RESEARCH ARTICLE

Persistent vs transient alteration of folliculogenesis and estrous cycle after neonatal vs adult exposure to Bisphenol A

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ABSTRACT

Background: Exposure to Bisphenol A (BPA), a ubiquitous endocrine disrupting chemical (EDC) is known to produce variable effects on female puberty and ovulation. This variability of effects is possibly due to differences in dose and period of exposure. Little is known about the effects of adult exposure to environmentally relevant doses of this EDC and the differences in effect after neonatal exposure.

Objectives: This study aims at comparing the effects of neonatal versus adult exposure to a very low or a high dose of BPA for two weeks on ovulation and folliculogenesis and exploring the hypothalamic mechanisms involved in such disruption by BPA.

Methods: One day-old and 90 day-old female rats received daily subcutaneous injections of corn oil (vehicle) or BPA (25 ng/kg/d or 5 mg/kg/d) for 15 days.

Results: Neonatal exposure to both BPA doses significantly disrupted the estrus cycle in adulthood and induced a decrease in primordial follicles. During adult exposure, both doses caused a reversible disruption of the estrous cycle associated with a delay and a decrease in the amplitude of the LH surge. Moreover, a reversible decrease in antral follicles and corpora lutea was also observed.

Conclusions: Adult exposure to a very low or a high dose of BPA for 2 weeks cause similar and reversible disruption of estrus cyclicity and folliculogenesis associated with disruption of the preovulatory LH surge. After neonatal exposure, effects on estrus cyclicity and folliculogenesis are persistent in adulthood, consistent with disturbed organizational effects.

Key terms: *endocrine disruption, neuroendocrinology, GnRH, ovary, puberty, female rat*

1. INTRODUCTION

Bisphenol A (BPA) is a ubiquitous endocrine disrupting chemical (EDC) used in the production of polycarbonate plastics and epoxy resins[1]. Despite its partial ban in some countries, it is currently one of the most largely used chemical compounds in the world with more than 8 billion tons produced each year[2]. Human exposure is nearly universal in developed countries and occurs mainly through contaminated beverages and food[3]. Several studies indicate widespread contamination of fetuses and neonates, leading to the questions as to whether such an EDC can affect development[4–6] and whether there is a limit for a safe exposure. Currently, the US Environmental Protection Agency (EPA) "safety level" of BPA is set at 50 µg/kg/d i.e. 1,000 times the average human exposure[1]. The European Food Safety Authority's tolerable daily intake was recently lowered to 4 µg/kg./d.[7].

Sex steroids play a crucial role perinatally in "organizing" the control of female reproduction. For that reason, the adult female estrus cycle is altered following exogenous exposure to sex steroids during that vulnerable perinatal period[8]. Therefore, the effects of early exposure to BPA on puberty and reproduction are a matter of concern. Recent evidence suggests that exposure to BPA during this sensitive developmental period could have long-term impacts on reproductive function[9]. Early exposure to BPA affects puberty onset with effects depending markedly on the window and dose of exposure, and possible non-linear dose–response relationship[10–25]. Effects of neonatal exposure to BPA on estrous cyclicity have produced inconsistent results. While a few studies did not show any effect on estrous cyclicity[26–29], others reported abnormal cyclicity[12–14,30–33]. Prenatal or perinatal exposure to BPA also decreases the number of preantral follicles in mice[34] and rats[35] and leads to a decline in fertility and fecundity[36]. Taken together, these studies suggest that prenatal or neonatal exposure to BPA doses in the mg or µg ranges affects several structures and functions of the neuroendocrine system and the ovaries. Whether the exposure to very low doses of BPA neonatally or during adulthood results in different effects on female neuroendocrine and ovarian functions remains largely unknown.

We have recently shown that neonatal exposure to a very low dose of BPA (25 ng/kg/day) delays the developmental changes in GnRH secretion before puberty while a high dose of BPA (5 mg/kg/day) results in early occurrence of those neuroendocrine changes[15]. This effect is followed by a delayed or

advanced vaginal opening after exposure to the low or high dose of BPA, respectively. Here, the aim is to elucidate whether such a neonatal exposure to a very low dose of BPA could produce persistent disruption of folliculogenesis and estrous cycle that could be consistent with disturbed organization. We also used the high BPA dose since opposing effects on GnRH secretion and pubertal timing were seen after using the low and the high doses of BPA neonatally[15]. Finally, the aim is to evaluate whether adult BPA exposure in similar conditions would produce persistent or transient effects on ovulation and folliculogenesis.

2. MATERIALS AND METHODS

Animal care and exposure

Adult Female Wistar rats from the animal facility of the University of Liège were housed individually in standardized conditions (12h dark/light phase from 4pm, 22.8ºC and food and water *ad libitum*). All animals were raised in BPA-free cages (Polypropylene cages, Ref 1291H006, Tecnilab, Netherlands) and fed EDC- and phytoestrogen-free chow (V135 R/Z low phytoestrogen pellets, SSNIFF Diet, Netherlands). Water was supplied in glass bottles.

Two timings of exposure were studied: female rats were exposed neonatally or during adulthood. Neonatal exposure to BPA started on postnatal day 1 (PND 1) and ended on PND 15. Animals received a daily s.c injection (0.05 ml) of corn oil (vehicle) or one of the 2 doses of BPA, a low environmental dose of BPA dose of 25 ng/kg/d or a high dose of 5 mg/kg/d (BPA Ref: 23,9658; Sigma–Aldrich, Saint Louis, USA). BPA was diluted in ethanol at an initial concentration of 100 mg/ml and then diluted in corn oil. The same concentration of ethanol was added to the control solution of corn oil. Once we obtained the final solution, tubes were opened to allow ethanol evaporation. Subcutaneous injections were given every 24h between 10.00 and 12.00 am. Adult exposure took place from PND 90 to PND 105 under the same conditions. All experiments were carried out with the approval of the Belgian Ministry of Agriculture and the Ethical Committee at the University of Liège.

Experimental design

Effects of exposure to BPA from PND 1 to 15 on estrous cycle and folliculogenesis.

Litters were homogenized for size and sex ratio on the first postnatal day of life in order to have 10-12 pups per litter and a 1:1 male:female ratio. Cross-fostering of maximum 2 pups per litter was used when homogenization was required. The day of birth was considered as PND 0. Pups were weaned on PND 21. Twenty-eight female pups born from 8 dams were exposed from PND 1 to 15 to 25 ng/kg/d (n=7) or 5 mg/kg/d of BPA (n=7) or corn oil (vehicle) (n=14). The animals were followed for estrous cyclicity from the time of vaginal opening until PND 105 when they were sacrificed to study ovarian folliculogenesis during the diestrus stage.

