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Environmentally Relevant Perinatal Exposures to Bisphenol A Disrupt Postnatal Kiss1/NKB Neuronal Maturation and Puberty Onset in Female Mice

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Background: The timing of puberty is highly sensitive to environmental factors, including endocrine disruptors. Among them, bisphenol A (BPA) has been previously analyzed as potential modifier of puberty. Yet, disparate results have been reported, with BPA advancing, delaying, or being neutral in its effects on puberty onset. Likewise, mechanistic analyses addressing the central and peripheral actions/targets of BPA at puberty remain incomplete and conflictive.

Objective: We aimed to provide a comprehensive characterization of the impact of early BPA exposures, especially at low, real-life doses, on the postnatal development of hypothalamic Kiss1/NKB neurons, and its functional consequences on female pubertal maturation.

Methods: Pregnant CD1 female mice were orally administered BPA at 5, 10, or 40 μg/kg body weight (BW)/d from gestational day 11 to postnatal day 8 (PND8). Vaginal opening, as an external marker of puberty onset, was monitored daily from PND19 to PND30 in the female offspring. Blood and brain samples were collected at PND12, 15, 18, 21, and 30 for measuring circulating levels of gonadotropins and analyzing the hypothalamic expression of Kiss1/kisspeptin and NKB.

Results: Perinatal exposure to BPA, in a range of doses largely below the no observed adverse effect level (NOAEL; 5 mg/kg BW/d, according to the FDA), was associated with pubertal differences in the female progeny compared with those exposed to vehicle alone, with an earlier age of vaginal opening but consistently lower levels of circulating luteinizing hormone. Mice treated with BPA exhibited a persistent, but divergent, impairment of Kiss1 neuronal maturation, with more kisspeptin cells in the rostral (RP3V) hypothalamus but consistently fewer kisspeptin neurons in the arcuate nucleus (ARC). Detailed quantitative analysis of the ARC population, essential for pubertal development, revealed that mice treated with BPA had persistently lower Kiss1 expression during (pre)pubertal maturation, which was associated with lower Tac2 (encoding NKB) levels, even at low doses (5 μg/kg BW/d), in the range of the tolerable daily intake (TDI), recently updated by the European Food Safety Authority.

Conclusions: Our data attest to the consistent, but divergent, effects of gestational exposures to low concentrations of BPA, via the oral route, on phenotypic and neuroendocrine markers of puberty in female mice, with an unambiguous impact on the developmental maturation not only of Kiss1, but also of the NKB system, both essential regulators of puberty onset. https://doi.org/10.1289/EHP5570

Introduction

The onset of puberty is a robust developmental trait that depends on the proper functional organization of the reproductive neuroendocrine system, also termed the hypothalamic–pituitary–gonadal (HPG) axis, at earlier stages of maturation. This intricate process starts *in utero* and progresses throughout postnatal development and is highly influenced by the dynamic interplay between genes and environment (Tena-Sempere 2010). When this delicate interplay is disrupted, the maturation of the HPG axis is altered and perturbation of the timing of puberty can occur, often as the first phenotypic sign of later reproductive disorders. Interestingly, different epidemiological studies have identified an advancement of the age of puberty onset, especially in girls, with younger cohorts starting puberty earlier (Aksglaede et al. 2009a, 2009b; Euling et al. 2008a; Parent et al. 2016). For instance, the Copenhagen puberty study reported an advancement of approximately 1 y in the mean age of thelarche in two cohorts of girls born and studied 15 y apart, between the early 1990s and 2006 (Aksglaede et al. 2009b). Given the similar genetic background and the short time elapsed, these observations support a key role of environmental factors in this phenomenon, as highlighted by various consensus reports (Buck Louis et al. 2008; Euling et al. 2008b). Indeed, the escalating prevalence of child obesity has been blamed as major contributor for this trend (Reinehr and Roth 2019). However, body mass index alone does not likely account for all the environmental contribution to the advancement of the age of puberty, which seemingly include also the effects of different endocrine-disrupting compounds (EDCs) (Castellano and Tena-Sempere 2016; Euling et al. 2008b; Özen and Darcan 2011; Parent et al. 2015).

Among EDCs, BPA is one of the most widely studied. BPA is an organic compound with estrogenic activity used in the production of polycarbonate plastics and epoxy resins (Vandenberg et al. 2007). BPA is present in many common products, including food and beverage containers, plastic eating utensils, toys and office products (Viñas et al. 2012). BPA is currently considered among the most important chemicals to be managed in terms of environmental health (Kot-Wasic et al. 2007; Petrović et al. 2003). This is mainly due to its increasing production, with more than 3.6 million tons/year (Rubin 2011), and its potential involvement in a wide

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array of health problems, including reproductive disorders related to altered maturation or function of the HPG axis (Dickerson et al. 2012; Patisaul et al. 2009; Rubin et al. 2001; Welshons et al. 2006). Regardless, the impact of BPA on puberty onset remains contentious. In female rodents, pre- and postnatal BPA exposure has been associated with either a normal or precocious age of vaginal opening (VO), considered to be an external index of puberty onset, depending on the timing, dose, and route of administration (Adewale et al. 2009; Fernández et al. 2009; Honma et al. 2002; Nikaido et al. 2004; Parent et al. 2015). Moreover, a recent study, involving subcutaneous administration of various of doses of BPA, suggested that postnatal exposure to very low doses of BPA [25 ng/kg body weight (BW)/d] delayed puberty onset in rats, whereas postnatal injections of very high doses (5 mg/kg BW/d) tended to advance the onset of puberty (Franssen et al. 2016). In the same vein, contradictory findings have also been reported in girls, where some studies have suggested an association between increased urinary levels of BPA and altered puberty (Durmaz et al. 2014, 2018; Kasper-Sonnenberg et al. 2017; McGuinn et al. 2015) and other studies have failed to detect changes in pubertal timing linked to BPA excretion (Buluş et al. 2016; Lee et al. 2014).

Despite numerous phenomenological studies, the mechanisms whereby BPA exposure might impact the timing of puberty in females remain poorly understood. Although initial studies suggested the involvement of peripheral mechanisms (Adewale et al. 2009; Nah et al. 2011; Nikaido et al. 2004), recent reports, involving the use of rodent models exposed to different doses of BPA, have highlighted the relevance of central neuroendocrine mechanisms (Franssen et al. 2016; Losa et al. 2011; Naulé et al. 2014; Oliveira et al. 2017; Roepke et al. 2016). In this context, compelling, but as yet incomplete, evidence has suggested a pathogenic role for the hypothalamic kisspeptin system as a potential target and mediator for the disrupting neuroendocrine actions of BPA on female pubertal development (Castellano and Tena-Sempere 2016; Patisaul 2013).

Kisspeptins, a family of structurally related peptides encoded by the *Kiss1* gene, are considered essential regulators of key aspects of reproductive function (Navarro and Tena-Sempere 2011; Pinilla et al. 2012). Kisspeptins play an essential role in the activation of the HPG axis at puberty (Tena-Sempere 2013; Terasawa et al. 2013). This is based on different studies showing that *a*) inactivating mutations of either *KISS1* or *KISS1R*, which encodes the canonical receptor for kisspeptins, cause a lack of pubertal maturation; *b*) kisspeptin tone and sensitivity increase along pubertal development; and *c*) the number of kisspeptin neurons and their projections to gonadotropin-releasing hormone (GnRH) neurons increase during the juvenile–pubertal transition (Pinilla et al. 2012). Importantly, neuroanatomical studies have shown the existence of two major populations of kisspeptin neurons that are differently regulated by sex steroids (Clarkson et al. 2009b; Pinilla et al. 2012). One of these populations is located in the rostral preoptic area of the third ventricle (RP3V) and responds to estrogen with an increased synthesis of kisspeptin. In contrast, the other population is located in the arcuate nucleus (ARC) and responds to estrogen with the inhibition of kisspeptin production (Marraudino et al. 2018; Smith et al. 2005a, 2005b). ARC kisspeptin neurons co-express neurokinin B (NKB), a tachykinin peptide encoded by the *TAC3/Tac2* gene (Lehman et al. 2010; Navarro et al. 2011). The release of NKB from these neurons, which are profusely interconnected and are termed KNDy because they also express dynorphin (i.e., they produce kisspeptins, NKB and dynorphin), can activate their kisspeptin output and, in this way, modulate GnRH neurosecretory activity (Navarro et al. 2011; Ramaswamy et al. 2010; Wakabayashi et al. 2013). In good agreement, it has been shown that expression of Tac2 increased earlier than Kiss1 during postnatal development in mice (Gill et al. 2012) and that NKB stimulated kisspeptin in male mice during the prepubertal period (Navarro et al. 2011), thus suggesting the potential role of NKB in the pubertal activation of kisspeptin–GnRH secretion.

The possibility that BPA might alter the maturation of the HPG axis in females by disrupting the hypothalamic kisspeptin system has been suggested by a limited number of studies that documented that neonatal injections of high doses of BPA significantly decreased *a*) *Kiss1* mRNA expression at the RP3V in infantile female rats (Cao et al. 2012); *b*) the hypothalamic levels of *Kiss1* mRNA in peripubertal female rats (Navarro et al. 2009b); and *c*) the number of kisspeptin fibers in the ARC of adult female rats (Patisaul et al. 2009). However, a comprehensive characterization of the impact of early BPA exposure, especially at low, environmentally relevant doses, on the postnatal development of hypothalamic Kiss1/NKB neurons and its functional consequences in terms of pubertal maturation of the female HPG axis is totally missing. Here, we explore the consequences of perinatal exposure to a wide range of doses of BPA, relevant in terms of environmental exposure and route of administration, on the infantile, juvenile, and pubertal development of hypothalamic Kiss1/NKB neurons in female mice and correlate such changes with relevant functional markers of pubertal onset such as the age of VO, which reflects the rise of ovarian estrogens and antecedes the first ovulation, as well as the developmental pattern of gonadotropin secretion.

