

Effects of Perinatal Administration of Bisphenol A on the Neuronal Nitric Oxide Synthase Expressing System in the Hypothalamus and Limbic System of CD1 Mice

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Bisphenol A (BPA) is a well-known plastic-derived pollutant that can bind to oestrogen receptors and is considered an endocrine-disrupting chemical. Its impact on different behaviours in rodents has been largely investigated, however, only a few data are available on its effects upon neural circuits. In the present study, we investigated the long-term effects of early exposure of mice of both sexes to BPA on the nitrinergic system, one of the neural systems involved in the control of sexual behaviour and under the control of gonadal hormones. Mice of both sexes were exposed for eight prenatal and eight postnatal days to BPA that was administered to the mothers. The maternally-exposed mice were sacrificed at the age of 2 months and their brains were sectioned and immunohistochemically treated for the detection of neuronal nitric oxide synthase (nNOS). Significant effects of BPA exposure were detected for the number of immunoreactive cells in the medial preoptic nucleus and in the ventromedial subdivision of the bed nucleus of the stria terminalis, in a sex-oriented and dose-dependent way. These results indicate that BPA has a powerful effect on specific portions of the nNOS-immunoreactive system belonging to the accessory olfactory system that are particularly important for the control of sexual behaviour. In addition, they confirm that perinatal exposure to endocrine-disrupting chemicals, in particular to BPA, may have a high impact on the organisation of specific neural pathways that can later affect complex behaviours and functions.

Key words: endocrine-disrupting chemicals, medial preoptic nucleus, bed nucleus of the stria terminalis, accessory olfactory system, xenoestrogens.

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The observation that early exposure to several pollutants could cause adverse effects on endocrine structures' development, such as the reproductive organs, induced public concern about the existence of endocrine active compounds that may affect humans, farm animals and wildlife (endocrine-disrupting chemicals; EDCs) (1, 2). Many of these EDCs are capable of interacting with the oestrogen (ER) or androgen (AR) receptors and are therefore named xenoestrogens or xenoandrogens. As a result of their nature, they could, even in very low concentrations, influence the development and the function of ER- or AR-dependent neural circuits and related behaviours (3, 4), reduce fertility, induce congenital malformations of the

reproductive tract, and increase the incidence of cancer in gonadal hormones-responsive tissue (1, 5). One of such compounds is bisphenol A (BPA), a monomer used in the manufacture of polycarbonate, epoxy and polyester-styrene resins, such as milk and food containers, baby formula bottles, water carboys (6), and the interior lining of food cans (7). BPA has been shown to leach from these materials as a result of incomplete polymerisation and the degradation of the polymers by exposure to high temperatures, as occurring under normal conditions of use (6).

BPA can bind to both isoforms of the ER receptor (ER α and ER β) *in vitro*, albeit with low relative binding affinity compared to

17 β -oestradiol (8, 9). In addition to its oestrogenic activity, there is some evidence that BPA also possesses anti-androgenic activity, but data on yeast reporter or on mammalian cell reporter assays are controversial (10–14).

Given the well-known ability of gonadal hormones to affect sexual differentiation of the brain during a critical period (i.e. perinatal life in rodents) (15), it is likely that some behaviours, such as reproductive behaviour, can be affected by perinatal exposure to EDCs. Many studies have been undertaken to check whether EDCs may interfere with the organisational effect of endogenous gonadal steroid hormones during prenatal and perinatal periods. Most of these studies suggest that, despite its low relative binding affinity for ER, exposure to BPA during development causes irreversible damage to reproductive organs (16). This compound has been found to influence adult reproductive physiology and behaviour when administered during development. For example, exposure to BPA during the pre- or postnatal period can increase ER α mRNA expression in the female mediobasal hypothalamus and in the male anterior pituitary (17), as well as decrease the expression of tyrosine hydroxylase in the female anteroventral periventricular nucleus (18), resulting in early, persistent oestrus in females (19). The brain structures controlling reproductive functions and behaviour are also likely to be affected by such an early exposure (20, 21). These data suggest that BPA is capable of disrupting ER-dependent processes in the developing brain, including sexual differentiation and the organisation of other behavioural and neuroendocrine circuits (4, 22, 23).

Nitric oxide (NO) is a gaseous regulatory molecule that acts both as a second messenger and as a neurotransmitter. It is synthesised from arginine by the enzyme nitric oxide synthase (NOS) and it has been implicated in the regulation of several functions (24–26). In rodents, the neuronal isoform of NOS (nNOS) is expressed in neural circuits implicated in the control of reproductive behaviour (27, 28), namely the medial preoptic nucleus (MPOM), the bed nucleus of the stria terminalis (BST), the ventromedial nucleus (VMH), the bed nucleus of the accessory olfactory tract (29, 30) and the medial amygdaloid nucleus (31, 32), structures that belong to the vomeronasal system (33, 34), and partially overlaps that of gonadal hormones' receptor (35–37). In mice, some of these structures show a sex dimorphism in the number of nNOS-positive cells, as well as fluctuations related to physiological changes of gonadal hormones level during the oestrous cycle (30). The regionally specific distribution of nNOS immunoreactive (nNOS-ir) elements and their co-existence with both gonadal hormones' receptors and NMDA receptors (38) suggest the existence of significant neuroendocrine relationships (39, 40) among NO-producing elements and sexual behaviour (41).