Effects of exposure to BPA from PND 90 to PND 105 on estrous cyclicity, GnRH and LH secretion, hypothalamic gene expression and folliculogenesis.

Eightly-one female rats were followed for estrous cyclicity from PND 60 onwards. Among these females, only those (n = 74) showing at least 3 regular cycles out of 4 consecutive cycles at PND 90 were selected for the exposure experiment. These adult female rats were exposed to 25 ng/kg/d of BPA (n = 27) or 5 mg/kg/d (n = 26) or corn oil (n = 21) for 15 days from PND 90 to PND 105. A group of females were sacrificed 24h after the last dose of BPA or corn oil to measure plasma and pituitary LH and FSH levels (n = 15-16/group), hypothalamic gene mRNA expression (CTL: n = 5; BPA-25 ng: n = 6; BPA-5 mg: n = 5) as well as folliculogenesis (n = 6/group). Only females on diestrus based on smear results were considered for analysis.

GnRH pulse frequency was analysed *ex vivo* 24h after the last dose of BPA by using a hypothalamic explant incubation (n = 4/group). Only females on diestrus were considered for analysis. Another group of BPA-exposed female rats was followed for estrous cyclicity (BPA-25 ng: n = 11; BPA-5 mg: n = 11) until four weeks after the exposure. Among those females, a subgroup undertook serial blood samples in order to determine LH surge (BPA-25 ng: n = 5; BPA-5 mg: n = 5). Finally, a set of females was sacrificed 4 weeks after exposure and one ovary per animal was collected to study folliculogenesis on diestrus (CTL: n = 6; BPA-25 ng: n = 6; BPA-5 mg: n = 6).

Estrous cyclicity

Animals exposed from PND 1 to PND 15 were examined daily to evaluate estrous cyclicity from the day of vaginal opening until PND 105. Estrous cyclicity was measured with vaginal smears taken every day in the beginning of the afternoon as described previously[37]. Rats exposed during adulthood were examined for estrous cyclicity from 2 weeks before the exposure to 4 weeks after the end of exposure to BPA.

We defined a regular cycle as a sequence of diestrus 1, diestrus 2, proestrus and estrus in 4 consecutive days[38]. The percentage of females having a regular cycle and the time spent in every stage of the cycle were calculated every period of 8 days corresponding to 2 full estrous cycles.

Hypothalamic explant incubation and GnRH assay

As previously shown, a neonatal exposure to BPA significantly affects GnRH pulse frequency at PND 20[15]. In order to determine whether GnRH frequency was affected after adult BPA exposure, GnRH secretion from hypothalamic explant was studied *ex vivo* 24h after the last s.c injection of BPA. As previously described[39,40], this method allow to reliably measure GnRH pulsatility. Briefly, after decapitation, the brain was placed ventral side up. Two sagittal incisions along the lateral hypothalamic sulci and two transversal incisions of 2 mm were made 2 mm ahead from the anterior boundaries of the optic chiasm and along the caudal margin of the mammillary bodies. Then, the hypothalamic region including both the medial basal hypothalamus (MBH) and the medial preoptic area (PoA) were transferred into an individual chamber, in a static incubator, submerged in MEM. The incubation medium was collected and renewed every 7.5 min for a period of 4 hours. The GnRH released into the incubation medium was measured in duplicate using a radioimmunoassay method with intra and inter-assay coefficients of variation of 14 and 18% respectively. The highly specific CR11-B81 (AB_2687904) rabbit anti-GnRH antiserum (final dilution 1:80,000) was kindly provided by Dr. V.D. Ramirez (Urbana, IL)[41]. Data below the limit of detection (5 pg/7.5-min fraction) were assigned that value.

Serum and pituitary LH and FSH radioimmunoassay

Blood samples and pituitaries were quickly collected 24h after the end of adult exposure to BPA or corn oil. Blood samples were stored overnight at 4ºC, followed by decantation of serum and stored at -20ºC until the assay was performed. Pituitary samples were stored in PBS at -20ºC before homogenization with ultrasound and centrifugation to obtain the supernatant used for the assays.

Serum and pituitary LH and FSH levels were determined using a double Ab method and a RIA kit (mLH RIA, rFSH RIA), kindly supplied by NIH (Dr. A.F. Parlow, National Institute of Diabetes and Digestive and Kidney Diseases, National Hormone and Peptide Program, Torrance, CA). Antibodies used were NIDDK-anti-rFSH-S-11 (AFP-C0972881, AB_2687903) and LH antiserum AFP-240580 (AB_2665533). Rat FSH antigen NIDDK-rFSH-I (AFP-5178B) and rat LH-I-8 (AFP-12066B) were

labelled with ^{125}I by the chloramine-T method, and the hormone concentration was calculated using the mouse LH and rat FSH reference preparation (LH: AFP-5306A; FSH: NIDDK-rFSH-RP-2, AFP-4621B) as standard. The intra- and interassay coefficients were 6-9% and 7-10% for LH and FSH respectively. The sensitivity of the assay was 4 pg/100µl for LH and 0.125 ng/100µl for FSH.

LH surge

Females were handled for habituation to tail-blood sampling daily during 2 weeks prior to the experiment. Pre- and post-exposure blood samples were collected 2 and 4 weeks before and after BPA exposure, respectively. During those periods, samples were collected every hour from 13h00 (3 hours prior to the beginning of the dark cycle) to 22h00 during proestrus defined by vaginal smear. Because cyclicity was disrupted during BPA exposure, blood samples were collected from 11h00 to 22h00 during two consecutive days prior to the expected oestrus stage during the second week of exposure LH was measured using an ultrasensitive ELISA LH assay[42]. Briefly, 96-well high affinity binding plates (Corning), were coated overnight with a bovine LHβ518B7 monoclonal antibody (in 0.015M Na_2CO_3 and 0.035M $NaCO_3$ coating buffer, pH 9.6). Samples and serial dilution of known concentrations of LH were incubated for 2 hours. After incubation, a rabbit polyclonal primary antibody for LH (1:10,000; rabbit antiserum AFP240580Rb; National Hormone and Peptide Program, USA) provided by Albert F. Parlow, a polyclonal goat anti-rabbit IgG secondary antibody (1:1000; DAKO) and 1-Step™ Ultra TMB-ELISA Substrate (ThermoFisher) were added to each well. The sensitivity of this assay was 0.06 ng/ml and intra- and interassay coefficients of variance were 6.3% and 10% respectively.

