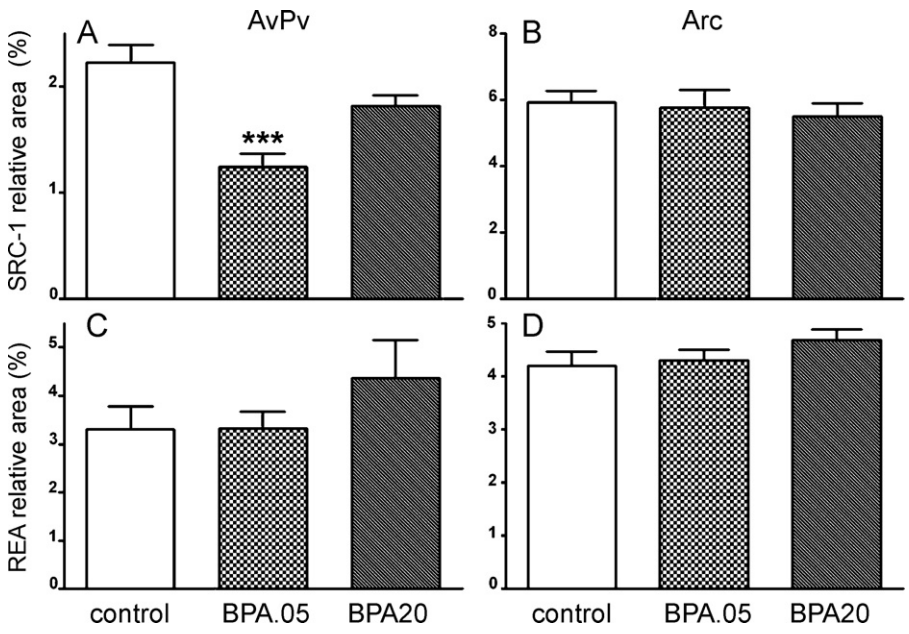

Fig. 6. Effects of neonatal xenoestrogen exposure on the expression of the transcription cofactors SRC-1 (A, B) and REA (C, D) in the AvPv (A, C) and Arc (B, D) hypothalamic nuclei of adult females. AvPv and Arc were delimited according to Paxinos and Watson [39]. Data are expressed as the relative area occupied by positive cells; each column represents the mean ± S.E.M. of at least four semi-serial sections per animal of six animals per group (***$P < 0.001$ *vs.* control females).

and expression of ERα, PR and cofactors SRC-1 and REA in the neuronal nuclei AvPv and Arc using a model of estrogenic induction of LH surge.

The model of neonatal exposure to xenoestrogens used in the present work has been extensively used in our laboratory [8,10,35,48] and has been established as a persuasive paradigm for the study of short- and long-term consequences of neonatal exposure to hormonally active substances. Generally, the *in vivo* estrogenic activity of an endocrine disruptor is determined by comparing the tested compound with a positive control substance, like estradiol benzoate or diethylstilbestrol (DES). In a previous work, we showed that the results obtained in the positive control group (DES treated animals) were difficult to be compared with what was observed in BPA-exposed rats [8], as the differences in the actions of these substances were of qualitative nature, fading away the role of DES as a positive control. Furthermore, the doses used in positive control groups are selected to effectively produce the classic and expected estrogenic response. However, the low dose effects of endocrine disruptor compounds are often very different in nature of the expected estrogenic action of classical estrogens at pharmacological or physiological doses [49,50]. In a previous report, we demonstrate that, at a molecular level of organization, an uterotrophic dose of estradiol produces different and sometimes opposite responses than a non-uterotrophic dose [51]. Therefore the use of a positive control of estrogenic effects would be conditioned to the dose selected, ultimately becoming into a new variable of analysis rather than a control of positive effects.