In the present study, we investigated the effects of perinatal exposure (before and after delivery, during a period that is critical for rodent brain organisation) to BPA on the nitrinergic system of hypothalamic and limbic nuclei belonging to the vomeronasal system and involved in the control of sexual behaviour. BPA concentrations were below the US–Environmental Protection Agency lowest observed adverse effect level (50 mg/kg/day) that was used to calculate a safe daily limit for human exposure of 50 μ g/kg/day (7, 42, 43).

Materials and methods

Animals and treatment procedures

CD-1 mice (*Mus musculus domesticus*) were initially purchased from Charles River Italia (Calco, Italy) and were maintained as an outbred colony at the University of Parma.

One day before the mating procedure, females were housed between cages of males to stimulate oestrous cyclicity and ovulation. Adult (3–4 months old) virgin female mice were time-mated by being placed into the cage of a stud male for 4 h beginning at 08.00 h (at the end of the dark phase of the light/dark cycle). Mating was verified by the presence of a vaginal plug (day 0 of gestation). After mating, pregnant females were housed three per cage (45 \times 25 \times 15 cm).

Food and water were available *ad libitum* (standard mouse chow 4RF21; Mucedola srl, Settimo Milanese, Italy). Rooms were maintained under a 12 : 12 h light/dark cycle (lights on 10.00 h) at 22 \pm 1 $^{\circ}$ C.

One week after the detection of vaginal plug, females were trained daily to suck tocopherol-stripped corn oil (Sigma Chemical, St Louis, MO, USA) from a modified syringe (i.e. without needle and with a large hole). This training ensured that the treatment procedures were not stressful. Female mice were randomly allocated to four groups and subjected to the following treatments administered daily from day 11 of gestation to day 8 post partum: (i) oil, receiving only the solvent (corn oil); (ii) BPA10, receiving 10 μ g/kg/day BPA (Sigma Chemical purity 99%) dissolved in corn oil; (iii) BPA20, receiving 20 μ g/kg/day BPA dissolved in corn oil; and (iv) BPA40, receiving 40 μ g/kg/day BPA dissolved in corn oil. Pregnant females consumed an average 0.10 ml/50 g body weight of different BPA solutions. Females were weighed every second day and the volume administered was reduced or increased accordingly.

The dams gave birth on day 19 of pregnancy, which is postnatal day (PND) 1. Within 12 h from birth, litters were weighed, sexed and culled to 10 pups (5 \pm 1 males and 5 \pm 1 females) and returned to their mothers. The offspring were weaned on PND 28–30 and housed in groups of three to five males or three to five females in 45 \times 25 \times 15 cm polypropylene mouse cages at 22 \pm 1 $^{\circ}$ C, under a natural light/dark cycle (light on 10.00 h). Food and water were available *ad libitum* (standard mouse chow 4RF21; Mucedola srl).

In the present study, we used 48 (24 females and 24 males) mice, offspring of mothers treated during gestation and lactation, as described above. The animals were randomly taken from different litters (one male and one female per litter) and categorised into four groups (six males and six females each) on the basis of their exposure to BPA during the perinatal period: (i) oil, offsprings of vehicle-treated mothers; (ii) BPA10, offsprings of mothers treated with 10 μ g/kg/day BPA; (iii) BPA20, offsprings of mothers treated with 20 μ g/kg/day BPA; and (iv) BPA40, offsprings of mothers treated with 40 μ g/kg/day BPA.

Fixation and tissue preservation

At the age of 2 months, a total of 48 male and dioestrus female mice, belonging to all experimental groups (n = 6 per group), were weighed and anaesthetised with tribromoethanol (250 mg/kg i.p.). Before sacrifice, and to minimise the potential variations of nNOS system as a result of the oestrous cycle (30), females were inspected by daily vaginal smears, and those in dioestrus after exhibiting two or more consecutive 4-day oestrous cycles were used for immunohistochemical processing.

Animals were intracardially perfused with saline solution (NaCl 0.9%) followed by 4% paraformaldehyde (PAF) in phosphate buffer (0.1 M, pH 7.4). The brains were removed and stored in a freshly prepared PAF solution for 2 h at 4 $^{\circ}$ C, followed by several washings in 0.01 M phosphate-buffered saline (PBS). Finally, they were stored in a 30% sucrose solution in PBS at

4 °C, frozen in liquid isopentane at -35 °C, and stored in a deep freezer at -80 °C until sectioning.

Brains were serially cut in the coronal plane at 25 µm thickness with a cryostat. The plane of sectioning was oriented to match the drawings corresponding to the coronal sections of the mouse brain atlas (44). Sections were collected in a cryoprotectant solution (45) at -20 °C. Every fourth section was processed for nNOS immunohistochemistry. Adjacent sections were Nissl-stained with toluidine blue, or used for controls. Brain sections were always stained in groups containing male and females sections of each treatment, so that between-assays variance would not cause systematic group differences.

nNOS immunohistochemistry

The sections were stained for nNOS by the biotin-avidin method according to our standard procedure (28). Briefly, to block endogenous peroxidase activity, sections, collected in multidish wells, were immersed in a solution of methanol/hydrogen peroxide (46) for 20 min. After washing, they were incubated overnight at room temperature with an anti-nNOS rabbit antibody (ImmunoStar, Hudson, WI, USA, diluted 1 : 12,000 in PBS, pH 7.3–7.4, containing 0.2% Triton X-100). The antigen-antibody reaction was revealed by the biotin-avidin system (BAS, Vectastain Elite kit; Labtek, Scotts Valley, CA, USA). The peroxidase activity was visualised with a solution containing 0.15 mg/ml 3,3'-diamino-benzidine (Sigma, Milano, Italy) and 0.025% hydrogen peroxide in 0.05 M Tris-HCl buffer pH 7.6. Sections were collected on chromalum-coated slides, air-dried, washed in xylene and coverslipped with Entellan (Merck, Milano, Italy).