Methods

Animals and Experimental Design

All experimental procedures involving the use of laboratory rodents were carried out according to the European Union regulations (EU 2010), current Italian legislation (DL 26/2014), and the institutional guidelines on animals' welfare of the University of Turin. Experiments were approved by the ethical committee of the University of Turin and designed to minimize the number of animals used.

Forty adult CD1 female mice (12-week-old) mice bred in the vivarium of the University of Turin were used as dams for BPA administration. A graphical presentation of the experimental design is shown in Figure S1. Animals were maintained under constant conditions of light (12-h light/dark cycle) and temperature (22–23°C), and housed in 45 × 25 × 15-cm BPA-free polypropylene cages in groups of three to four mice per cage with free access to food and water. The standard mouse chow, 4RF25-GLP (Mucedola S.R.L.), with certified nondetectable estrogenic activity, was used. During a preexperimental phase of 10 d, animals with adequate weight gain and without clinical abnormalities were trained to spontaneously drink tocopherol-stripped corn oil (Sigma-Aldrich) through a nonstressful procedure (Palanza et al. 2002). Briefly, a micropipette tip was placed into the mouse mouth with the pipette tip gently touching the roof of the mouth, and the oil was ejected from the pipette. The mice readily consumed the oil. After this period, female mice were housed between cages of males to induce ovulation and then paired with CD1 male mice for up to a week. Mating was verified by the presence of a vaginal plug, which was considered gestational day 0 (GD0).

After mating, pregnant females were housed singly and were randomly divided into four experimental treatment groups ($n = 10$ pregnant mice per group): OIL, receiving only the vehicle (corn oil); BPA 5, 10, and 40, receiving 5, 10, and 40 μg/kg BW/d of BPA (BPA purity 99%; SIGMA-Aldrich), respectively. Dosing solutions were freshly prepared and adjusted to mouse weight (final volume: 30 μL). Treatments were orally administered daily

from GD11 to postnatal day 8 (PND8). Oral dosage was selected to mimic human exposure routes to BPA (Arambula et al. 2016; Rebuli et al. 2014). The indicated age-window of BPA exposure was selected to cover the critical period of prenatal brain organization, starting from GD10–11 (Howdeshell 2002) and the postnatal period of organization of hypothalamic sexually dimorphic circuits in rodents (Toran-Allerand 1976).

Within 12 h of birth, litters were weighted, sexed, and culled to 10 pups (5 ± 1 males and 5 ± 1 females) and returned to their mother. In total, 40 litters were generated. Due to logistic reasons related to the large number of litters/animals being handled simultaneously and given our primary interest on the physiological and environmental determinants of female puberty, the experiments were performed selectively in female mice. Hence, subsets of female mice were euthanized at the following age points of early postnatal development: PND12, 15, 18, 21, and 30, for hormonal, *in situ* hybridization (ISH), and immunohistochemical (IHC) analyses. Given the differential processing of brain tissues for ISH and IHC (see below), for each age point and BPA treatment, 10 female mice were euthanized and allocated for ISH and IHC procedures ($n = 5$/each). For PND30, larger groups, of 15 mice each, were generated for monitoring the age of VO. Allocation of pups to each age point of sampling was conducted randomly. However, to avoid any potential litter effect, only one pup per litter was included in each experimental group, as defined by the age of sampling and dose of BPA.

The rationale for selection of the different age points for blood and brain sampling was as follows. PND12 was chosen as representative of the infantile period, when sex differences in the pattern of hypothalamic *Kiss1* mRNA expression start to emerge (Semaan et al. 2010). Later age points were selected because they cover the transition from the late-infantile to the juvenile period, between PND15 and 21, corresponding to the third week of postnatal life, when clear changes in Kiss1/kisspeptin expression become fully detectable in female rodents (Clarkson and Herbison 2006). An additional age point (PND30), corresponding to the pubertal period, was also included (Clarkson and Herbison 2006). As a general procedure for termination of experiments, the animals were anesthetized and euthanized in the morning (at 1000 hours), either by direct decapitation (for ISH) or after perfusion (for IHC), at the age points indicated above. Trunk blood samples were collected, centrifuged for serum isolation and stored at -20°C for hormonal determinations.

For the PND30 groups, weaning was conducted at PND21, when female pups of the same group of treatment were housed in single mouse cages with free access to food and water. In these mice, VO, considered to be an external sign of puberty onset, was monitored daily from PND19 to 30, in OIL- and BPA-treated mice ($n = 15$/group). VO was considered positive only when complete canalization of the vagina was confirmed upon visual inspection, following standard protocols (Gaytan et al. 2017). Considering our experimental design, with the latest age point being PND30, and the fact that in mice, first estrus usually occurs around 7 d after VO (Gaytan et al. 2017), we decided not to obtain vaginal smears for detection of cytological signs of first ovulation given that this would have occurred several days after PND30. This procedure minimized manipulation, and potential interference, of the experimental animals.

Hormonal determinations were conducted for each age point and treatment in samples from 6–10 independent mice, whereas ISH and IHC analyses were applied in brain sections from at least 5 different mice. These numbers of individual determinations are in line with previously published studies analyzing analogous parameters (Arambula et al. 2016; Navarro et al. 2009b) and, according to statistical power calculations (G*Power; University of Dusseldorf, Germany), were anticipated to be sufficient to reliably detect differences, with statistical significance ($p < 0.05$), for the hormonal [$n = 6$–10; luteinizing hormone (LH) and follicle-stimulating hormone (FSH)], mRNA ($n = 5$; *Kiss1* and *Tac2*), and protein ($n = 5$; kisspeptin) end points being analyzed.

Kisspeptin Immunohistochemistry and Quantitative Analysis

For IHC analyses, female mice ($n = 5$ from each of the OIL or the BPA groups, at the various age points between PND12 and 30) were deeply anesthetized with 2,2,2-tribromoethanol [250 mg/kg intraperitoneal (i.p.); SIGMA-Aldrich] and perfused first with 0.9% saline solution until blood was flushed, followed by perfusion with fixative solution (4% formaldehyde in 0.1 M phosphate buffer, pH 7.4). Brains were dissected, post-fixed overnight in the same fixative, and stored in 30% sucrose–phosphate buffered saline (PBS) solution for 2–4 d at 4°C. Brains were frozen in a dry ice/isopentane bath at -35°C and four sets of 25-µm-thick coronal sections were cut containing the Anteroventral Periventricular Nucleus/Periventricular Nucleus (AvPV/PeN) and the ARC nuclei, respectively, and kept as free-floating sections in antifreeze solution at -20°C. The plain of sectioning matched the drawings corresponding to the coronal sections of the mouse brain atlas (Paxinos and Franklin 2001). All sections were processed simultaneously, and the individual slides were coded to blind the treatment for the evaluator.

Brain sections were processed for the avidin–biotin immunohistochemical method, according to previous references (Franceschini et al. 2006). Briefly, after overnight rinsing in 0.01 M PBS, sections were exposed to Triton X-100 (0.2% in PBS), treated in 1% methanol/hydrogen peroxide solution for 20 min to block endogenous peroxidase activity, then incubated for 30 min with normal goat serum (Vector Laboratories). Sections were then incubated at 4°C for 24 h with a polyclonal rabbit antiserum AC#566 against rodent kisspeptin-10, diluted 1:10,000, as previously defined (Clarkson and Herbison 2006; Franceschini et al. 2006). The antibody AC#566, used for immunohistochemical detection of kisspeptin, was a generous gift from I. Franceschini (Nouzilly, France). After washing in PBS, the sections were incubated for 60 min in biotinylated secondary goat anti-rabbit IgG (Vinci), washed, and incubated for 60 min with the avidin–biotin complex (VECTASTAIN® Elite® kit; Labtek). Finally, they were incubated in 0.15 mg/mL diaminobenzidine (DAB; SIGMA-Aldrich), and 0.025% hydrogen peroxide in 0.05 M Tris-HCl buffer (pH 7.6) for 5 min and then washed twice in distilled water and PBS. The free-floating brain sections were mounted on chrom-alum coated slides, air dried, washed in xylene, and cover-slipped in Entellan mounting medium (Merck).

For quantitative analysis of kisspeptin-immunoreactive (Kp-IR) cell bodies and fibers, we selected two hypothalamic areas: RP3V (Bregma 0.50 to 0.02) and ARC (Bregma -1.46 to -1.70). A variable number of standardized sections of comparable levels were chosen for each nucleus: four for ARC, and six for RP3V. Selected levels were digitized with a NIKON Digital Sight system connected to a NIKON Eclipse 80i microscope (Nikon Italia S.p.S.), by using a 10× (RP3V) or a 20× (ARC) objective. Digital images were processed and analyzed by means of a freeware (ImageJ 1.42q; Schneider et al. 2012). Total measurements were performed within predetermined areas [areas of interest (AOIs)]. The AOI selected for each nucleus was a box of fixed size and shape, selected to cover positive material only within the boundaries of each nucleus ($\sim 600.000\ \mu m^2$ for RP3V, and 280.000 for ARC). Kp-IR structures, both cell bodies and processes, were measured by calculating, in binary transformations of the images (the threshold function of the software), the fractional area, defined as the percentage of pixels covered by immunoreactive elements. Due to differences in the immunostaining, the range of the

threshold was individually adjusted for each section. In line with our previous studies (Marraudino et al. 2018; Panzica et al. 2011), the Kp-IR cell bodies were clearly visible only in the RP3V, whereas they were not clearly discernible in the ARC due to dense fiber accumulation. Therefore, the number of Kp-IR cell bodies was measured only in the RP3V, by the Cell Counter plugin of ImageJ, and expressed as total number of cells in the reference area. The analyses were performed blinded with regard to treatment group.