It has been demonstrated that androgenized females rats [52] or, female rats exposed neonatally to *p-tert*-octylphenol or estradiol-17β [15] showed diminished afternoon LH surges in response to estrogenic stimulation. In the present study, we demonstrated that our estrogenic stimulation model induced a robust LH surge at 16:00 h in response to estradiol-17β and that this surge was affected in females exposed to the high dose of BPA. Furthermore, expression of hypothalamic LHRH RNA species also showed temporal dependence within this estrogenic stimulation model. LHRH gene consists of four exons and three intervening introns (A, B and C) [18]. Excision of intron A (but not B or C) from the LHRH transcript is highly cell type- [22] and developmental stage specific [53], and the presence of intron A in LHRH pre-mRNA transcripts completely blocks the translation initiation of downstream sequences both *in vivo* and *in vitro*, pointing out that precise and efficient excision of intron A is likely to be a critical regulatory step for the post-transcriptional regulation of LHRH synthesis [24]. Our results suggest that the expression of the hypothalamic LHRH mRNA levels necessary to promote the spontaneous afternoon LHRH surge is at least partially provided by an increase in the excision rate of intron A from unprocessed cytoplasmic LHRH RNA. In this work, we demonstrate that exposure to both low and high doses of BPA during the early postnatal period alter hypothalamic LHRH mRNA processing. A recent work reported that LHRH neurons of females neonatally exposed to levels of BPA similar to those used herein retained the capacity to respond to hormones [54], as these neurons expressed the wide-ranging transcription factor FOS. Determination of FOS expression in LHRH neurons reveals the global transcriptional status of these neurons. In addition, our findings suggest that LHRH neurons of females exposed to the high dose of BPA are responsive to hormonal stimuli to a lesser extent, as evidenced by the specific overall decrease in the hypothalamic transcription of the LHRH gene. We theorize that the decline observed in the expression of cytoplasmic LHRH mRNA could be at least partly responsible for the incapacity of BPA20 females to produce a normal LH surge. Furthermore, we hypothesize that in females exposed to the low dose of BPA an internal compensatory mechanism augments mature LHRH mRNA levels by means of an increase in the intron A excision rate, probably leading to the production of an LH surge similar to that observed in control females. In support of this hypothesis, our previous observations [48,8] indicate that animals exposed neonatally to xenoestrogens present overcompensation mechanisms in the control of estrogen-dependent genes in the hypothalamus and hippocampus.

Our results show that exposure to low environmentally relevant and high doses of BPA during the early postnatal period induces long-term changes in hypothalamic ERα protein expression, as evidenced by the opposite site-specific effects on ERα expression in two functionally distinct hypothalamic nuclei. In the AvPv, both BPA doses were associated with an increase in ERα expression, while the exact opposite effect was observed in neu-