The commercial antibody against nNOS was generated in rabbit against a C-terminal synthetic peptide sequence (1419–1433) of human nNOS. The manufacturer (Dr Jeffrey Spangenberg, IncStar, Stillwater, MN, USA) tested the specificity of the antibody by western blot analysis and pre-adsorption with synthetic human nNOS (5 mg/ml antibody at working dilution). No cross-reactivity with other forms of NOS was reported (47, 48). The nNOS antiserum has been successfully used in human, rat, mouse, cat and monkey tissue. In particular, the specificity of this antibody for mouse central nervous tissue was tested in nNOS knockout mice where cerebellar and amygdala staining was totally abolished (49). We have performed the following controls in our material: (i) the primary antibody was omitted or replaced with an equivalent concentration of normal serum (negative controls); (ii) the secondary antibody was omitted. In these conditions, cells and fibres were totally unstained.

Quantitative analysis

Quantification procedure was performed according to our previously published method (30, 50). Briefly, in the present study, we investigated six different limbic-hypothalamic nuclei identified on the basis of the stereotaxic mouse brain atlas (44). For each animal, two standardised sections of comparable levels of MPOM (anteroposterior: 0.14 and -0.10 mm relative to bregma), paraventricular nucleus (PVN) (anteroposterior: -0.58 and -0.82 mm relative to bregma), ventrolateral subdivision of the ventromedial nucleus (VMHvl) (anteroposterior: -1.46 and -1.70 mm relative to bregma), arcuate nucleus (Arc) (anteroposterior: -1.46 and -1.70 mm relative to bregma), posteromedial (BSTmpm) and ventromedial (BSTmv) subdivision of the bed nucleus of the stria terminalis (anteroposterior: -0.10 and -0.22 mm relative to bregma), and one section including the nucleus caudate putamen (CPu), as a control nucleus devoid of sex hormones' receptors (anteroposterior: 0.14 mm relative to bregma), were chosen.

Positive neurones were identified for the presence of a clearly-labelled cell body. Cell counting was performed with a Leitz Laborlux microscope (Leica SpA, Milano, Italy), equipped with a camera lucida, using a ×10

objective. The use of the microscope instead of photographs granted us more flexibility (i.e. higher enlargements) to count positive elements in greater detail, even if they are overlapped. For each level of these nuclei, we selected a squared region with a fixed extension covering a large part of the considered nucleus (288 800 µm² for MPOM; 144 400 µm² for BSTmpm and BSTmv; 192 533 µm² for VMHvl; 48 133 µm² for each subdivision of PVN; 144 400 µm² for Arc; 451 250 µm² for CPu) and counted immunoreactive cells within this frame. For the analysis of PVN, nNOS-positive elements were sampled within three subdivisions of the nucleus: the ventral part, the lateral magnocellular part, and the anterior parvicellular part.

During the immunohistochemical reaction, a few sections were lost and, consequently, some animals were excluded from quantitative analysis. Therefore, the different experimental groups had n = 5 or n = 6 mice.

The results for each nucleus were analysed by two-way ANOVA for repeated measures with a mixed design using the treatment and the sex as independent factors and the anteroposterior neuroanatomical levels as repeated factor. When preliminary analyses revealed no significant effects of anteroposterior levels, the levels were collapsed and the average number (calculated using the average values from two sections) was analysed by a two-way ANOVA (treatment and sex as independent factors). P < 0.05 was considered statistically significant. These analyses were followed, when appropriate, by a post-hoc test (Fisher's protected least significant difference; PLSD) to test the interactions between the independent variables, and were all performed by using STATVIEW, version 5.0 (Abacus Concepts, Berkeley, CA, USA).

Sections were photographed with a Zeiss Axioplan microscope (Zeiss Italia SpS, Milano, Italy), equipped with a Leica DFC 320 digital telecamera connected to an Apple G4 Macintosh (Apple, Cupertino, CA, USA). Digital images were processed using Adobe Photoshop 7.0 (Adobe Systems Incorporated, San Jose, CA, USA).

Results

In the overall study, BPA-exposed animals did not differ from controls with respect to body weight of dams before and after delivery, the number of pups born and sex ratio, whereas a tendency to a significant effect of exposure was found for body weight of litters at birth, with BPA20 exposed litters weighing less than control litters. However, no differences were found for growth rates and body weight at weaning (data not shown). In addition, we did not observe changes in the oestrous cycle of adult BPA-exposed females compared to control mice.

In male and female mice in the present study, the distribution of nNOS-ir system is in accordance with our previous studies; for a complete description of nNOS distribution within the mouse hypothalamus, see (30).