Detection of Kiss1 and Tac2 mRNA Expression by in Situ *Hybridization*

Female mice exposed to OIL or the range of BPA doses were anesthetized with an i.p. injection of chloral hydrate (400 mg/kg) and euthanized by decapitation in the morning (at 1000 hours), at the age points indicated above (PND12, 15, 18, 21, and 30; $n = 5$ animals per BPA dose and age). Upon decapitation, brain samples were collected on dry ice and stored at −80°C until processed for analysis of *Kiss1* and *Tac2* mRNA expression using ISH. Five sets of 20-μm sections in the coronal plane were cut on a cryostat from the diagonal band of Broca to the mammillary bodies, thaw-mounted onto SuperFrost™ Plus slides (VWR Scientific), and stored at −80°C.

To quantitatively assess *Kiss1* and *Tac2* mRNA levels, ISH procedures were performed in hypothalamic tissues, with special attention to the analysis of the regions comprising most of the expression of Kiss1 (ARC and RP3V) and Tac2 (ARC). For detection of mouse *Kiss1* mRNA, a specific antisense riboprobe (5′-ACCTGCCTCCTGCCGTAGCG-3′) located between nucleotides 352 and 371 of the circular DNA (cDNA) sequence (GenBank accession no. AF472576) was used. In the case of *Tac2* mRNA, the specific antisense riboprobe (5′-ATTTGAGGATGCCAAAGCTG-3′) is located between nucleotides 444 and 463 of the cDNA sequence (GenBank accession no. NM_009312).

Single-Label ISH of Kiss1 and Tac2 mRNA

Radiolabeled *Kiss1* and *Tac2* mRNA probes were transcribed using T7 polymerase (Bio-Rad Laboratories) and *Kiss1* or *Tac2* cDNA templates containing the T7 polymerase region, as described previously (Ruiz-Pino et al. 2012; Sánchez-Garrido et al. 2014, 2015). Briefly, radiolabeled RNA probes were synthesized by adding the

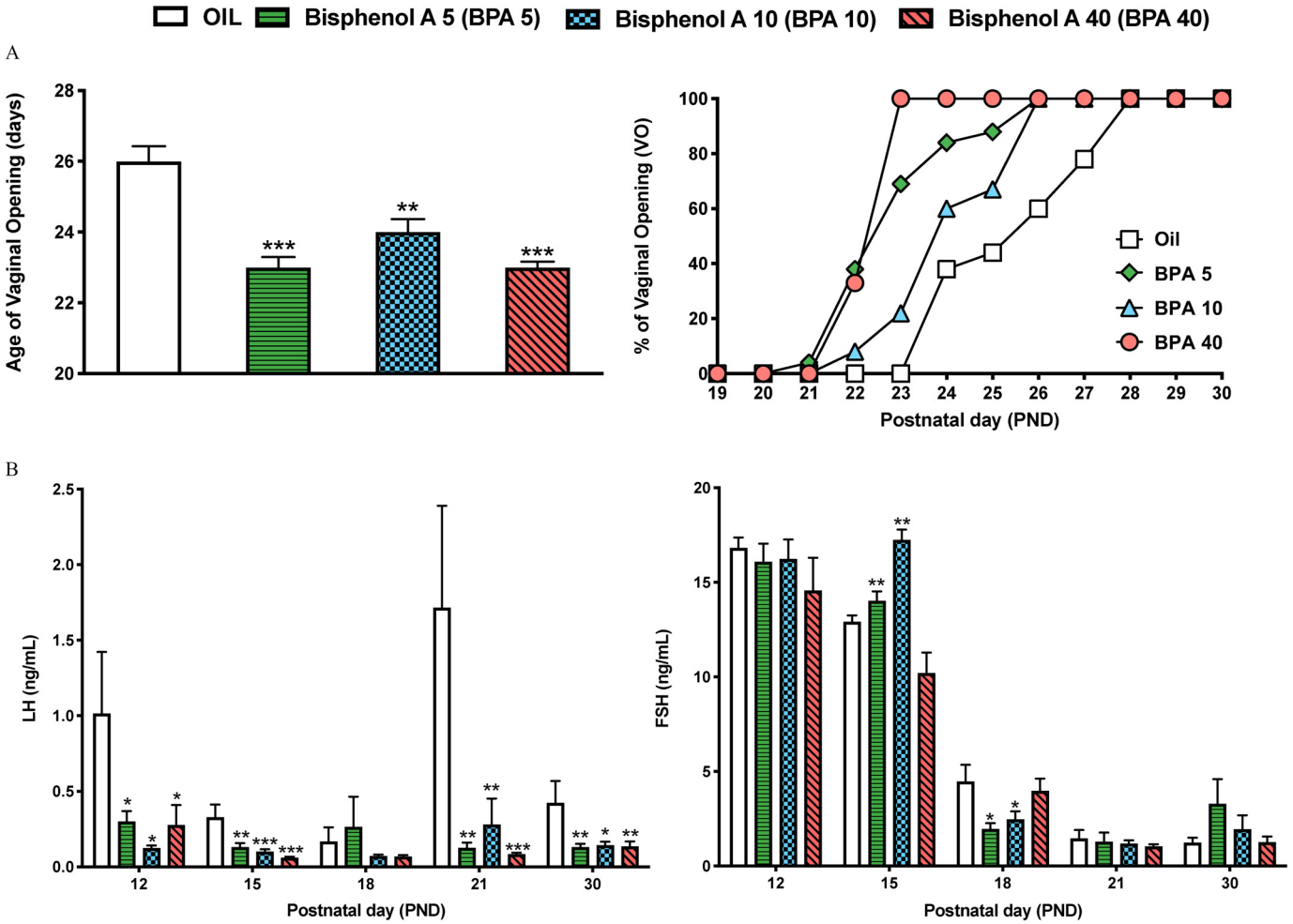

Figure 1. Impact of perinatal exposure to a range of doses of BPA on the age of VO and the pattern of gonadotropin secretion. Female mice exposed to increasing doses of BPA: 5, 10, or 40 μg/kg BW/d (BPA 5, 10, or 40 groups) from GD11 to PND8 were monitored between days 19 and 30 for canalization of the vagina; the day of complete canalization being considered to be vaginal opening (VO). An OIL-exposed group was used as a control. (A) Average age at VO (presented as mean ± SEM; $n = 15$ mice/group), and (B) percentage curves of VO are shown for each experimental group. (C,D) Gonadotropin levels were assayed for each experimental group at different time points of postnatal development: PND12, 15, 18, 21, and 30. (C) LH and (D) FSH values are represented as the mean ± SEM of 6–10 independent determinations. For VO data, $^{**}p < 0.01$, $^{***}p < 0.001$ vs. corresponding OIL-exposed (control) group (one-way ANOVA followed by Student-Newman-Keuls multiple range test). For hormonal data, $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$ vs. corresponding OIL-exposed group for each age [one-way ANOVA followed by Fisher least significant difference (LSD) test]. ANOVA, analysis of variance; BPA, bisphenol A; BW, body weight; FSH, follicle-stimulating hormone; GD, gestational day; LH, luteinizing hormone; PND, postnatal day; SE, standard error.

following reagents in a final volume of 25 μL: 250 Ci [33P]-uridine triphosphate (Perkin Elmer); 1 μg of template for Kiss1 or Tac2; 0.5 mM rATP, rCTP, and rGTP; and 40 U T7 RNA polymerase. Residual DNA was digested with 4 U DNase (Bio-Rad Laboratories), and the reaction was terminated by the addition of 2 μL of 0.5 M EDTA, pH 8.0. The riboprobes were separated from unincorporated nucleotides using Illustra™ ProbeQuant™ G-50 MicroColums (GE Healthcare).

Slides with mouse hypothalamic sections from the different treatment groups and ages were processed before hybridization, as described previously (Manfredi-Lozano et al. 2016). Briefly, slides were *a*) fixed in paraformaldehyde at 4% for 15 min, *b*) stabilized with 0.1 M phosphate buffer (pH 7.4) at room temperature for 20 min, *c*) acetylated for 10 min in 0.25% acetic anhydride in 0.1 M triethanolamine to prevent nonspecific binding of probes, *d*) dehydrated in increasing concentrations of ethanol, *e*) delipidated with chloroform, and *f*) air dried at room temperature for 1 h. After these steps, hybridization with Kiss1 and Tac2 riboprobes was performed during 16 h at 55°C. Hybridization solution contained deionized formamide (SIGMA), 50% dextran sulfate (SIGMA), 5 M NaCl, 1 M Tris base (pH 8.0), 0.5 M EDTA (pH 8.0), 50× Denhardt's solution (SIGMA), and 0.03 pmol/mL of radiolabeled ribo-probe along with 10 mg/mL yeast tRNA (SIGMA).

The following day, slides were washed with 4× sodium saline citrate (SSC) for 30 min, treated with RNase A (10 g/mL; Sigma) for 30 min and washed under conditions of increasing stringency including two 30-min washes in 0.1× SSC (1× SSC: 150 mM NaCl, 15 mM sodium citrate) at 65°C, dehydrated in increasing ethanol series and air dried at room temperature for 1 h. Finally, slides were dipped in Kodak Autoradiography emulsion type NTB (Eastman Kodak), air dried, and stored at 4°C for 1 week in a dark room. After this period, the sections were developed and fixed following the manufacturer instructions (Kodak): 3-min in Kodak Dektol developer and 5 min in Kodak Fixer. Slides were previously dehydrated and rinsed with Sub-X™ clearing medium and Sub-X mounting medium (SIGMA).