For each detected pulse, the amplitude was determined by subtracting the highest LH value from the basal value immediately prior to the onset of the pulse. Overall basal levels of LH secretion were determined by combining a minimum of the 5 lowest LH measurements from each mouse.

Ovarian histology

When the ovaries were removed, they were weighted on PND 105, 90 days after the end of neonatal exposure to BPA or corn oil or 24 h after the adult exposure to BPA. In order to determine whether folliculogenesis was affected by BPA, the ovaries were removed on PND 105 after neonatal exposure

to BPA and either on PND 105 or PND 135, corresponding to 24h or 30 days after the end of adult exposure to BPA. The ovaries were fixed in 4% paraformaldehyde overnight, dehydrated in 70% EtOH and paraffin-embedded. For histological analysis, 8 µm coronal sections were cut using a microtome. Every other section was deparaffinised, stained with hematoxylin and eosin, covered with a coverslip and examined for quantification of folliculogenesis.

For quantification, images of every other section throughout the whole ovary were acquired using an automated digital microscopy system DotSlide (Olympus, BX51TF, Aartselaar, Belgium). Dotslide images taken at a magnification of 10x which were in a proprietary format were converted into a standard TIFF format and 3-fold decimated, easier to handle. Thereafter, quantification of follicles and corpora lutea was carried out manually with Aperio ImageScope v12.3.2.8013 software (SCR_014311, Leica Biosystems) by an experimenter blinded to treatment. Total ovarian volume was automatically calculated using an original program developed using the image analysis toolbox of the MatLab (SCR_001622, 2016a, The Mathworks Inc., Natick, MA, USA) software.

Folliculogenesis was analysed by quantifying follicles at every stage of folliculogenesis: primordial, primary, secondary, antral and atretic follicles. In addition, cysts and corpora lutea were identified. The follicles were classified according to well-established criteria[43,44]. Double-counting of late stage follicles was avoided by digitally marking each follicle throughout the consecutive images. Each follicle was counted once whenever the oocyte was present. For quantification of early stage follicles (primordial and primary follicles), a 2-fold correction factor was added to compensate for the sections that were not analysed. Measurements are expressed as number of follicles or corpora lutea per volume (mm^3).

Real-time PCR

Because neonatal exposure to BPA altered the expression of hypothalamic genes involved in the GABAergic pathway (Glutamic Acid Decarboxylase 2 (*GAD2*) and Glutamic Acid Transporter 2 (*GAT2*)) [15], we studied the expression of those genes after adult exposure to corn oil or BPA. Quantitative PCR (qPCR) analysis was carried out in the MBH and the PoA. After decapitation, the PoA and the MBH were rapidly dissected. The brain was placed ventral side up. The dissection began by 2

sagittal incisions along the lateral hypothalamic sulci. Two transversal incisions of 2 mm were made 2 mm ahead from the anterior boundaries of the optic chiasm and along the caudal margin of the mammillary bodies. Finally, a frontal incision was made 2 mm under the ventral surface of the hypothalamus. The expression of estrogen receptor 1 (*ESR1*), estrogen receptor 2 (*ESR2*) and *KISS1* mRNA levels was also measured in both MBH and PoA.

Total RNA was extracted from the MBH, medial PoA and total ovarian tissue using the Universal RNA Mini kit (Quiagen, Netherlands). Prior to extraction, ovarian tissue was homogenized using a Mikro-Dismembrator S (SartoriusStedim, Germany). Five hundred ng of RNA for each sample were reverse transcribed using the Transcriptor first strand cDNA synthesis kit (Roche, Germany). For real-time quantitative PCR reactions, the cDNA of our samples were diluted 10 fold and 4 µl were added to a mix of 5 µl FastStart Universal SYBR Green Master (Roche, Germany), 0.4 µl of nuclease-free water and 0.3 µl of forward and reverse primer (see primer sequences in Table 1 and Supplemental Table 3[45]). The samples were run in triplicate using a LightCycler 480 thermocycler (Roche, Germany). Ct values were obtained from each individual amplification curve and the average Ct was calculated for each target gene in each sample. Quantification of relative gene expression was performed using an original program developed on Python 2.7.13 according to the ΔΔCt method implemented with the Pfaffl equation which takes into account reaction efficiency depending on primers[46]. All assays had efficiencies between 1.9 and 2.1. β-actin was used as housekeeping gene.

Statistics

Data analysed with non-parametric test were expressed in median and interquartile range (IQR). Numeric values of data analysed with a two-way ANOVA were expressed as mean ± SEM. When homogeneity and homoscedasticity were not accomplished, a two-group comparison Mann-Whitney non-parametric test was carried out. The U value in the Mann Whitney test is consistent with the formula $U = n_1 n_2 + n_x\,(nx - 1)/2 - Tx$ where n_1 and n_2 are the sample size, n_x is the group with the larger sample size and T_X is the group with the greatest sum rank. . A U value of zero is consistent with the maximum value where all values from one group are greater than the other group. Effect sizes were calculated using the equation $r = Z/\sqrt{N}$, where the Z is consistent with the adjusted normally

distributed variable value. Estrous cyclicity after adult BPA exposure was analysed using a McNemar test comparing the pre-exposure period versus the exposure period (PRE vs EXP) and the exposure period versus the post-exposure period (EXP vs POST) using each group as their own control. When the conditions for this test were not fulfilled, a mid-p McNemar test based on the binomial test was carried out. Estrous cyclicity after neonatal exposure and LH surge data after adult exposure were analysed by using a repeated-measures ANOVA followed by the Tukey's test for multiple comparisons and η^2 and Cohen's d as an indicator of effect size. The level of statistical significance was a p value lower than 0.05. Data were analysed using Prism 6.01 (SCR_002798, Graph Pad, Inc.).

3. RESULTS

Adult exposure to BPA transiently disrupts LH surge and estrus cycle

Adult exposure to BPA for 15 days caused significant alterations of the estrous cycle (Figure 1.B and Supplemental Table 1[45]). During exposure to 25 ng/kg/d or 5 mg/kg/d of BPA, the proportion of females with regular cycle decreased markedly and similarly to 12% and 9%, respectively (χ^2 = 13.1 and 12.1, p<0.001). This effect persisted for one week after the end of BPA exposure. Subsequently, the percentage of regularly cycling females was restored to 100% four weeks after the end of exposure to the two doses of BPA, indicating the reversibility of the effect. The alteration in cyclicity was characterized by a significant decrease of the time spent in proestrus (BPA-25 ng: χ^2 = 12.5, p <0.001; BPA-5 mg: χ^2 = 16.1, p <0.001) as well as an increase of the time spent in diestrus (BPA-25 ng: χ^2 = 8.5, p <0.01; BPA-5 mg: χ^2 = 4.92, p <0.05) (Figure 1.C-E). Time spent in estrus was not affected by exposure to BPA. Time spent in proestrus was restored to 23-25% after the end of exposure to both doses but EXP vs POST comparisons by using the mid-p McNemar test did not reach significance. Ninety-two percent of the control females showed regular cycles during the pre-exposure (PRE) and the exposure periods (EXP).