In particular, nNOS-ir small neurones are largely present in the medial part of the MPOM, whereas several positive fibres are present in the lateral aspect of this nucleus (Fig. 1). A relevant group of positive cell bodies was observed in the BST, mainly clustered within its ventromedial subdivision; these cells were small, round, intensely or weakly stained with a relatively large nucleus (Fig. 1). A large number of intensely-stained neurones are present in the magnocellular hypothalamic nuclei (PVN and supraoptic nucleus).

In the posterior hypothalamus, nNOS-ir neurones are present in VMH and Arc nuclei. Within the VMH, intensely-stained neurones are only present in the ventrolateral part of the nucleus (VMHvl). The other sub-regions of the nucleus are intensely positive for the

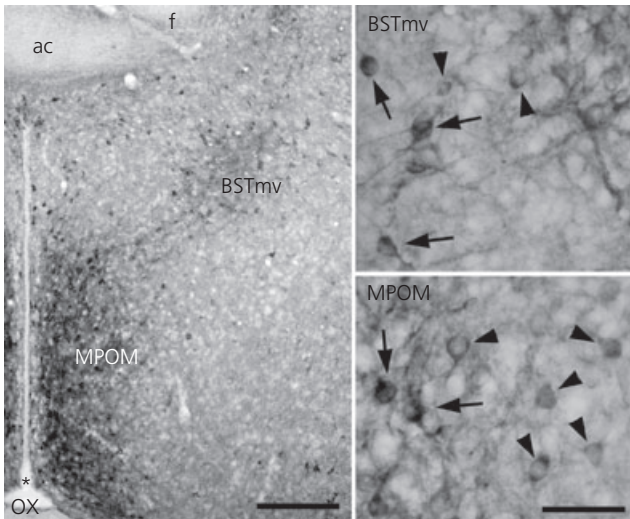


Fig. 1. Left: low power enlargement of a section including the medial pre-optic nucleus (MPOM) and the bed nucleus of the stria terminalis, ventromedial subdivision (BSTmv). ac, anterior commissure; f, fornix; OX, optic chiasma; *, third ventricle (scale bar = 200 μm). Right: high power enlargements of MPOM and BSTmv nuclei to show the morphology of neuronal nitric oxide synthase-immunoreactive elements. Arrowheads: weakly stained neurones (in many cases with a large and visible cell nucleus); arrows: more intensely stained cells, also showing labelled processes (scale bar = 50 μm).

presence of a high number of positive fibres. In the Arc, round and weakly-stained cell bodies are present in the whole extension of the nucleus. Scattered intensely positive cells are also present in the dorsal hypothalamic nucleus.

In our specimens, we observed qualitative differences in the nNOS immunoreactivity only in very specific regions, corresponding to the MPOM and the ventral part of the BST (Figs 2, 3 and 4). In the other regions, we did not observe any qualitative difference. These observations were confirmed by the quantitative analysis (Table 1).

For all nuclei, the preliminary two-way ANOVA for repeated measures did not reveal a statistically significant effect for the antero-posterior levels; therefore, we repeated the two-way ANOVA (with treatment and sex as the independent factors) considering the average number of nNOS-ir cells. In the MPOM, the results of two-way ANOVA revealed a significant effect of sex ($P < 0.05$) and treatment ($P < 0.05$). In BSTmv, we detected a significant difference only for sex ($P < 0.02$), whereas, in BSTmpm, the difference for sex is close to significance ($P = 0.06$). Considering the whole PVN, two-way ANOVA revealed a significant global effect of sex ($P < 0.05$). For all the other examined nuclei (Arc, VMHvl, CPu), the results of the ANOVA were not significant. We therefore applied the post-hoc Fisher's PLSD test for two-by-two comparisons of the independent variables to the results obtained for the MPOM, BSTmv, BSTmpm and PVN.

In the MPOM (Fig. 5), a statistically significant sexual dimorphism ($P < 0.05$) was observed only in control animals, with oil males having a significantly higher number of cells than oil females. The dimorphism is not present in all the other experimental groups (i.e. BPA-treated males and females do not significantly differ). For