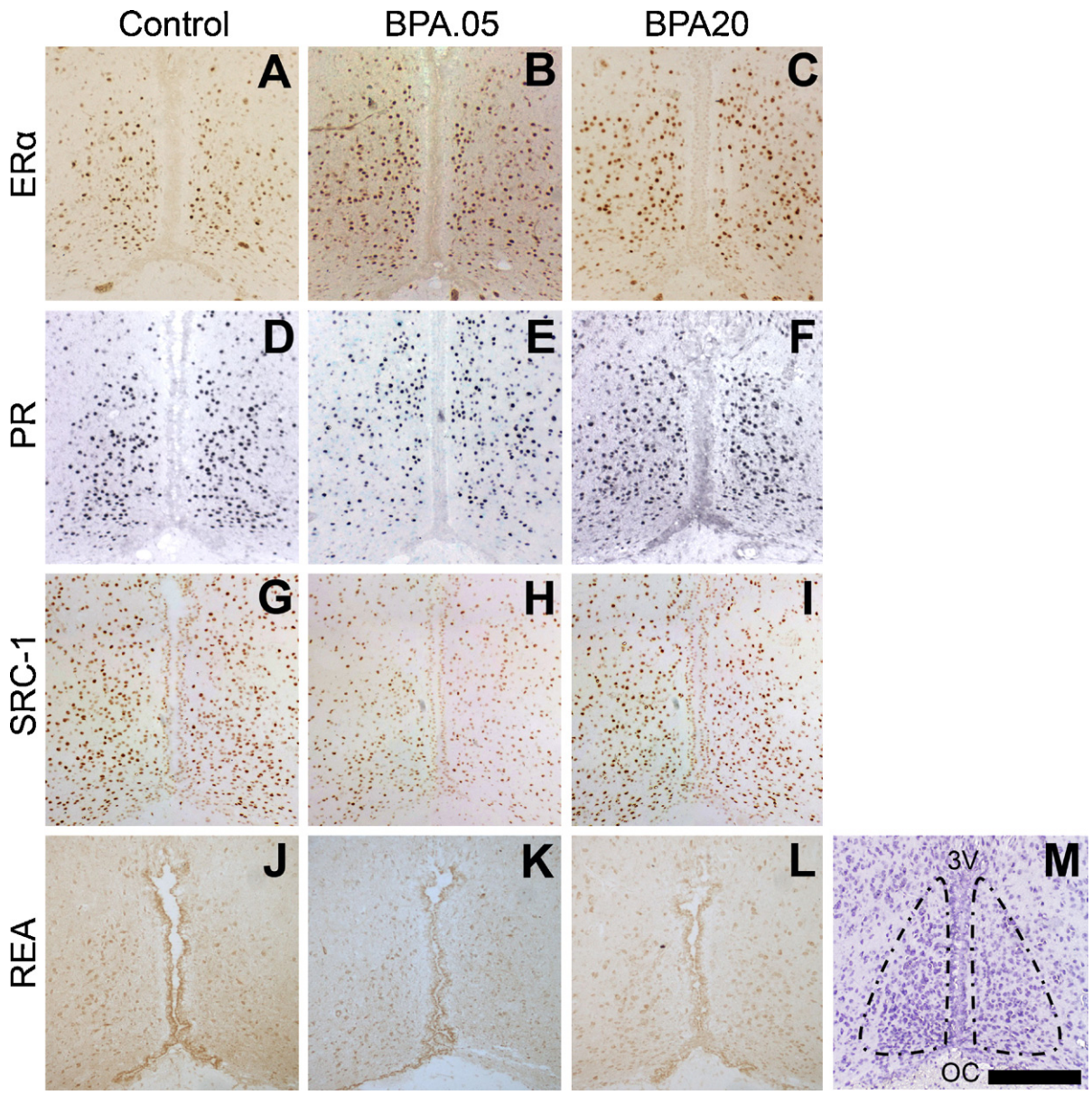

Fig. 7. Representative photomicrographs of immunohistochemical staining of ERα (A, B, C), PR (D, E, F), SRC-1 (G, H, I) and REA (J, K, L) in the AvPv nucleus of control (A, D, G, J), BPA.05 (B, E, H, K) and BPA20 (C, F, I, L) female rats. The AvPv was identified by Nissl staining of one slide per series (M) and delimited according to Paxinos and Watson [39] (M, *dotted line*). 3*V*, third ventricule; *OC*, optic chiasm. *Scale bar*, 200 μm.

rons from the Arc nucleus. Evidence indicates that expression of ERα, but not ERβ, in the AvPv is essential in the pathways controlling the estrogen positive-feedback that generates preovulatory gonadotropin surges [25]. Moreover, ERα expression and steroid-sensitive projections from the Arc have also been demonstrated to be indispensable in the estrogen-mediated negative-feedback control of LH secretion [28,55].

Our results suggest that both positive and negative regulatory mechanisms could be affected by neonatal BPA treatment because of BPA-mediated disruption of ERα expression in the AvPv and Arc, but the molecular mechanisms by which BPA elicits inverse effects on ERα expression in these nuclei are unknown. We hypothesize that something intrinsic to each cell type could be responsible for the observed opposite effects, such as the expression of nuclei-specific coactivators or repressors other than those studied here. Supporting data for this theory are our previous observations that higher levels of REA in the ventromedial hypothalamic nucleus of BPA-treated females were associated with lower ERα levels within the same nuclei [10]. An archetypal example of an estrogen-responsive gene in the brain is the PR gene [56,57], which contains several consensus sequences for estrogen response elements [58,59]. Throughout life, PR expression in the brain depends on estrogenic stimulation [60,61] and ERα in particular is necessary for the sexually dimorphic induction of PR in the hypothalamus of the developing brain [62]. According to previous reports, the magnitude of the LH surge induced by estrogenic stimulation is relatively low compared with that observed in proestrous intact-ovary females, probably due to lower estradiol-17β levels, a lack of progesterone in OVX rats, or both [15,63]. In our work, BPA.05 females showed irregular estrous cycles even though they were able to produce an apparently normal LH surge in response to estradiol. On the other hand, females exposed to the high dose of BPA failed to produce a normal LH surge in response to estrogens, which might be expected to limit reproductive fertility and decrease overall reproductive success. We hypothesize that a clue to explain such contradictory results could be the altered expression of PR