Because the proestrus timing appeared to be disrupted by BPA exposure, we characterized the spontaneous LH surge before, during and after a 15-day exposure to BPA. LH secretion was measured during 2 consecutive afternoons 48 hours after estrus in order to identify the spontaneous LH surge. The LH surge was significantly delayed during the exposure to both doses of BPA. A systematic delay of 2.6 and 2.8 hours on average was observed during exposure to BPA-25ng (p<0.05) and BPA-5mg group (p<0.01) respectively (Figure 2.C). The timing of the LH surge was restored one month after the end of exposure. Additionally, the high BPA dose significantly blunted the LH surge during the second week of exposure compared to the pre-exposure period (p<0.01) (Figure 2.B). The effect was reversible as the LH surge amplitude was restored after exposure. The amplitude of the LH surge was not significantly affected by the low BPA dose.

Adult exposure to BPA transiently disrupts the late stages of folliculogenesis

The number of antral follicles was significantly decreased 24 hours after the last day of adult exposure to the low dose ($U = 0.0$, $z = 2.5$, $p < 0.05$, $r = 1.1$) or high dose of BPA ($U = 0.0$, $z = 2.3$, $p < 0.05$, $r = 1.0$) (Figure 3.C). The number of corpora lutea was also decreased after exposure to BPA, however only significantly for the high BPA dose ($U = 0.0$, $z = 2.5$, $p < 0.05$, $r = 1.1$). The number of atretic follicles tended to increase though not significantly in the animals exposed to the two doses of BPA compared to controls. Cystic follicles were found in the ovaries of females exposed to the low and the high dose of BPA but were never observed in control animals (Figure 3.D). The effect of BPA on ovarian histology was reversible, since, 30 days after BPA exposure, the number of follicles per volume at the different stages of folliculogenesis was no longer significantly different among the control and exposed animals (Figure 3.E). However, the animals previously exposed to the low and high BPA dose still showed cystic follicles which were not observed in the control group.

Adult BPA exposure does not significantly affect GnRH or LH secretion during diestrus

The frequency of GnRH secretion from hypothalamic explants was measured *ex vivo* during diestrus 24h after the last day of exposure to BPA (Figure 2.A). The GnRH interpulse interval was not significantly different between the control group (41.1 ± 0.2 min) and BPA exposed females to the contrary of what was observed after neonatal exposure[15]. GnRH interpulse interval was however significantly longer after exposure to the low dose when compared to the high dose of BPA ($U = 0.0$, $z = 2.2$, $p < 0.05$, $r = 0.5$), with an average difference of 3.1 minutes. Serum and pituitary levels of LH and FSH measured during diestrus 24h after the last day of adult exposure (figure 4.A-D) were not significantly affected by BPA exposure.

Consistent with the lack of effect of BPA on GnRH and LH secretion during diestrus, the hypothalamic mRNA expression of *GAT2*, *GAD2*, *ESR1*, *ESR2* or *Kiss1* was not affected 24 hours after the end of adult BPA exposure (see Supplemental Figure 1[45]). Those genes had been previously shown to be sensitive to neonatal exposure to BPA[15].

Neonatal exposure to BPA persistently alters estrus cycle

As we had previously shown, pubertal onset was affected by neonatal exposure to BPA with opposing effects depending on the dose[15]. Vaginal opening occurred 3 days later on average after neonatal exposure to BPA 25 ng/kg/d and 3 days earlier after BPA 5 mg/kg/d. The time period from the day of vaginal opening (VO) to the first full regular cycle was significantly increased after exposure to both the low BPA dose ($U = 7$, $z = 2.0$; $p < 0.05$) and the high BPA dose ($U = 6.5$, $z = 2.2$; $p < 0.05$; $r = 0.8$) in 5.5 and 6.1 days, respectively (Figure 1.A left). Subsequently, exposure to both the low and high dose of BPA significantly decreased the percentage of cycling females (Figure 1.A right, see Supplemental Table 2[45]). At PND 90-105, while the control group showed regular cycles in 71% of the females, the BPA exposed groups showed only 43% (BPA-25 ng) and 14% (BPA-5 mg) of regularly cycling females. While the BPA treated groups showed a trend towards less time spent in proestrus and more time in diestrus, most values were not significantly different except on PND 82-89 when comparing CTL and the high BPA dose (Figure 1.C-E; see Supplemental Table 2[45]).

Neonatal exposure to BPA persistently disrupts the early stages of folliculogenesis

Ovarian weight was significantly reduced after neonatal exposure to the low BPA dose ($U = 6.5$, $z = 2.2$, $p < 0.05$, $r = 0.8$). While a similar average weight reduction was observed after exposure to the high BPA dose, difference was not found to be significant (Figure 3.B). The number of primordial follicles in the ovaries evaluated at PND 105 in the control group was significantly decreased after neonatal exposure to both the low dose ($U = 6.0$, $z = 3.4$, $p < 0.001$, $r = 1.5$) and the high dose ($U = 9.0$, $z = 2.7$, $p < 0.001$, $r = 1.2$) (Figure 3.A). Moreover, the number of atretic follicles per ovary was increased after neonatal exposure to the high dose of BPA ($U = 12.0$, $z = -2.4$, $p < 0.05$, $r = 1.1$). The low dose of BPA did not affect the number of atretic follicles. Cystic follicles were absent in the control ovaries and present after neonatal exposure to both doses of BPA.

4. DISCUSSION

In the present study, we provide the first evidence that adult exposure to a low environmentally relevant dose of BPA, in the range of nanograms, disrupts the preovulatory LH surge and leads to abnormal estrous cycle and folliculogenesis. Such disruption is reversible after adult exposure to BPA whereas it persists into adulthood following neonatal exposure, indicating a disruption of ovarian programming.