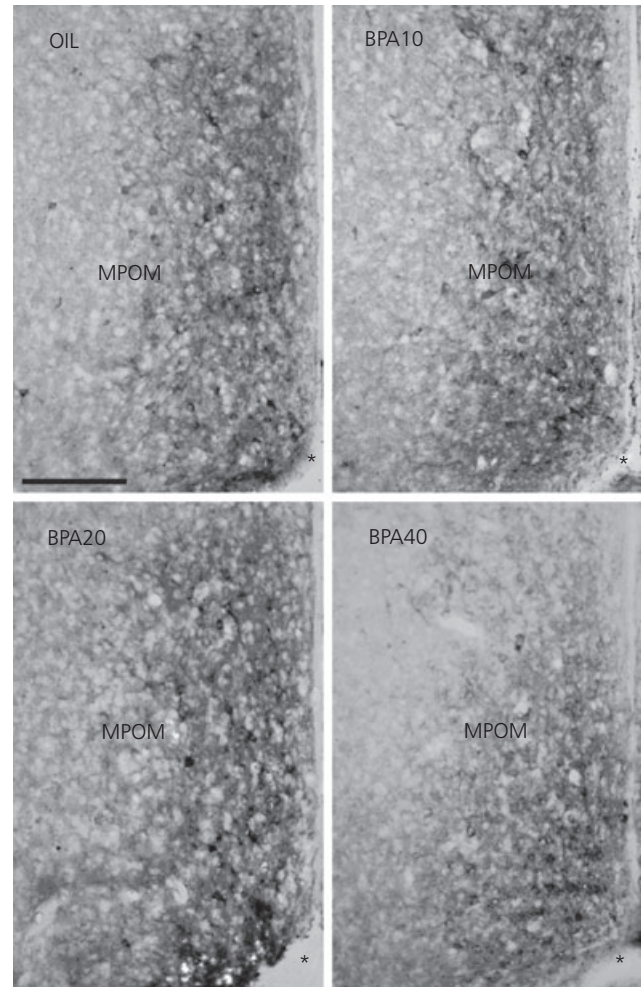


Fig. 2. Distribution of neuronal nitric oxide synthase positive elements within the medial preoptic nucleus (MPOM) of male mice exposed perinatally to bisphenol A (BPA). OIL, male mice perinatally treated with corn oil; BPA10, male mice perinatally treated with 10 $\mu\text{g}/\text{kg}/\text{day}$ of BPA; BPA20, male mice perinatally treated with 20 $\mu\text{g}/\text{kg}/\text{day}$; BPA40, male mice perinatally treated with 40 $\mu\text{g}/\text{kg}/\text{day}$. *, third ventricle (scale bar = 150 μm).

BPA10 (10 $\mu\text{g}/\text{kg}/\text{day}$) or BPA20 (20 $\mu\text{g}/\text{kg}/\text{day}$) treatments, the lack of dimorphism is the result of an increase in the number of female cells (BPA20 females versus oil females; $P < 0.05$). In BPA10 and BPA20 males, the mean number of positive cells is stable, whereas we detected a significant decrease in BPA40 (40 $\mu\text{g}/\text{kg}/\text{day}$) compared to BPA20 males ($P < 0.05$), thus determining the disappearance of the sex dimorphism.

In BSTmv (Fig. 5), we did not observe sex dimorphism in the oil group, whereas dimorphism (i.e. females having a higher number of cells than males) is present and significant ($P < 0.01$) in animals treated with 20 $\mu\text{g}/\text{kg}/\text{day}$ of BPA. This is the result of a significant decrease in nNOS-ir cell number in males ($P < 0.05$ versus oil males; $P < 0.01$ versus BPA10 males). In females, the treatments did not induce any significant alteration of cell number. In BSTmpm, as well as in PVN, the post-hoc test did not detect any significant difference among sexes and experimental groups.

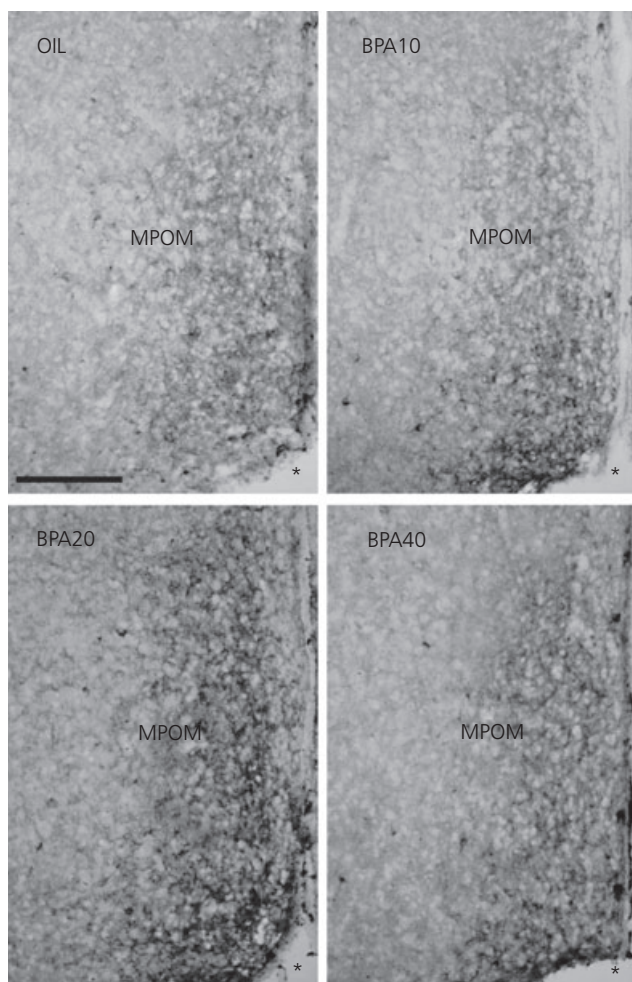


Fig. 3. Distribution of neuronal nitric oxide synthase positive elements within the medial preoptic nucleus (MPOM) of female mice exposed perinatally to bisphenol A (BPA). OIL, female mice perinatally treated with corn oil; BPA10, female mice perinatally treated with 10 $\mu\text{g}/\text{kg}/\text{day}$ of BPA; BPA20, female mice perinatally treated with 20 $\mu\text{g}/\text{kg}/\text{day}$; BPA40, female mice perinatally treated with 40 $\mu\text{g}/\text{kg}/\text{day}$. *, third ventricle (scale bar = 150 μm).

Discussion

The data obtained in the present study demonstrate that perinatal administration (up to day 8 of lactation) of BPA to the dams, in concentrations lower than that considered safe for human beings (50 $\mu\text{g}/\text{kg}/\text{day}$), results in a permanent alteration of some nitrinergic circuits of the BPA-exposed offspring when they become adults.