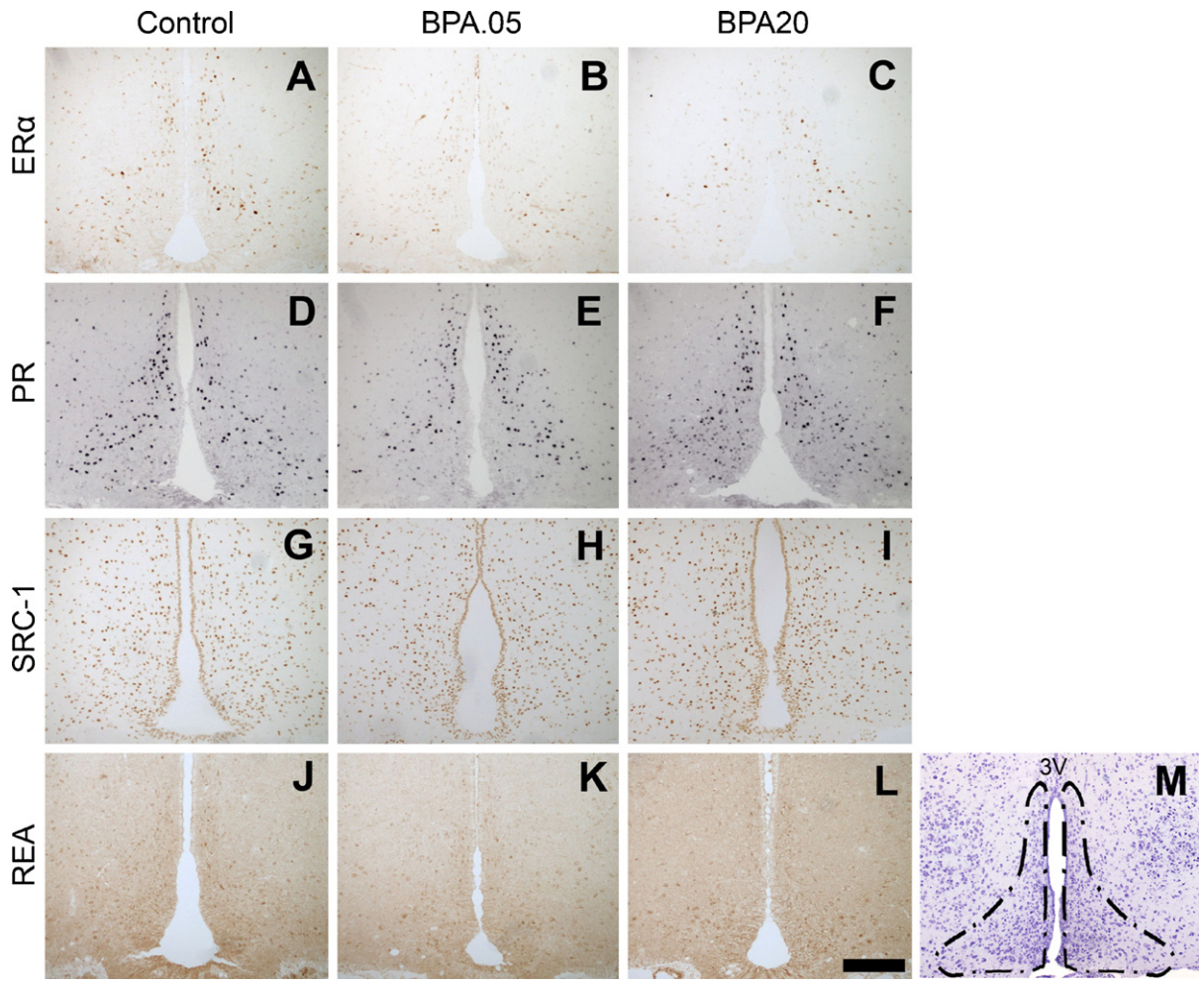

Fig. 8. Representative photomicrographs of immunohistochemical staining of ERα (A, B, C), PR (D, E, F), SRC-1 (G, H, I) and REA (J, K, L) in the Arc nucleus of control (A, D, G, J), BPA.05 (B, E, H, K) and BPA20 (C, F, I, L) female rats. The Arc was identified by Nissl staining of one slide per series (M) and delimited according to Paxinos and Watson [39] (M, *dotted line*). 3*V*, third ventricule. *Scale bar*, 200 μm.

and SRC-1 in the AvPv nucleus of BPA.05-exposed animals. Experimental models are intended to isolate single processes from their complex biological realm, thus finding answers to specific questions; however, estrous cycles in intact females are highly complex endpoints controlled by both estrogen dependent and independent pathways. In this context, deficient expression of PR in the AvPv of intact BPA.05-exposed females could lead to inadequate amplification of LHRH [64] and/or LH surges [65], and subsequent failure of estrous cyclicity. Moreover, insufficient levels of SRC-1 protein within the same nucleus could add to the overall negative effect, as this molecule is an enhancer of PR transcriptional activity [66] and is co-expressed with PR in hypothalamic neurons [67].

In summary, our results clearly demonstrate that BPA exposure during early postnatal life alters the normal estrogenic responsiveness of the neural network that controls estrous cyclicity during adulthood. In addition, an increase in the excision rate of intron A from LHRH pre-mRNA was described as a possible mechanism of LHRH surge prompting. Long-term effects of neonatal BPA exposure were described as estrous cyclicity failure, altered LHRH pre-mRNA splicing regulation and altered expression of steroid receptors and transcription cofactors in two important hypothalamic nuclei as AvPv and Arc. Furthermore, some of the long-term effects observed were dose-dependent and restricted to females treated with the low dose of BPA, which was deemed an acceptable daily intake by the US EPA [5]. These findings should be cause for concern regarding public health, confirming that exposure to low doses of weak environmental estrogens during the early stages of development affects brain differentiation and could limit adult reproductive performance.

Conflict of interest statement

The authors declare that there are no conflicts of interest.

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