Few studies have directly compared the windows of sensitivity to BPA. Nikaido and coworkers have used BPA during and after the organizational period of sex steroids for reproduction in female mice[22,47]. CD-1 female mice were exposed to 10 mg/kg/d of BPA for 4 days either prenatally during the last week of gestation or prepubertally starting at PND 15. In both conditions, a reduced presence of corpora lutea was observed at 4 weeks of age, by the time of vaginal opening. This effect had disappeared at 8 and 24 weeks of age. These data indicate some reversibility of BPA effects on luteinisation following exposure during and after the organizational fetal window. Though we did not study ovarian histology close after the time of vaginal opening, we report here that the time from vaginal opening to the first complete estrus cycle is markedly increased after neonatal exposure to BPA. This is consistent with a reduced likelihood of ovulation and corpora lutea formation. Also, the presence of corpora lutea by 18 weeks of age is not reduced after neonatal exposure in the present study, in agreement with Nikaido's findings. The importance of the selected endpoints is emphasized by our data since the reduced representation of primordial follicles appears here to be the major expression of disrupted ovarian organization.

While the alterations of estrous cyclicity persist after neonatal exposure, they appear to be transient during adult exposure to BPA. During two weeks of adult exposure to a very low or high dose of BPA, altered estrous cyclicity occurs together with disruption of the late stages of folliculogenesis (antral follicles and corpora lutea). Importantly, all these effects appear to have disappeared one month after stopping the exposure to BPA.

Both neonatal and adult exposure lead to alterations characterized by a decrease in the percentage of time spent in proestrus and an increase in the time spent in diestrus. Wang et al. reported that proestrus was reduced and diestrus increased in 3 month-old mice after fetal exposure to a relatively low dose of

BPA (500 ng/kg)[48], in agreement with our findings after neonatal exposure of rats to 25 ng/kg. In our study, as the time spent in estrus was not affected by BPA, we hypothesized that the timing of ovulation during proestrus could be affected. To verify this hypothesis, we have measured the LH surge using serial blood sampling on the day of the expected proestrus and our results showed a systematic delay of the LH surge caused by both BPA doses together with a decrease in LH surge amplitude. Gestational exposure to much higher concentrations of BPA in sheep has been previously shown to lead to a dampened[49] and slightly delayed LH surge[50]. Other studies have reported decreased amplitude of the LH surge after gestational or prepubertal exposure to p-tert-octylphenol[51], perfluorooctanesulfonic acid[52], atrazine[53] or polychlorobyphenyls[54]. However, our study is the first one showing such a systematic delay during adult exposure to a low dose of BPA.

Prenatal exposure to BPA has been shown to modify hypothalamic gene expression and behavior in mice and rats[55,56], supporting central mechanisms for BPA effects. We also found that GABA neurotransmission was involved in the neonatal effects of BPA on the neuroendocrine control of GnRH secretion[15]. In our previous studies using a model of pulsatile GnRH secretion by hypothalamic explants *ex vivo*, we have shown that neonatal exposure to BPA results in opposing effects on the GnRH interpulse interval depending on the dose[15]. In the present study, adult exposure also leads to some opposing central effects of BPA on the GnRH interpulse interval during diestrus studied *ex vivo.* While a low BPA dose slightly increases the GnRH interpulse interval, the high BPA dose results in a decrease of the interpulse interval. While adult exposure to BPA results in the same pattern of change in GnRH secretion than neonatal exposure, the effect is quantitatively less important. However, both doses significantly disrupted the LH surge, which brings more evidence regarding the neuroendocrine disruption of ovulation caused by BPA. Additionally, Veiga Lopez et al. (2013) have shown that the preovulatory estradiol rise in prenatal BPA-treated female sheep was similar to that of controls which indicates that the ovarian signal is normal and the defect involves the neuroendocrine control of the LH surge generation[50].

The anteroventral periventricular (AVPV) nucleus is a region critical for the occurrence of the LH surge and is known to be sensitive to endocrine disruptors. Decreased Kiss1 and ESR1 mRNA expression in

the AVPV was observed on postnatal day 10 after exposure to BPA[57] while adult exposure might increase Kiss1 mRNA expression in the AVPV[58]. Altogether, a decreased hypothalamic-pituitary sensitivity, caused by a failure of the AVPV to respond to peripheral signals could explained the impaired LH surge caused by BPA. However, mRNA levels of oestrogen receptors and Kisspeptin were not affected neither in the mPoA nor the mediobasal hypothalamus in our model. Further studies should look at specific AVPV expression. The occurrence of the preovulatory LH surge depends on the master circadian clock within the suprachiasmatic nucleus together with rising ovarian oestrogen levels. The clock genes Per1 and Bmal1 in the AVPV play a critical role as integrator of ovarian and circadian signals to time the LH surge[59,60] and appear to be sensitive to endocrine disruption[61]. Recently, Loganathan et al. (2019) showed that BPA was able to alter Bmal1 and Per2 in immortalized hypothalamic neurons. Such data suggests that clock genes could be the central link explaining the effect of BPA on the LH surge timing.

The exposure to BPA during gestation or neonatal life can affect ovarian structure and function[62]. Fetal exposure to BPA was shown to increase cells in germ cell nests and to reduce primordial follicles[48]. The neonatal period is critical for ovarian differentiation since formation of primordial follicles are not completed until PND 3-4 and initial recruitment takes place during neonatal life. Thus, disturbances in early stages of folliculogenesis can also occur after neonatal exposure to BPA[63]. Our findings indicate that reduced pools of primordial follicles or antral follicles could reflect insults during or after development, respectively. In the CLARITY-BPA study[35], exposure to BPA 2.5 and 250 µg/kg/d from GD 6 to PND 21 resulted in reduced primordial, primary and preantral follicles at PND 21. However, irrespective of stopping exposure to BPA at PND 21 or continuing till the end of experiment, there were no longer any alteration of folliculogenesis at 3 and 6 months of age. By 1 year of age, cystic follicles were found only after exposure to 25 mg/kg/d of BPA till PND 21. These data indicate possible developmental effects of BPA though they appear to be transient and reversible, even during sustained exposure. Among the factors possibly accounting for discrepancies between the CLARITY-BPA study and the present one, the dose of BPA, the route of administration and the age window of exposure could play some role as well as differences in rat strain (Wistar versus Sprague-Dawley). In some studies, the

number of primary follicles was either reduced[29] or increased[64] after early postnatal exposure to BPA in the µg/kg dose range. Interestingly, fetal exposure of mice (from GD 6 to birth) to a lower dose of BPA (500 ng/kg) than in the CLARITY-BPA study resulted in reduced presence of antral follicles at PND 21[65] and at 3 months of age[34]. These findings are consistent with ours regarding persistent effects in adulthood after early life exposure though those authors did not find any reduction in primordial follicles[34]. In similar conditions, however, the number of primordial follicles was reduced on PND 4, indicating that both the age at exposure and the age at evaluation matter[48]. Taken together, those data point to the requirement of additional studies involving postnatal and sustained exposure to very low doses of BPA in the ng/kg range.