The action of BPA is very specific. Indeed, only two of the six nuclei that we have investigated [i.e. MPOM and BSTmv, both involved in the control of male (51, 52) and female (53) sexual behaviour and belonging to the accessory olfactory pathway] were affected by perinatal treatment. The detected effects were sex- and dose-specific.

In the control group of mice, we found a sexual dimorphism with respect to nNOS-positive cell number in the MPOM (i.e. higher number of nNOS-ir cells in males compared to females) and a lack

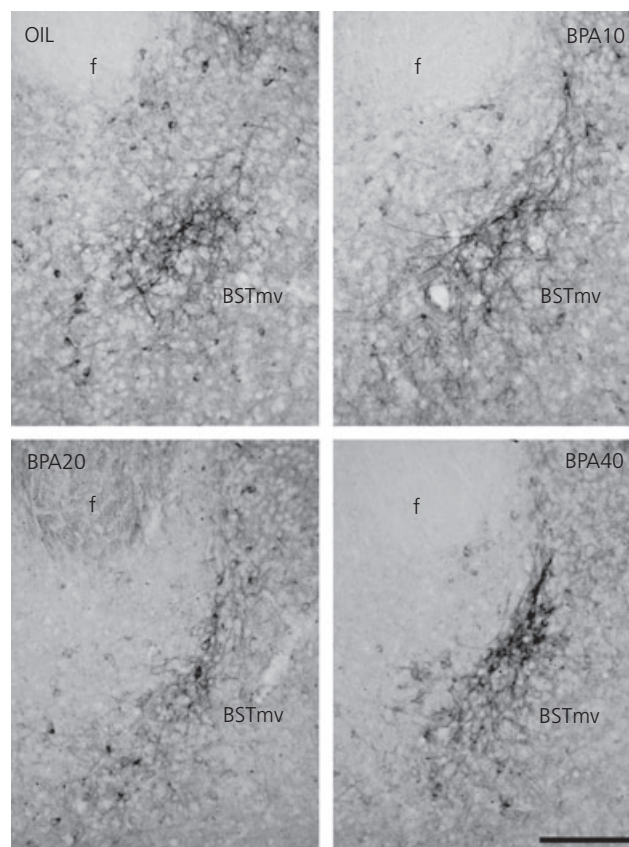


Fig. 4. Distribution of neuronal nitric oxide synthase positive elements within the bed nucleus of the stria terminalis, ventromedial subdivision (BSTmv) of male mice exposed perinatally to bisphenol A (BPA). OIL, male mice perinatally treated with corn oil; BPA10, male mice perinatally treated with 10 $\mu\text{g}/\text{kg}/\text{day}$ of BPA; BPA20, male mice perinatally treated with 20 $\mu\text{g}/\text{kg}/\text{day}$; BPA40, male mice perinatally treated with 40 $\mu\text{g}/\text{kg}/\text{day}$. f, fornix.

of dimorphism in both subdivisions of BST, thus confirming previous data obtained in our laboratory (30).

In the MPOM, the perinatal administration of BPA induced two opposing effects in males and females. In females, the intermediate dose of BPA (20 $\mu\text{g}/\text{kg}/\text{day}$) induced a significant increase of the number of nNOS elements, whereas, in males, the administration of the highest dose of BPA (40 $\mu\text{g}/\text{kg}/\text{day}$) induced a significant decrease of nNOS elements compared to BPA20 males. Consequently, the significant sex difference in nNOS cell number observed in MPOM of control mice disappears in BPA-exposed mice.

A similar effect is present also in male BSTmv, where we detected a decrease of the nNOS immunoreactive cells in BPA20 exposed males compared to oil males. By contrast, in females, we did not detect any significant difference. In this case, although control animals did not show sexual dimorphism in BST nNOS cells number, BPA20 exposed mice did. Finally, the nitrinergic population of the other examined nuclei was not affected by the treatments.

These results indicate that the mechanism of action of BPA on the differentiation and expression of nNOS-ir system is more complex than that considered on the basis of its classification as a

Table 1. Number of Neuronal Nitric Oxide Synthase-Immunoreactive Cells (Mean \pm SE) in different experimental groups.