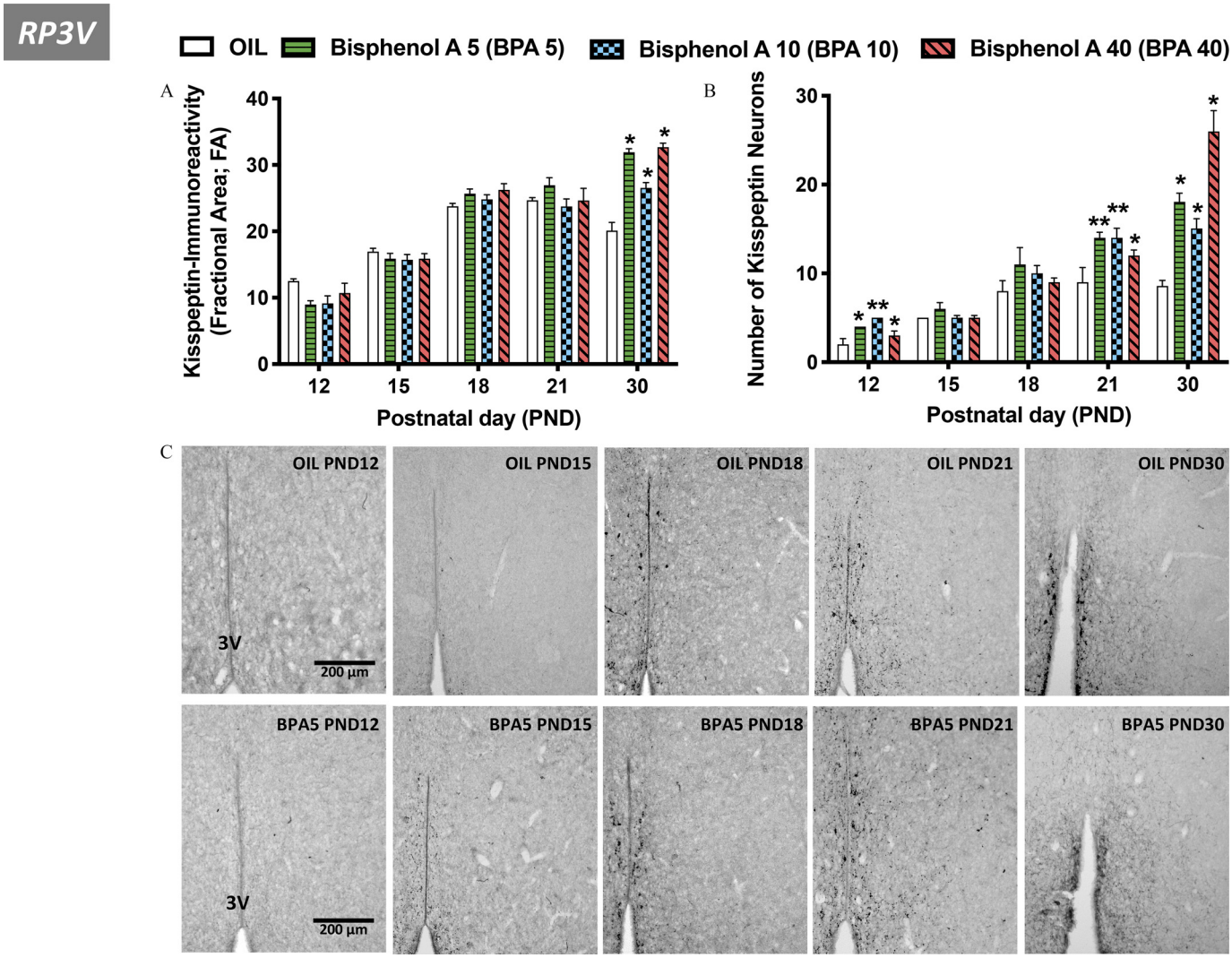

Figure 2. Impact of perinatal exposure to a range of doses of BPA on postnatal development of RP3V Kisspeptin neurons. (A) Immunohistochemical analyses of Kp-IR structures. (B) The number of Kp-positive neurons were assayed in the RP3V of female mice perinatally exposed to BPA 5, 10, or 40 μg/kg BW/d (BPA 5, 10, or 40 groups). An OIL-exposed group was used as a control. Quantitative data were collected for each experimental group at different age points of postnatal development: PND12, 15, 18, 21, and 30, representative of the infantile, juvenile, and pubertal stages of postnatal maturation. (C) Representative photomicrographs of RP3V Kp-positive neurons for OIL and BPA 5 groups at PND12, 15, 18, 21, and 30 are shown. The density of Kp-IR fibers, expressed as fractional area (FA), and the number of Kp-positive neurons are represented as the mean ± SEM of six serial sections of RP3V per animal ($n = 5$ mice per group). $^{*}p < 0.05$, $^{**}p < 0.01$ vs. corresponding OIL-exposed group for each age [one-way ANOVA followed by Fisher least significant difference (LSD) test]. ANOVA, analysis of variance; BPA, bisphenol A; BW, body weight; Kp-IR, kisspeptin-immunoreactive; PND, postnatal day; RP3V, rostral periventricular area of the third ventricle; SE, standard error; 3V, third ventricle.

Quantification and Analysis of Kiss1 and Tac2 mRNA

Similar anatomical regions were analyzed using the mouse brain atlas (Paxinos and Franklin 2001), and specific hybridization signals were quantified by densitometry using a digital imaging system (Image-Pro® Plus 4.5; Media Cybernetics Inc.). Brain sections were divided into five sets, composed of a total of 32–40 sections each. For each set, sections were collected every 100 µm, with a thickness of slice of 20 µm. For quantification, a single set was analyzed unilaterally (adjacent sections at 100 µm intervals).

Hormone Measurements

Serum LH and FSH levels were measured in 25–50 µL aliquots, using radioimmunoassay (RIA) kits supplied by the National Institutes of Health (A.F. Parlow, National Hormone and Peptide Program, Torrance, CA). Rat LH-I-10 and FSH-I-9 were labeled with ^{125}I using Iodo-gen tubes, following the instructions of the manufacturer (Pierce Chemical Co.). Hormone concentrations were expressed using reference preparations LH-RP-3 and FSH-RP-2 as standards. For each sample, hormone measurements were conducted in duplicate. Intra- and interassay coefficients of variation were 8% and 10% for LH and 6% and 9% for FSH. The sensitivity of the assay was 5 pg/tube for LH and 20 pg/tube for FSH. Group sizes for hormonal determinations were 6–10 samples per group. Accuracy of hormone determinations was confirmed by assessment of mouse serum samples of known concentrations (used as internal quality controls).

Presentation of Data and Statistical Analysis

Data are presented as means ± standard error of the mean (SEM). Results were analyzed for statistically significant differences using unpaired one-way analysis of variance (ANOVA) followed by Fisher least significant difference (LSD) post hoc tests [SPSS software (version 16; IBM)] or by Student-Newman-Keuls multiple range test [Prism (version 6.0; GraphPad). In addition, Student's nonparametric *t*-test was used to assess variation among experimental groups (Prism). Values of $p \leq 0.05$ were considered significant.