Human exposure to BPA is sustained throughout life and provides the rationale for lifelong exposure as done in the CLARITY-BPA study[66]. We elected to expose the animals to BPA transiently for 2 weeks because a transient exposure was required to evaluate whether effects persisting into adulthood could result from neonatal exposure. Likewise, transient exposure in adulthood was necessary to study possible reversibility of the effects after exposure in conditions comparable with those used neonatally. In contrast to the CLARITY-BPA study in rats and other studies in mice, our conditions did not include fetal exposure[34,48,65,67]. The subcutaneous route of administration was indispensable for reliable administration of BPA to neonatal rats, particularly using such a very low dose as discussed previously[15]. This required control for contamination by other BPA sources through the use of low-phytoestrogen pellets, glass-bottles and BPA-free cages. The oral route could not allow reliable administration of BPA doses in the range of ng/kg. This possibly explains that such very low doses were not used in the CLARITY-BPA study[66]. While the oral route is consistent with the human conditions of exposure, oral gavage as done in the CLARITY-BPA study can account for confounding factors such as stress and bypass of oral absorption[68]. Comparable serum levels of BPA and UDP-glucuronosyltransferase, the enzyme that conjugates BPA have been reported after oral and subcutaneous administration neonatally[69]. However, in another more extensive study in neonatal mice, the systemic levels of free BPA were found to be 3-4 times higher after subcutaneous injection than after oral administration[70]. Assuming that the pups in our study would have been exposed to BPA levels 4 times higher than using the oral route, such

levels (equivalent to 100 ng/kg orally) would still be consistent with human exposure and 25 times less than the lowest dose used in the CLARITY-BPA study[66]. For consistency and to ensure precision in the low levels administered, we also used the subcutaneous route of administration in the adult females. The reversibility of the effects of the low BPA dose after resumption of control conditions supports the evidence that the effects of the very low dose are unlikely resulting from a contaminant since all the management conditions except BPA (vehicle, food, drink and cages) were identical in the control and treatment settings. That very low dose is far below the No-Observed-Adverse-Effects Level (NOAEL) and below the EFSA "safe dose". It represents half the average exposure of the general population[1]. Following neonatal or adult exposure to BPA, the ovulatory cycle and folliculogenesis are impaired and the effects are similar using a very low dose of BPA or a high dose in the range of milligrams. Altogether, the present study suggests that the toxicity of BPA on the ovaries is more dependent on the period of exposure in life than the dose of BPA though only two doses were studied and the effects of intermediate doses warrant further studies.

CONCLUSIONS

In conclusion, we show that both adult and neonatal exposure to a very low dose of BPA in the range of nanograms can result in alteration of estrous cyclicity and folliculogenesis. Similar alterations are observed using a high dose of BPA. Neonatal exposure leads to effects occurring after exposure and persisting on the long-term suggesting that BPA is able to reprogram the reproductive axis at early stages, particularly by affecting the early follicular development. By contrast, adult exposure to BPA causes effects to occur transiently during exposure since normal cyclicity and folliculogenesis are restored within one month after resuming control conditions. Moreover, estrous cyclicity during adulthood seems to be altered by a central mechanisms involving the disruption of the LH surge. Our findings imply that when further evaluating BPA adverse effects on the female reproductive axis, very low doses in the range of average environmental exposure should be used with inclusion of the critical neonatal period and addressing both neuroendocrine and ovarian endpoints.

5. ACKNOWLEDGEMENTS

We will be forever grateful for Pr Jean-Pierre Bourguignon's mentorship. Until the end of his life, he brought critical ideas and insights regarding this project. His sharp scientific mind and generous guidance will be greatly missed. We are indebted to Professor P. Delvenne for assistance with Papanicolaou staining and to Dr V. D. Ramirez (Urbana, IL) for providing the CR11-B81 anti-GnRH antiserum. We thank Dr R.T. Zoeller for helpful comments.

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661 **TABLES**

Table 1. Primer Sequence						
Gene		Sequence	Annealing Temp. (Tm)	Length (bp)	Accession Number	Conc. (μM)
β-Actin	F	5'−CGCGAGTACAACCTTCTTGC−3'	59.6	200	NM 031144.3	10
	R	5'− ATACCCACCATCACACCCTG-3'	59.1			
GAT2	F	5'−TTCATCGGGCTCATTATGCTCA−3'	59.9	193	NM 133623.1	10
	R	5'−TGATAAGAGGCCACGGCTTG−3'	60.1			
GAD2	F	5'−GCACCTGTGACCAAAAACCC−3'	59.9	73	NM 012563.1	10
	R	5'−AGGTCTGTTGCGTGGAGAAG−3'	60.0			
Notes: qRT-PCR primers (F: forward; R: reverse), annealing temperature, PCR product size and concentration used in qRT-PCR. Experiments were run at 45 cycles and 60◦C as Tm						