Nucleus	OIL M (n = 5)	OIL F (n = 5)	BPA10 M (n = 5)	BPA10 F (n = 5)	BPA20 M (n = 6)	BPA20 F (n = 5)	BPA40 M (n = 5)	BPA40 F (n = 5)	ANOVA
MPOM	166.70 + 41.16	80.40 + 23.03^o	147.50 + 21.77	115.20 + 24.50	191.68 + 24.68	164.80 + 28.24[†]	110.90 + 15.01*	105.50 + 11.40	$F_{3,33} = 4.46$ P < 0.05 (sex) $F_{3,33} = 2.66$ P < 0.05 (treatment)
BSTmv	20.00 + 2.53*	24.20 + 2.66	22.30 + 3.21**	21.80 + 2.66	12.75 + 1.99	22.70 + 3.35	15.60 + 1.81	20.42 + 1.47	$F_{1,33} = 7.32$ P < 0.01 (sex)
BSTmpm	13.68 + 0.99	18.20 + 1.87	12.70 + 2.46	16.70 + 1.12	13.50 + 2.48	16.30 + 1.25	16.40 + 3.72	16.08 + 1.45	$F_{1,33} = 3.53$ P = 0.069 (sex)
VMHvl	49.10 + 5.34	52.30 + 2.93	49.80 + 10.23	63.20 + 3.31	45.60 + 5.44	50.30 + 6.29	51.10 + 6.66	58.60 + 7.24	NS
Arc	36.60 + 2.00	34.10 + 2.52	33.50 + 2.34	33.30 + 2.29	32.90 + 1.86	38.90 + 0.97	36.30 + 1.99	36.80 + 2.73	NS
PVN	43.83 + 7.04	50.00 + 5.55	35.30 + 5.71	46.83 + 4.94	37.33 + 7.27	49.08 + 3.55	47.80 + 5.01	53.75 + 5.59	$F_{1,33} = 4.72$ P < 0.05 (sex)
CPu	89.80 + 9.63	71.60 + 17.86	76.80 + 10.35	85.00 + 7.41	65.00 + 4.89	77.20 + 4.34	66.20 + 10.96	92.40 + 7.10	NS

The last column reports the F and P values (if significant) of one-way ANOVA for the corresponding nucleus. NS, not significant. The data demonstrating significant differences after the two-by-two comparisons with the Fisher's protected least significant difference test are reported in bold. ^oP < 0.05 compared to oil males; *P < 0.05 compared to BPA20 males; **P < 0.01 compared to BPA20 males; [†]P < 0.05 compared to oil females.

M, males; F, Females; n, number of examined animals; MPOM, medial preoptic nucleus; BSTmv, ventromedial subdivision of the bed nucleus of the stria terminalis; BSTmpm, posteromedial subdivision of the bed nucleus of the stria terminalis; VMHvl, ventrolateral subdivision of the ventromedial nucleus; Arc, arcuate nucleus; PVN, paraventricular nucleus; CPu, nucleus caudate putamen.

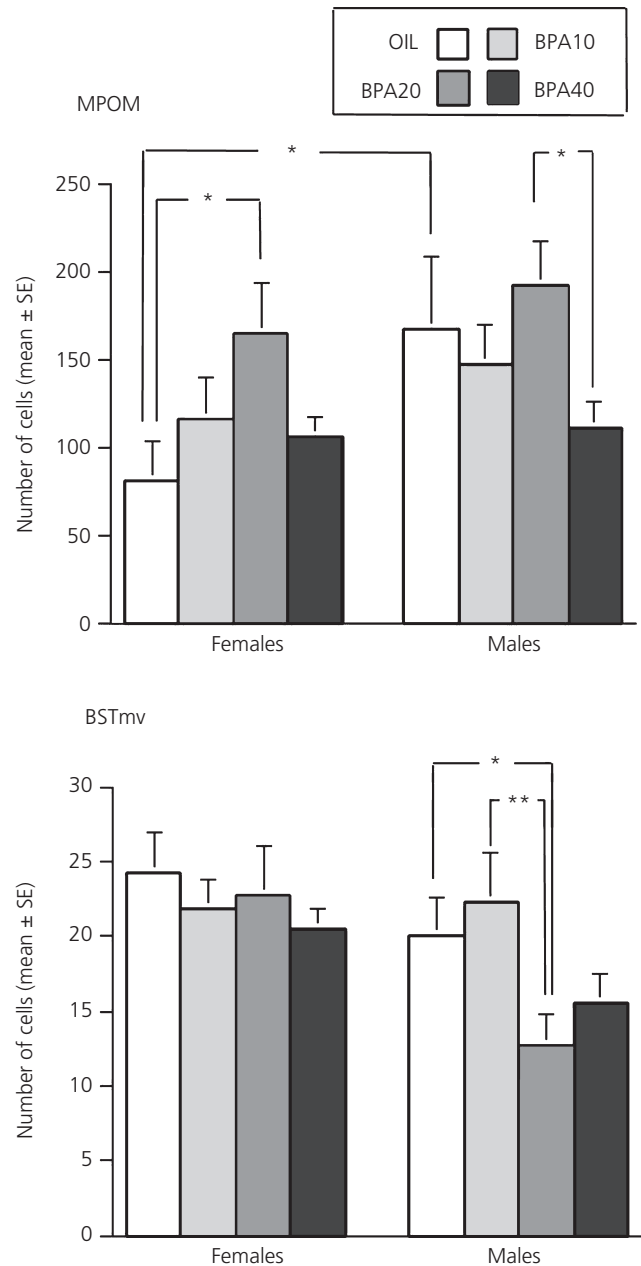


Fig. 5. Histograms reporting the differences in neuronal nitric oxide synthase-immunoreactive cells number (\pm SE) in the medial preoptic nucleus (MPOM) and in the bed nucleus of the stria terminalis, ventromedial subdivision (BSTmv) of male and female mice exposed perinatally to bisphenol A (BPA). OIL, male mice perinatally treated with corn oil; BPA10, male mice perinatally treated with 10 μ g/kg/day of BPA; BPA20, male mice perinatally treated with 20 μ g/kg/day; BPA40, male mice perinatally treated with 40 μ g/kg/day. Asterisks indicate significant differences (Fisher's protected least significant difference test). *P < 0.05, **P < 0.01.

xenoestrogen. On one side, all the nuclei that we examined express ERs, although the effects of BPA are significant only in MPOM and BSTmv. The simplest explanation for this behaviour is linked to our poor knowledge of the degree of colocalisation among ERs and nNOS within these regions. A partial quantification of this coexis-

tence has been published only for male mouse MPOM, BST and PVN (37), but not for the other nuclei that we have here investigated. According to these previous data, within the male MPOM, the nNOS-ir cells show a 90% degree of coexistence with ER α , whereas, in the BST, only 16% of cells coexist, and even less in the PVN (<10%); no data are available, even for these two nuclei, for ER β immunoreactivity and for the females, and no data are available regarding the situation during development. Therefore, we may only hypothesise that differences observed in the present study among these and the other investigated regions may be based on differences in the degree of coexistence for ER α , ER β and nNOS during the embryonic and perinatal period.