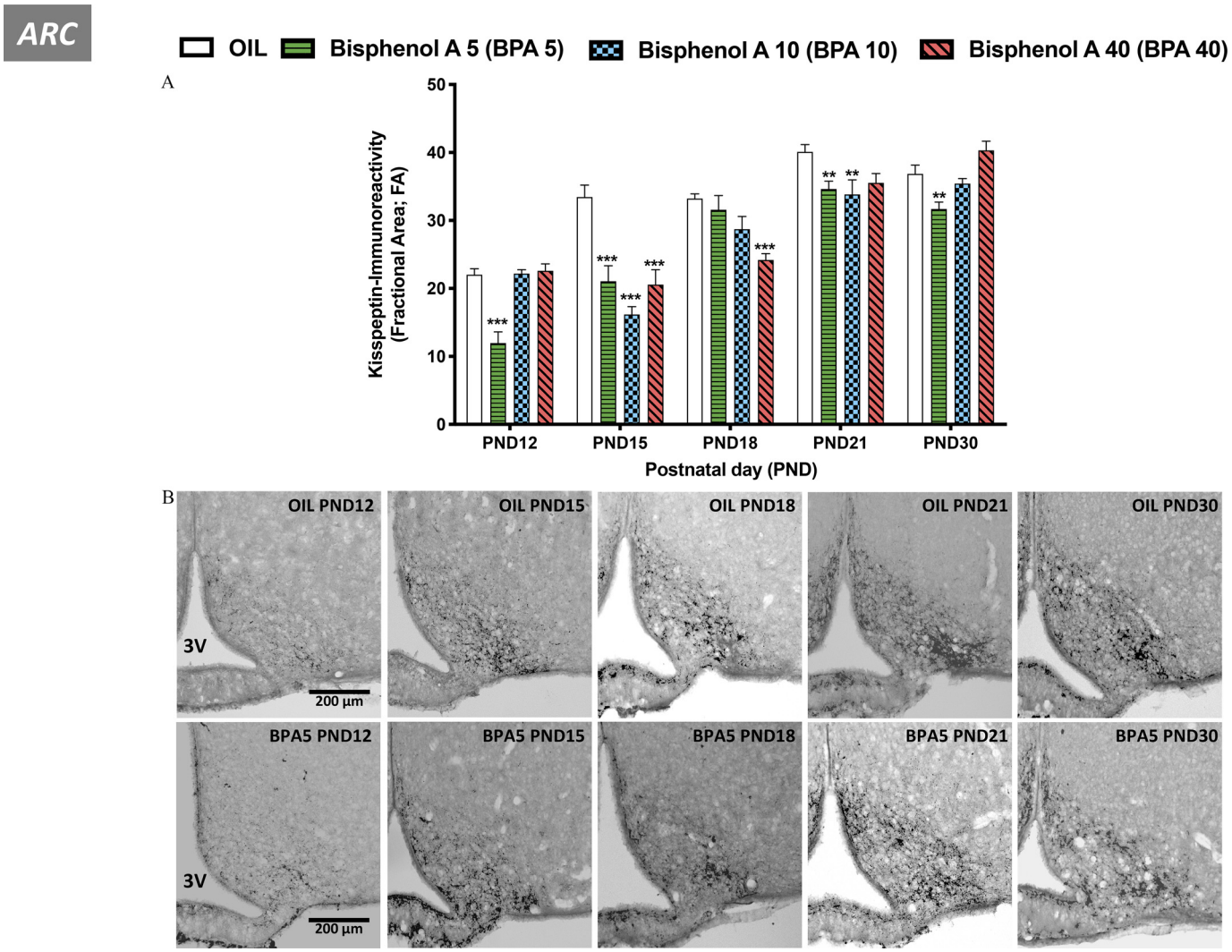

Figure 3. Impact of perinatal exposure to a range of doses of BPA on postnatal development of ARC Kisspeptin immunoreactivity. (A) Immunohistochemical analyses of Kp-IR positive structures (fibers and cell bodies), expressed as fractional area (FA), were assayed in the ARC of female mice perinatally exposed to BPA 5, 10, or 40 µg/kg BW/d (BPA 5, 10, or 40 groups). An OIL-exposed group was used as a control. Quantitative data were collected for each experimental group at different time points of postnatal development: PND12, 15, 18, 21, and 30, representative of the infantile, juvenile, and pubertal stages of postnatal maturation. (B) Representative photomicrographs of Kp-IR structures in ARC from OIL and BPA 5 groups at PND12, 15, 21, and 30 are shown. The density of Kp-IR structures is represented as the mean ± SEM of four serial sections of ARC per animal ($n = 5$ mice per group). $^{**}p < 0.01$, $^{***}p < 0.001$ vs. corresponding OIL-exposed group for each age [one-way ANOVA followed by Fisher least significant difference (LSD) test]. ANOVA, analysis of variance; ARC, arcuate nucleus; BPA, bisphenol A; BW, body weight; Kp-IR, kisspeptin-immunoreactive; PND, postnatal day; SE, standard error.

Results

Timing of Puberty Onset following Perinatal Exposure to BPA

The impact of BPA exposure on the age of VO was evaluated in female mice perinatally exposed to increasing doses of BPA: 5, 10, or 40 μg/kg BW/d. All BPA doses induced a significant advance in the mean age of VO compared with the control OIL group (Figure 1A). This phenomenon was especially evident at PND23, where all BPA 40-exposed animals already displayed VO while no VO was observed in the OIL-exposed group (Figure 1A). However, no dose–response effect was observed for BPA impact on pubertal timing, as illustrated by the fact that the age of VO was statistically similar between the groups exposed to the lowest and the highest dose of BPA (Figure 1A).

Postnatal Secretion Patterns of LH and FSH following Perinatal Exposure to BPA

Serum LH and FSH levels were assessed in the groups of female mice perinatally exposed to increasing doses of BPA, at different time points of postnatal development: PND12, 15, 18, 21, and 30, representative of the infantile, juvenile, and pubertal stages of postnatal maturation. Perinatal exposure to BPA resulted in significantly lower serum LH levels at all ages and doses tested as compared with the age-matched control animals, except for PND18, when no significant differences were detected. In OIL-exposed animals, LH levels fluctuated during the study period: They were high at PND12, declined at PND15–18, and increased before the onset of puberty at PND 21. However, serum levels of LH were relatively constant and persistently low in all BPA-exposed animals and ages tested (Figure 1B).

Regarding FSH secretion, variable but significant responses to BPA exposure were only detected at PND15 and 18. Although FSH secretion was higher in the BPA 5 and 10 groups versus the OIL group at PND15, FSH levels were significantly lower in these BPA groups at PND18 (Figure 1B). In contrast to developmental LH responses to BPA exposure, fluctuations in circulating levels of FSH were similar between OIL and BPA-exposed groups, with a decline along postnatal development, especially from early infantile (PND12 and 15) to late-infantile period (PND18; Figure 1B). In addition, although significant differences in LH and FSH levels were observed with various BPA doses and time points, no dose–response effect was detected at any age. Of note, early exposure to BPA 5, the lowest dose of BPA tested,

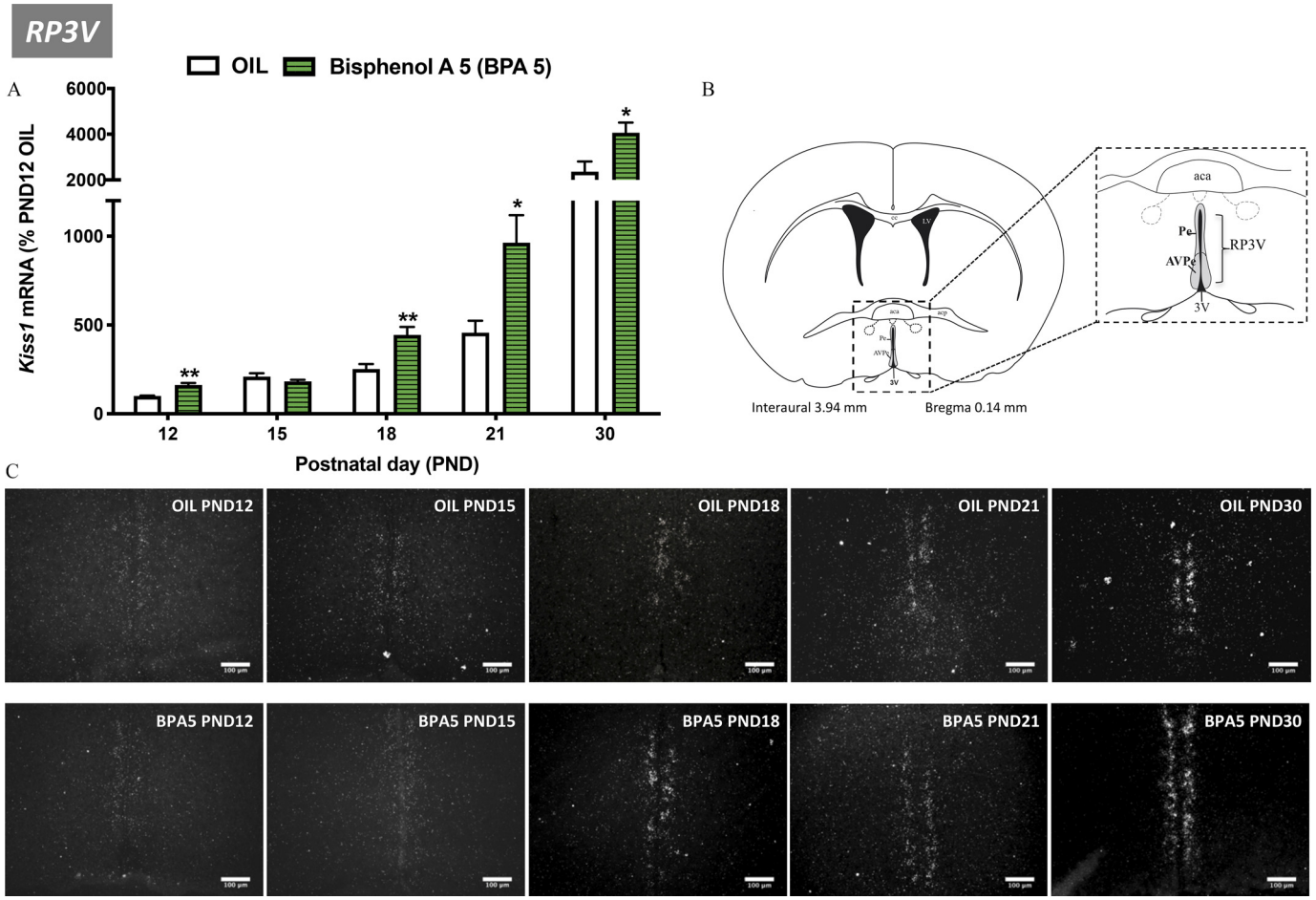

Figure 4. Impact of perinatal exposure to BPA 5 on postnatal expression of Kiss1 in the RP3V. *Kiss1* mRNA levels in the RP3V were assessed by ISH in female mice perinatally exposed to a low dose of BPA: 5 μg/kg BW/d (BPA 5 group). An OIL-exposed group was used as a control. (A) Quantitative *Kiss1* mRNA expression data are presented at infantile (PND12, 15, 18), juvenile (PND21), and pubertal ages (PND30) for each experimental group (OIL and BPA 5). (B) The neuroanatomical area selected for quantitative analyses is displayed in the representative cartoon. (C) Representative photomicrographs of RP3V *Kiss1* mRNA expression for each experimental group at PND12, 15, 18, 21, and 30 are shown. Quantitative data of Kiss1 expression in the RP3V are presented as the mean ± SEM of the hybridization signals, obtained by densitometry, in individual sections from 5 different mice per group (A). $^{*}p < 0.05$, $^{**}p < 0.01$ vs. corresponding OIL-exposed group for each age (Student's nonparametric *t*-test). aca, anterior commissure, anterior part; AVPe, anteroventral periventricular nucleus; BPA, bisphenol A; BW, body weight; ISH, *in situ* hybridization; Pe, periventricular hypothalamic nucleus; PND, postnatal day; RP3V, rostral periventricular area of the third ventricle; SE, standard error; 3V, third ventricle.

was sufficient to alter the pattern of gonadotropin secretion along the postnatal development.

Postnatal Development of RP3V and ARC Kisspeptin Neurons following Perinatal Exposure to BPA

Immunohistochemical analyses were conducted in female mice perinatally exposed to the various doses of BPA, at the different stages of postnatal development, in order to evaluate changes in Kp-IR in the hypothalamic RP3V and ARC areas. Consistent with previous studies, kisspeptin positive fibers and neurons were detected at the RP3V and ARC in all control animals along the study period (Clarkson and Herbison 2006). In the RP3V, Kp-IR in control (OIL) animals, both in terms of fiber density and number of neurons, increased from early infantile period (PND12 and 15) to PND18 and remained high from PND18 to 30 (Figure 2; see also Figure S2). A similar pattern of Kp-IR was also observed in BPA-exposed animals. However, at PND30, the number of both Kp-positive fibers and neuronal cell bodies was significantly higher in BPA-treated groups, whereas the number of Kp-IR cells was already significantly increased by perinatal BPA exposure, at all doses, on PND12 and 21 (Figure 2; see also Figure S2).