662

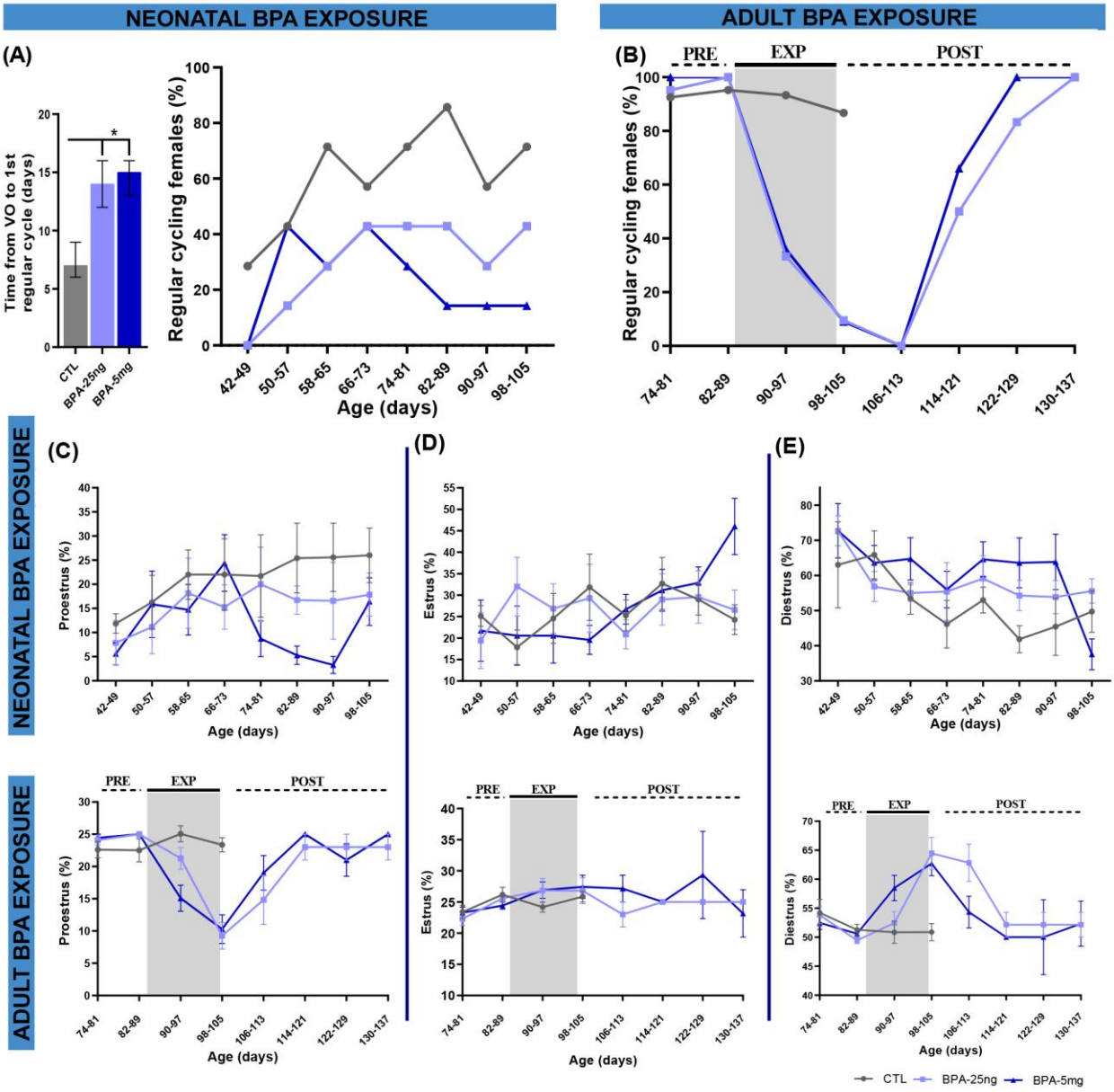
663 **FIGURES**

664 **Figure 1**. Characteristics of estrous cycle after neonatal (PND 1 to 15) and adult (PND 90 to 105)
665 exposure to BPA (25 ng/kg/d or 5 mg/kg/d) or corn oil (CTL). (A): Left: Average time from day of
666 vaginal opening to first regular cycle. Right: Percentage of females exhibiting regular cycling from 42
667 to 105 days of age after neonatal BPA exposure. (B): Percentage of females exhibiting regular cycling
668 2 weeks before, during and 4 weeks after adult BPA exposure. (C-E): Percentage of time spent in
669 proestrus (C), estrus (D) and diestrus (E) after neonatal (top) or adult (bottom) exposure to BPA. Data
670 are expressed as mean [IQR] (A) and percentage ± SEM (C-E).

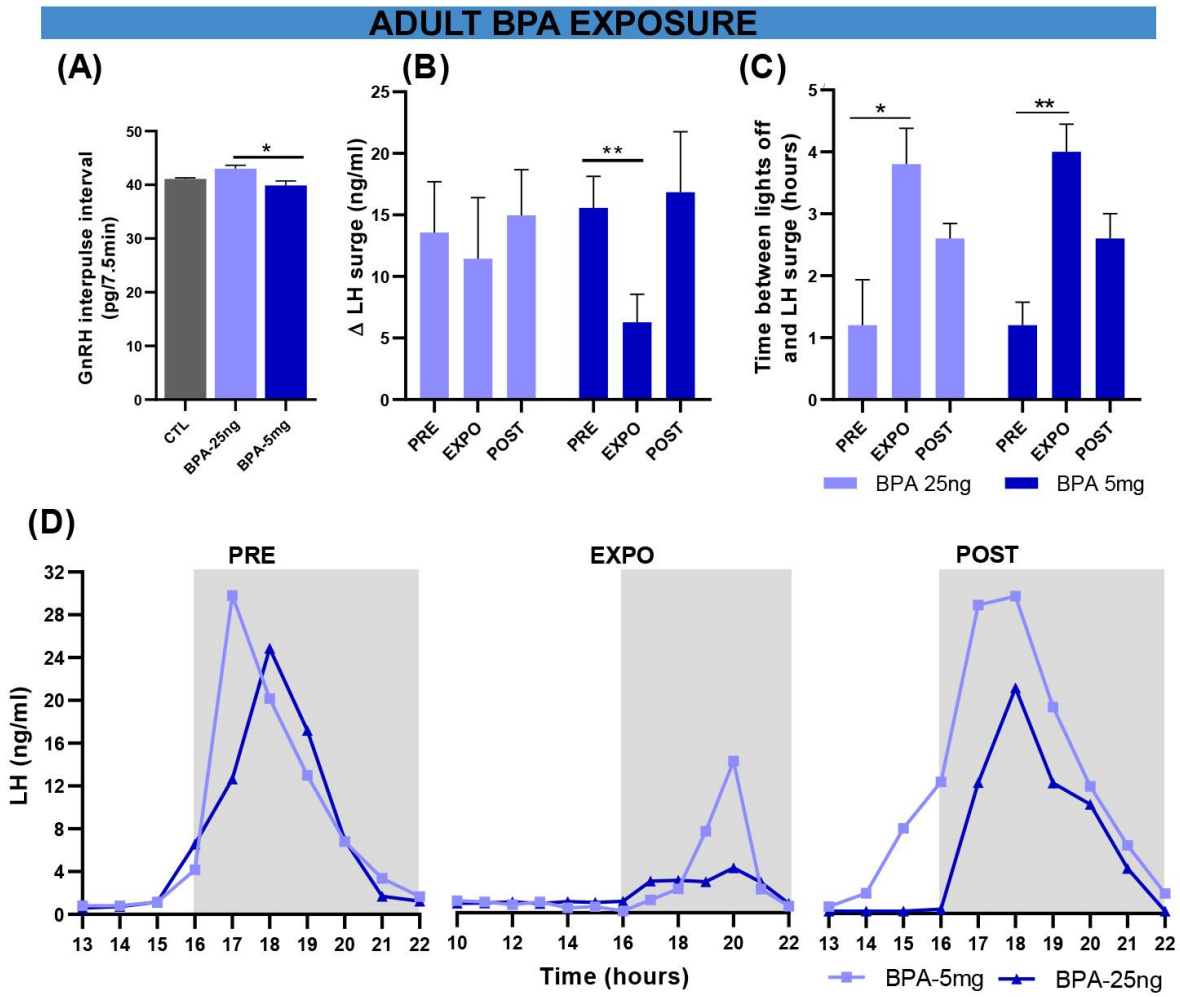
671 **Figure 2**. Effects of adult (PND 90 to 105) exposure to BPA (25 ng/kg/d or 5 mg/kg/d) or corn oil (CTL)
672 on GnRH pulsatile secretion and the preovulatory LH surge. (A) GnRH interpulse interval *in vitro* using
673 hypothalamic explants obtained on PND 106, i.e. 24h after the last administration of BPA or corn oil
674 (CTL) in adult female rats. (B) LH surge amplitude before (PRE), during (EXPO) and after (POST)
675 adult BPA exposure. (C) LH surge timing after beginning of the dark phase (16h00) (D) Representative
676 LH surge from 2 females exposed to either the low or high BPA dose. * $p<0.05$, ** $p<0.01$ vs CTL.
677 Data are mean ± SEM.