In addition, the results obtained in the present study show a masculinising effect of BPA in the female MPOM; this effect is therefore in agreement with the putative xenoestrogenic activity of this compound. By contrast, in the male MPOM and BSTmv, we detected a demasculinising effect that is not in accordance with the previously mentioned xenoestrogenic classification. Rather, we should hypothesise an anti-oestrogenic activity of BPA on the male MPOM and BSTmv nNOS-ir system. Clearly, these two hypotheses for the action of BPA are antagonist and it is difficult to explain them only through the interaction of BPA and classical ERs. Therefore, based also on the previous literature (54), we should consider other mechanisms that could be involved in the action of BPA. For example, it has been demonstrated that perinatal BPA can directly influence ER α expression (55) within the MPOM, and both ER α and ER β in the brainstem (56). In particular, some studies suggest that BPA can interfere with ER α expression via nongenomic mechanisms (55, 57).

Because sexual differentiation of the hypothalamus is ER-dependent and mediated at least partly through ER α (58), BPA deregulation of this receptor could affect the natural ER-directed dimorphisms of MPOM circuits. On the basis of these results, we can suggest that BPA exposure during critical periods of development disrupts hypothalamic gene expression, thus interfering with the normal maturation of sexually dimorphic areas of the forebrain.

The results of the present study relative to the female MPOM are in accordance with recent data pointing to the occurrence, in some cases, of a non-monotonic, inverted U-dose-response of the endocrine-disrupting compounds (54, 59–61). Indeed, in female MPOM, significant alterations of the nitrinergic system have been detected with the intermediate dose of BPA (20 $\mu\text{g}/\text{kg}/\text{day}$) and not with the highest dose (40 $\mu\text{g}/\text{kg}/\text{day}$).

Finally, the results obtained with respect to the effects of perinatal administration of low doses of BPA altering the nNOS system in MPOM and BST are in agreement with the behavioural results showing that perinatal exposure of BPA in rats and mice of both sexes affects socio-sexual behaviours (62, 63) that are under the control of the nitrinergic system (64). Although sexual behaviours of male mice did not appear to be altered after perinatal exposure to low-dose BPA (P. Palanza, unpublished data), male rats perinatally exposed to 40 $\mu\text{g}/\text{kg}/\text{day}$ of BPA showed less efficient sexual behaviours than controls (62).

A number of studies have recently shown that sexually dimorphic behaviours, either reproductive or nonreproductive, are particularly sensitive to developmental interferences produced in different ani-

mal models by chemicals with endocrine-disrupting properties (4, 22). A common issue emerging from developmental studies in mice and rats is that exposure to BPA, as well as to other oestrogenic EDCs, can diminish, eliminate, reverse or widen sex differences in brain and/or behaviours, thus interfering with sexual differentiation processes. In particular, the catecholaminergic system appears to be a preferential target as demonstrated by studies on the locus coeruleus (21, 65) and the anteroventral periventricular nucleus (18, 66, 67).

The data demonstrating the disappearance of sexually dimorphic differences in the nitrinergic system of MPOM and BST in mice are therefore in agreement with the idea that early administration of BPA may heavily impact the organisation of some dimorphic nervous circuits in the limbic-hypothalamic system of rodents, providing a structural basis for understanding the behavioural effects.

Recent studies (63) reported that perinatal exposure to low-dose BPA (10 $\mu\text{g}/\text{kg}/\text{day}$) reduced or reversed sex differences in exploration and emotional behaviours in different experimental paradigms, before and after puberty. Developmental exposure to BPA induced subtle behavioural alterations mainly in females, so that their behaviour was more similar to control males' behaviour than to control females' behaviour. This finding may well be considered as an index of the higher emotionality of exposed females in response to novel stimuli and is consistent with the possible 'masculinisation' effects of perinatal exposure to this oestrogenic compound. However, BPA exposed males also showed female-type behaviour on a few measures. The overall result was a reduction or a reversal of sex differences in exposed mice, relative to those displayed by controls, which is in good agreement with previous and present data showing alterations of the sexual dimorphism of the catecholaminergic and nitrinergic systems after embryonic and postembryonic BPA exposure. Finally, these findings emphasise the need to employ a comprehensive approach that addresses anatomical, functional and behavioural endpoints when evaluating the potential effects of EDC exposure (4, 68, 69).

In conclusion, the data obtained in the present study confirm that perinatal exposure to EDCs, in particular to BPA, may have a high impact on the organisation of specific neural pathways in a selective and non-unidirectional way, and this can later affect complex behaviours or functions such as those related to reproduction.

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