In the ARC, an increase in Kp-IR fiber density was detected along postnatal development in both control and BPA-exposed mice. However, animals exposed to BPA showed a significantly lower density of kisspeptin immuno-positive structures at all ages as compared with controls, with a variable interaction between the dose and age (Figure 3; see also Figure S3). Interestingly, the lowest dose of BPA tested (BPA 5) was consistently associated with a reduction in the total Kp-IR density at all ages studied, except for PND18; this dose also altered Kp-IR in the RP3V, both in terms of Kp fibers (at PND30) and number of Kp cells (at PND12, 21, and 30) (Figure 2). Quantification of Kp-positive neurons in the ARC was unviable due to the large amount of fibers detected, which made it very difficult to discern between kisspeptin positive fibers and cell bodies at this nucleus.

Postnatal Expression of Kiss1 mRNA in RP3V and ARC following Perinatal Exposure to BPA 5

Considering the impact of the lowest dose of BPA on neuroanatomical distribution of Kp-IR along postnatal development, we selected this experimental group for further quantitative ISH analyses of *Kiss1* mRNA expression at the R3PV and ARC of

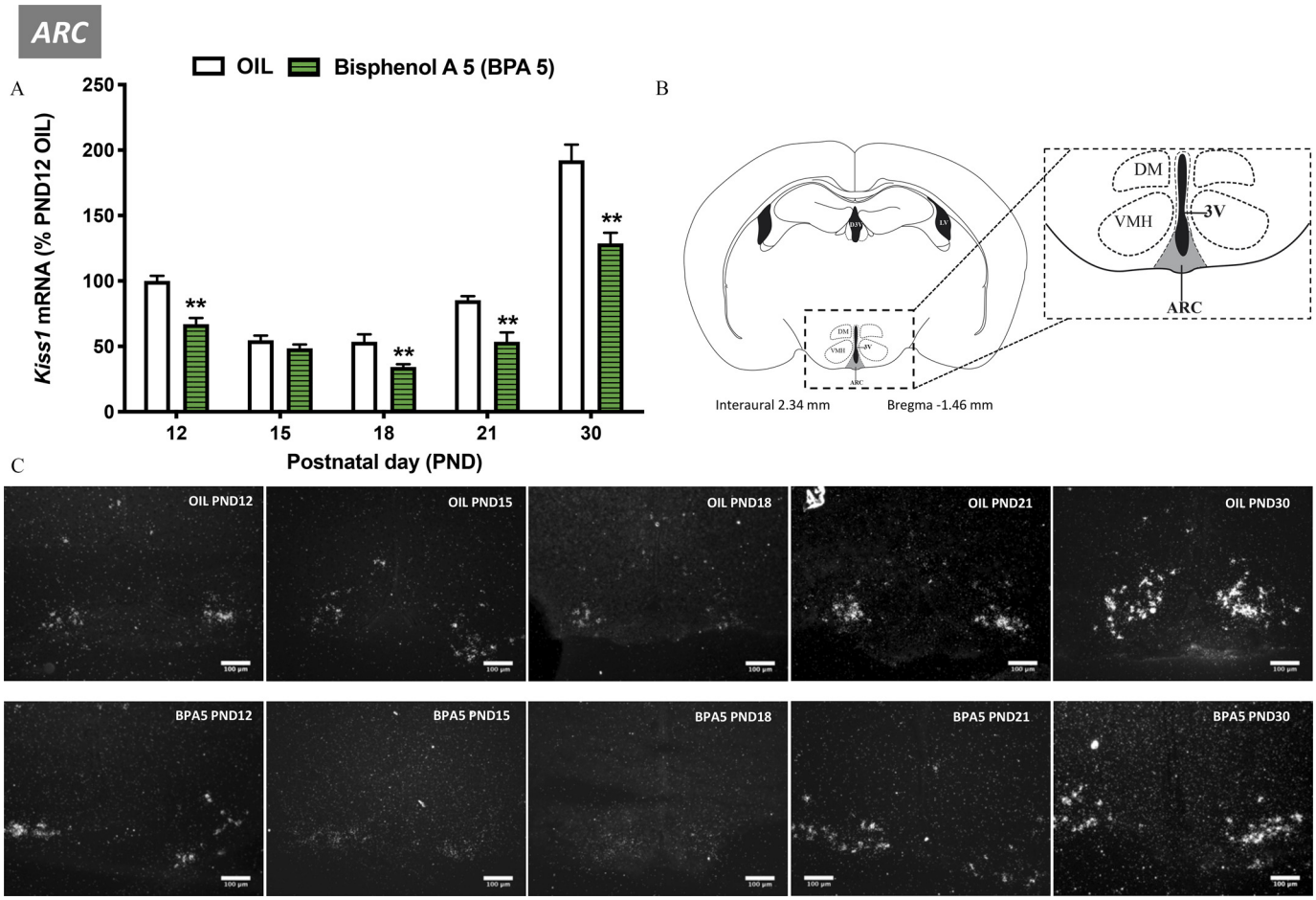

Figure 5. Impact of perinatal exposure to BPA 5 on postnatal expression of Kiss1 in the ARC. *Kiss1* mRNA levels in the ARC were assessed by ISH in female mice perinatally exposed to a low dose of BPA: 5 µg/kg BW/d (BPA 5 group). An OIL-exposed group was used as a control. (A) Quantitative *Kiss1* mRNA expression data are presented at infantile (PND12, 15, 18), juvenile (PND21), and pubertal ages (PND30) for each experimental group (OIL and BPA 5). (B) The neuroanatomical area selected for quantitative analyses is displayed in the representative cartoon. (C) Representative photomicrographs of ARC *Kiss1* mRNA expression for each experimental group at PND12, 15, 18, 21, and 30 are shown. Quantitative data of Kiss1 expression in the ARC are presented as the mean ± SEM of the hybridization signals, obtained by densitometry, in individual sections from 5 different mice per group (A). $^{**}p < 0.01$ vs. corresponding OIL-exposed group for each age (Student's nonparametric *t*-test). ARC, arcuate nucleus; BPA, bisphenol A; BW, body weight; DM, dorsomedial nucleus; ISH, *in situ* hybridization; PND, postnatal day; SE, standard error; VMH, ventromedial nucleus; 3V, third ventricle.

infantile (PND12, 15, and 18), juvenile (PND21), and pubertal (PND30) female mice. An increase in *Kiss1* mRNA expression during the juvenile–pubertal transition was observed at the R3PV and ARC in all experimental groups (Figures 4 and 5). However, perinatal BPA 5 exposure divergently impacted the profile of Kiss1 expression at these two sites during postnatal maturation. Thus, although BPA 5 females displayed significantly higher *Kiss1* mRNA levels than the control group in the RP3V at PND12, 18, and 21 (Figure 4), a significant drop in *Kiss1* mRNA levels over the control group was observed at the ARC at all ages, except for PND15, in BPA-treated animals (Figure 5).

Developmental Expression of Tac2 in the ARC following Perinatal Exposure to BPA

Considering the proven co-expression of Kiss1 and Tac2 in the ARC (Navarro et al. 2009a), and our findings on the *Kiss1* mRNA expression profile in the ARC of BPA-exposed animals, we selected two representative ages, PND15 (late infantile) and PND30 (pubertal), to assess the impact of the range of doses of BPA on the expression of *Tac2* mRNA at the ARC. An increase in Tac2 levels was detected in control females between the infantile and pubertal ages (Figure 6), in line with previous references (Navarro et al. 2012). Interestingly, perinatal exposure to a range of doses of BPA was associated with a consistent drop of ARC Tac2 levels at both ages tested, except for the BPA 10 dose at PND15. In fact, at PND30, Tac2 expression levels in the ARC were significantly lower in all BPA groups; perinatal BPA exposure was able to substantially attenuate (BPA 5 and BPA 40) or fully prevent (BPA 10) the developmental rise of Tac2 expression in the ARC detected in control animals (Figure 6).

Because the lowest dose of BPA, 5 μg/kg BW/d, was sufficient to induce significant alterations in the expression of *Tac2* mRNA at the ARC during the juvenile–pubertal transition, we used female mice perinatally exposed to BPA 5 to conduct a detailed analysis of the expression pattern of *Tac2* mRNA at the ARC of infantile (PND12, 15, and 18), juvenile (PND21), and pubertal (PND30) animals. In good agreement with *Kiss1* mRNA profiles (Figure 5) and our initial results (Figure 6), *Tac2* mRNA levels increased along pubertal development in the ARC of control and BPA-exposed mice. However, perinatal exposure to BPA 5 was associated with a significant suppression of *Tac2* mRNA expression, which was fully

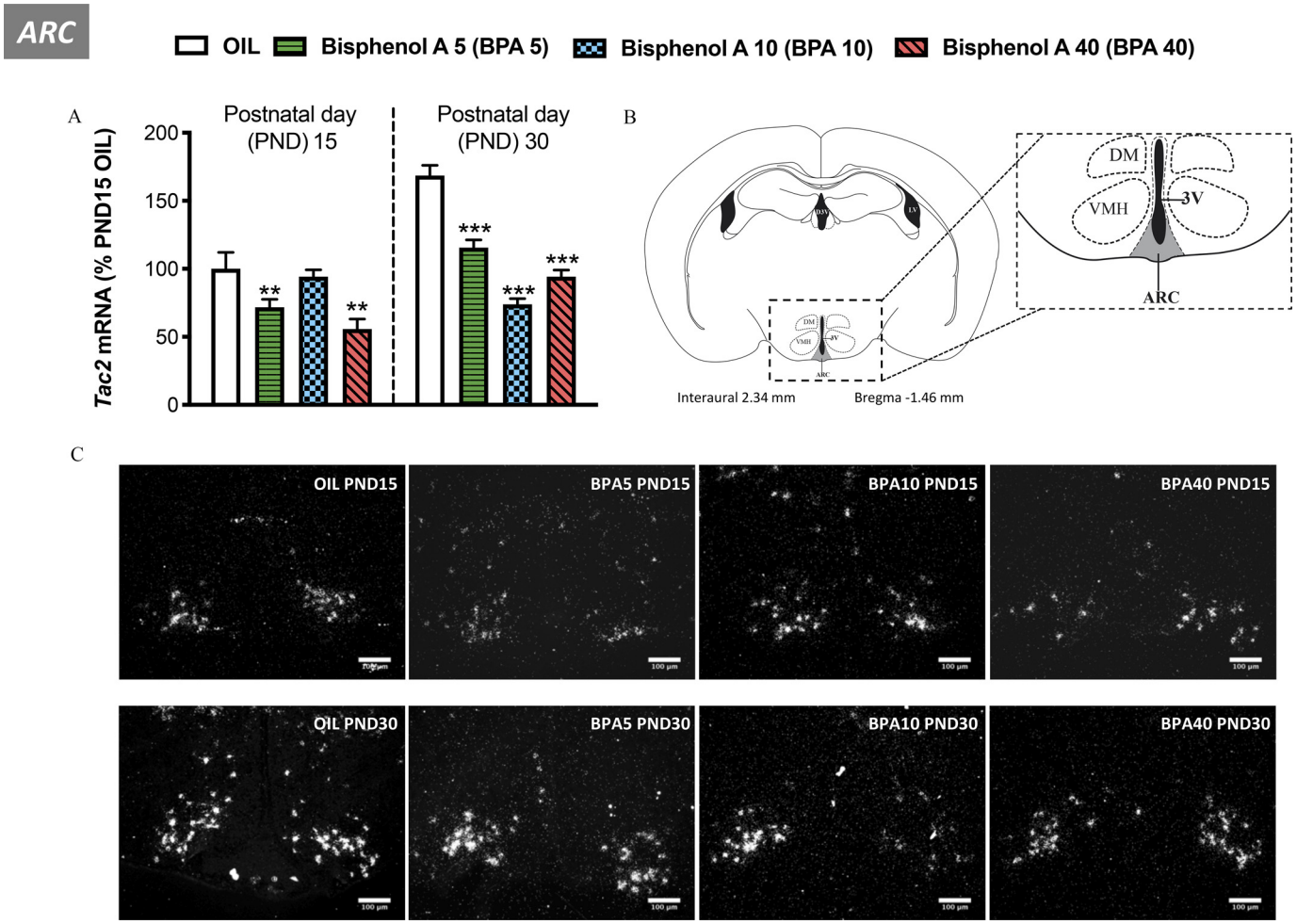

Figure 6. Impact of perinatal exposure to a range of doses of BPA on developmental expression of Tac2 in the ARC. *Tac2* mRNA levels in the ARC were assessed by ISH in female mice perinatally exposed to BPA 5, 10, or 40 μg/kg BW/d (BPA 5, 10, or 40 groups). An OIL-exposed group was used as a control. (A) Quantitative *Tac2* mRNA expression data are presented at PND15 and 30 for each experimental group. (B) The neuroanatomical area selected for quantitative analyses is displayed in the representative cartoon. (C) Representative photomicrographs of ARC *Tac2* mRNA expression at PND15 and 30 for each experimental group are shown. Quantitative data of Tac2 expression in the ARC are presented as the mean ± SEM of the hybridization signals, obtained by densitometry, in individual sections from 5 different mice per group (A). $^{**}p < 0.01$, $^{***}p < 0.001$ vs. corresponding OIL-exposed group for each age (one-way ANOVA followed by Student-Newman-Keuls multiple range test). ANOVA, analysis of variance; ARC, arcuate nucleus; BPA, bisphenol A; BW, body weight; DM, dorsomedial nucleus; ISH, *in situ* hybridization; PND, postnatal day; SE, standard error; VMH, ventromedial nucleus; 3V, third ventricle.

detectable at the mid infantile (PND15), juvenile (PND21), and pubertal (PND30) stages of postnatal maturation (Figure 7).

Discussion

Puberty onset is highly sensitive to environmental promptings, ranging from nutritional cues to hormone-mimics (Castellano and Tena-Sempere 2016). Interest in pubertal alterations has been boosted by recent worrying epidemiological reports of disturbed puberty onset in humans (Aksglaede et al. 2009a; De Leonibus et al. 2014; Herman-Giddens et al. 2012). Such perturbations might, on one hand, serve as a sentinel for the deterioration of environmental inputs onto reproductive health, and may, on the other, have adverse long-term consequences, including higher risk of different cancers as well as musculoskeletal, gynecological, neurocognitive, and metabolic disorders (Day et al. 2015) together with reduced life expectancy (Lakshman et al. 2009). In this context, the potential pubertal impact of BPA has been addressed by numerous clinical and preclinical studies. Yet, conflictive results have been reported, possibly due to differences in time windows, doses, and routes of exposure. Of note, most of these studies *a*) did not provide a tenable substrate, or even the neurohormonal or gonadal basis for the observed phenotypes, and *b*) did not consider the pubertal impact of real-life BPA exposure given that they involved the use of very high doses and more invasive routes of administration (e.g., injections) that do not match the expected patterns of environmental exposure.

In line with some previous studies (Adewale et al. 2009; Fernández et al. 2009; Losa-Ward et al. 2012; Nikaido et al. 2004), our data show that early exposures to BPA can advance VO as a consensus phenotypic sign of puberty onset. It must be noted, however, that most of the previous studies consisted of either exposures to high doses, in the range of >10- to 200-fold higher than the present study (Nikaido et al. 2004), or parenteral administration of the compound to the pups postnatally (Adewale et al. 2009; Fernández et al. 2009; Losa-Ward et al. 2012) and that the windows of exposure and doses are critically determinant for the net impact of BPA on pubertal timing (Franssen et al. 2016). Our analyses document an intergenerational influence of BPA, given orally to the mother over a range of doses, on pubertal timing of the female offspring, with a consistent advancement of the age of VO in all BPA-treated groups. Notably, no dose response was detected, and a trend for a nonmonotonic effect was even observed given that the lowest and the highest BPA dose appeared to impact the age of VO more than the intermediate dose. Although we do not have a definitive explanation for this

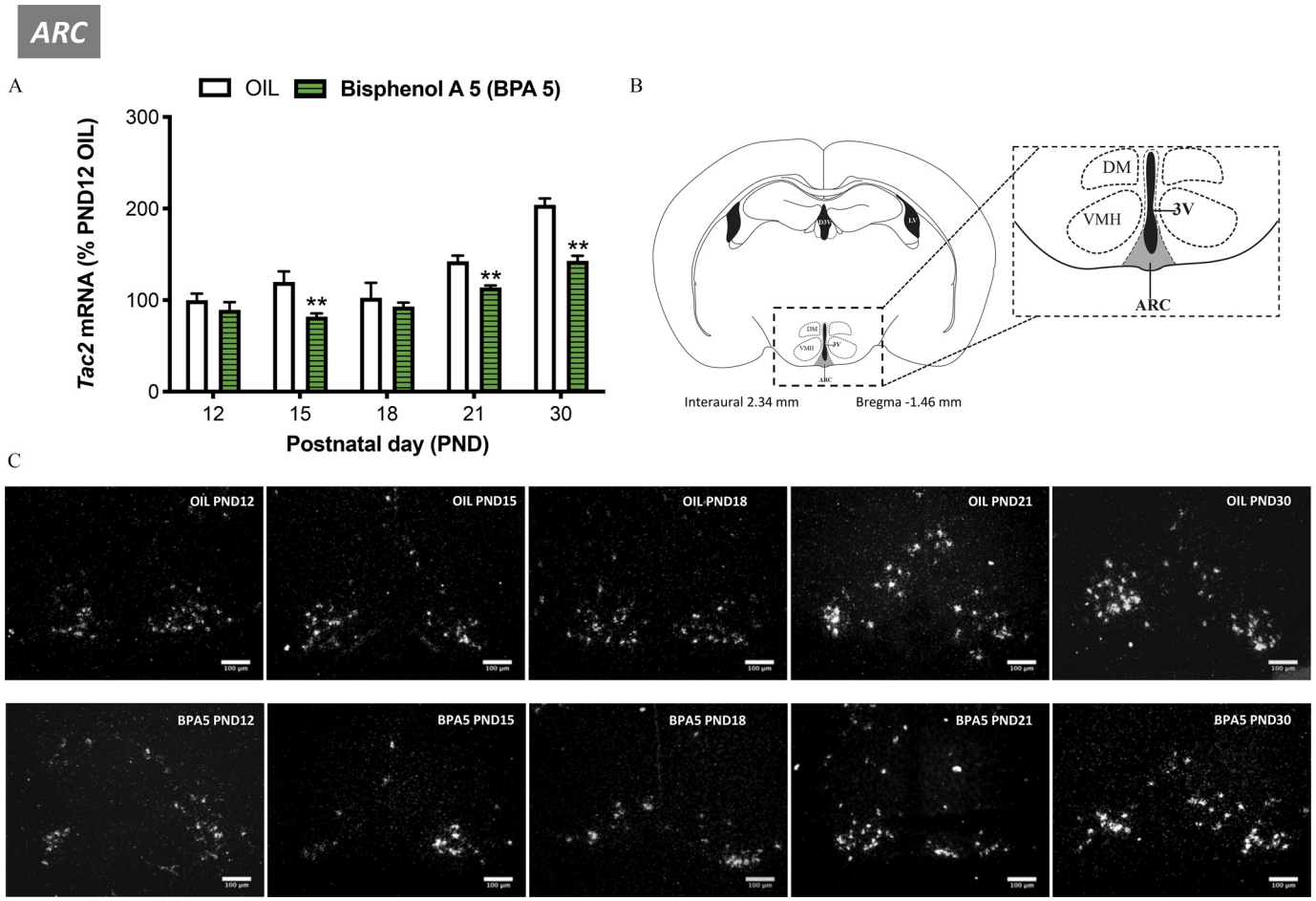

Figure 7. Impact of perinatal exposure to BPA 5 on postnatal expression of Tac2 in the ARC. *Tac2* mRNA levels in the ARC were assessed by ISH in female mice perinatally exposed to a low dose of BPA: 5 µg/kg BW/d (BPA 5 group). An OIL-exposed group was used as a control. (A) Quantitative *Tac2* mRNA expression data is presented at infantile (PND12, 15, 18), juvenile (PND21), and pubertal ages (PND30) for each experimental group (OIL and BPA 5). (B) The neuroanatomical area selected for quantitative analyses is displayed in the representative cartoon. (C) Representative photomicrographs of ARC *Tac2* mRNA expression for each experimental group at PND12, 15, 18, 21, and 30 are shown. Quantitative data of Tac2 expression in the ARC are presented as the mean ± SEM of the hybridization signals, obtained by densitometry, in individual sections from 5 different mice per group. $^{**}p < 0.01$ vs. corresponding OIL-exposed group for each age (Student's nonparametric *t*-test). ARC, arcuate nucleus; BPA, bisphenol A; BW, body weight; DM, dorsomedial nucleus; ISH, *in situ* hybridization; PND, postnatal day; SE, standard error; VMH, ventromedial nucleus; 3V, third ventricle.

phenomenon, the fact that VO is a binary qualitative marker, rather than a continuous variable, might have contributed to the lack of dose responsiveness to BPA. In any event, this nonmonotonic relationship, which is frequently observed in other BPA dose–response studies, seems to apply to other end points studied, such as gonadotropin levels, and has been previously related to the induction of different ligand–receptor interactions (Villar-Pazos et al. 2017). Importantly, the lowest dose tested (5 μg/kg BW/d, between GD11 and PND8) is 10-fold below the tolerable daily intake (TDI) proposed by the European Food Safety Authority (EFSA) in 2006 and 1,000-fold lower than the no observed adverse effect level (NOAEL) in humans (Picot et al. 2014). Even assuming the new recommendations of EFSA for a temporary TDI of 4 μg/kg BW/d (EFSA Panel on Food Contact Materials, Enzymes, Flavourings and Processing Aids 2015), the lowest dose tested in our study is fully aligned with this tentative precautionary value, which is nonetheless provisional and pending confirmation by a long-term BPA exposure study in rats undertaken by the U.S. Food and Drug Administration (FDA). All these features, together with the oral dosage, attest to the potential environmental relevance of our findings.

Despite phenotypic signs for earlier pubertal maturation, perinatal BPA exposure resulted in a consistent suppression of circulating LH levels, with a flattened pattern of secretion in all BPA groups that was in clear contrast with the dynamic fluctuation of endogenous LH levels seen in vehicle-treated prepubertal and pubertal females. Assumedly, changes in LH levels likely reflect changes in GnRH neurosecretory activity, therefore suggesting that the pulsatile secretion of GnRH is suppressed after perinatal exposure to BPA, even at low doses. This hypothesis is further supported by the lower *Kiss1* mRNA levels and kisspeptin content detected in the ARC of BPA-exposed mice; a Kiss1 neuronal population that is regarded as a key element of the GnRH pulse generator (Herbison 2016). Considering the more constitutive and less-kisspeptin responsive pattern of secretion of FSH, the fact that the secretory profiles of this gonadotropin were not equally affected by BPA exposures is not surprising and may help to reconcile the differential central versus peripheral impact of BPA; the former was clearly inhibitory, whereas the later was defined by an advancement of the age of VO. In any event, perinatal BPA might also operate as estrogenic compound directly at the vaginal level, where it might contribute to its precocious canalization, which is known to be an estrogen-dependent phenomenon (Rodriguez et al. 1997).

Previous studies have evaluated the potential impact of BPA on Kiss1/kisspeptin expression in the hypothalamus (Cao et al. 2012; Navarro et al. 2009b; Patisaul et al. 2009), although to our knowledge no study addressing the effects of environmentally relevant doses and routes of administration, and their impact on puberty, had been reported so far. Moreover, some of these initial studies lacked neuroanatomical resolution, which might explain the divergent results showing that early BPA exposure, at high doses, caused either a decrease (Navarro et al. 2009b) or an increase (Xi et al. 2011), in whole hypothalamic Kiss1 expression. Interestingly, a protocol of perinatal exposure to BPA grossly similar to ours was previously applied to evaluate the capacity of this EDC to induce feminization of adult male rats in terms of generation of the preovulatory surge of gonadotropins. In that study, which did not address puberty, an expansion of the RP3V population of Kiss1 neurons was documented in adult male rats after perinatal dosage of BPA (Bai et al. 2011). In addition, oral administration of BPA either perinatally, to mouse dams between G15 and PND21, or to adult female mice, at doses in the range of those of this study, caused an increase in the number of kisspeptin neurons in the rostral hypothalamic area at adulthood (Naulé et al. 2014; Wang et al. 2014). Our study nicely complements those previous observations by showing for the first time an expansion of the RP3V Kiss1 neuronal population during the pubertal transition following developmental exposures to low doses of BPA. The mechanism behind this consistent stimulatory effect may be related to the proven ability of BPA to directly activate Kiss1 expression *in vitro* (Mueller and Heger 2014), although it is equally tenable that BPA may mimic the ability of endogenous estrogens to induce plastic changes in the RP3V Kiss1 neuron population, causing its enlargement, as occurs during normal puberty (Clarkson et al. 2009a).

Surprisingly, in contrast to the attention devoted to the impact of BPA on the rostral population of Kiss1 neurons, little experimental evidence has been produced regarding the potential impact of this EDC on the ARC Kiss1 neuronal population, and no information is available on its potential disruption at puberty. One study reported that neonatal exposure to very high doses of BPA (50 mg/kg BW), which is 10,000-fold higher than the lowest dose used in our study, caused a reduction in kisspeptin-IR fibers in adult female rats (Patisaul et al. 2009). Our study, using a combination of analytical techniques for measurement, with anatomical resolution, of kisspeptin content (fibers) and Kiss1-expressing neurons conclusively documents that, contrary to the observed changes at the RP3V, there was a consistent suppression of the ARC Kiss1 neuronal population that was detectable even at the lowest dose of BPA tested. In fact, quantitative analyses by ISH revealed that such a suppression of Kiss1 was persistently detectable from the infantile period to the pubertal stage. It is well accepted that ARC Kiss1 neurons are sensitive to the inhibitory action of sex steroids, including estrogens, on Kiss1 expression (Marraudino et al. 2018; Pinilla et al. 2012; Smith et al. 2005a, 2005b). Although the effects of perinatal BPA are compatible with this action, it must be stressed that such an effect was intergenerational given that BPA was administered to the mothers (not to the pups) during a restricted time window, therefore suggesting potential developmental or organizing actions of this EDC on the maturation of the population of ARC Kiss1 neurons. Alternatively, early exposures to BPA may impact Kiss1 neurons, at least partially, via changes in gamma-amino aminobutyric acid (GABA) neurotransmission because postnatal administration of very low doses of BPA was recently shown to enhance the GABAergic tone (Franssen et al. 2016), and GABA transmission to Kiss1 neurons has been reported in mice (DeFazio et al. 2014). This possibility, however, seems less feasible because the effects of GABA on Kiss1 neurons seem to be transient or of excitatory nature (DeFazio et al. 2014).

In good agreement with the observed changes in Kiss1 levels, perinatal exposure to low doses of BPA caused a persistent suppression of Tac2 expression in the ARC. Despite the relevance of the Tac2-product, NKB, in the control of kisspeptin output and, thereby, GnRH pulsatility (Navarro and Tena-Sempere 2011; Pinilla et al. 2012), and the reported role of NKB signaling in the control of puberty (Gill et al. 2012; Navarro et al. 2012), the putative effect of BPA on this tachykinin remained totally unexplored. Our data demonstrate that, at all doses tested, BPA was able to blunt the levels of Tac2 detected in the ARC on PND30, that is, at the time of puberty. This inhibitory effect was consistently observed even for the lowest dose of BPA, at the infantile (PND15), juvenile (PND21), and pubertal (PND30) stages of postnatal maturation. Considering that NKB has been proposed to operate as a stimulator of the kisspeptin output (Navarro and Tena-Sempere 2011; Pinilla et al. 2012), whose increase in expression actually precedes that of Kiss1 during pubertal maturation (Navarro et al. 2012), our data open up the possibility that changes in Tac2 levels in the ARC might mechanistically

contribute to the dampening of Kiss1/kisspeptin expression caused by perinatal BPA exposures in (pre)pubertal female mice.

Although much emphasis has been placed recently on the trends for earliness in the timing of female puberty, detailed analyses have suggested that the observed changes are likely toward precocity for initial pubertal stages but toward lateness concerning the final stages of pubertal maturation in both sexes (Parent et al. 2016). Such a dichotomy, which has been considered to illustrate the complex nature of the environmental influences on the timing of puberty, is epitomized by our present findings that show the divergent intergenerational impact of low doses of BPA advancing the age of the initial sign of puberty onset in rodents, namely VO, but consistently suppressing the ARC Kiss1/NKB system and LH secretion, as a surrogate marker of GnRH neurosecretion, in pubertal female mice. These observations attest to the translational relevance of our current data and call for the application of precautionary measures concerning the prevention of gestational/perinatal exposures even to low doses of BPA given that they may disrupt key maturational events of the reproductive axis such as puberty onset.

Acknowledgments

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