678 **Figure 3**. Effects of neonatal (PND 1 to 15) and adult (PND 90 to 105) exposure to BPA (25 ng/kg/d or
679 5 mg/kg/d) or corn oil (CTL) on ovarian weight and folliculogenesis. (A) Quantification of follicles and
680 corpora lutea (A) and ovarian weight (B) after neonatal BPA exposure. Quantification of follicles and
681 corpora lutea 24 hours (C) or 30 days (D) after adult BPA exposure. Representative ovarian sections
682 obtained from animals 24h after adult exposure (D). Arrows depicts the presence of some cystic follicles.
683 Follicles were quantified in every other section and normalized by ovarian volume (mm^3). * $p<0.05$, **
684 $p<0.01$, *** $p<0.001$ vs CTL. The data are median and IQR.

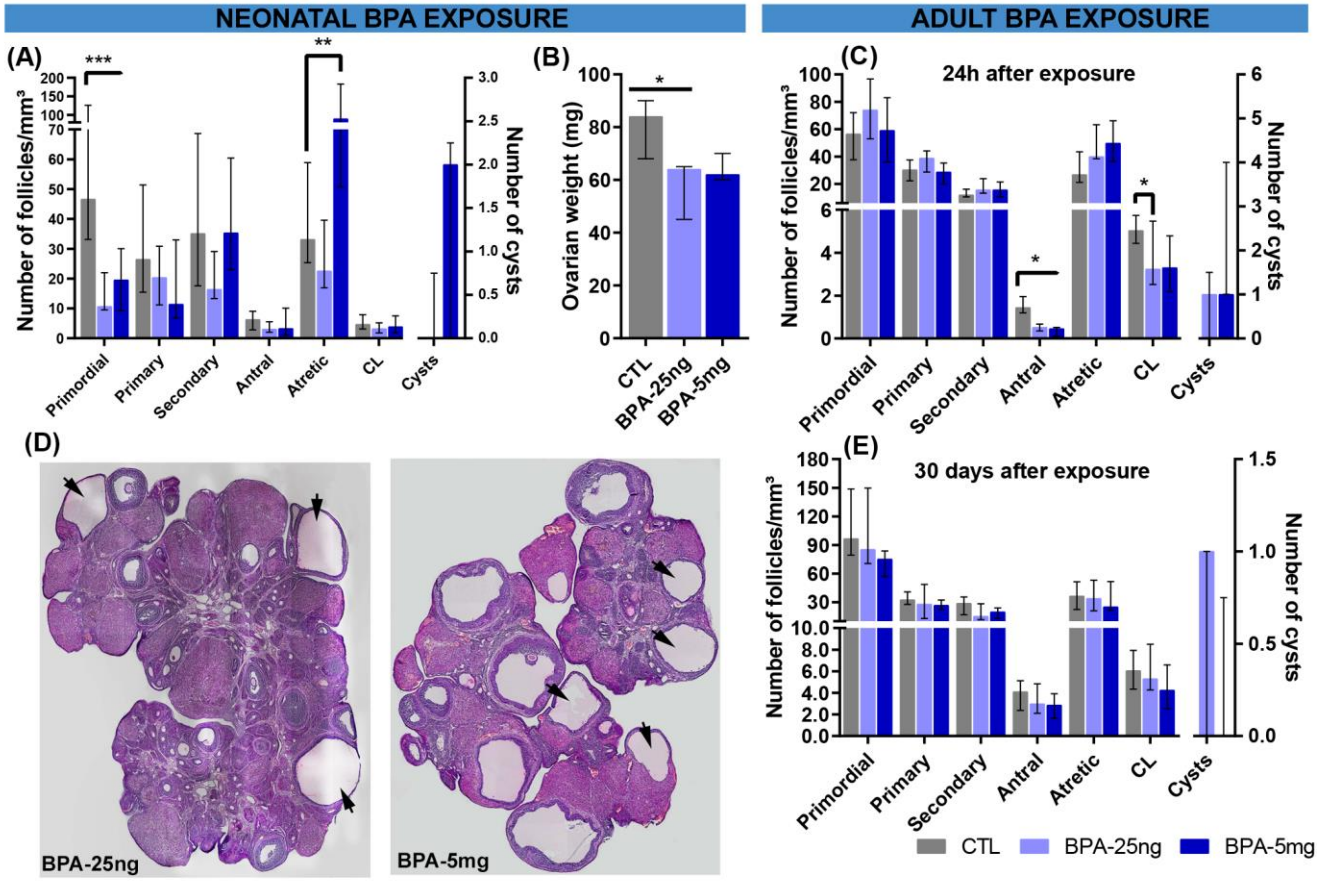
685 **Figure 4**. Effects of adult exposure to BPA on LH and FSH levels in serum (A-B) and pituitary (C-D)
686 gland. Samples were collected 24h after the last BPA or corn oil administration. The levels of pituitary
687 LH (diluted 2000x) and FSH (diluted 500x) were multiplied by their dilution factor to obtain an amount
688 of µg/gland. The data are represented as median and IQR.


NEONATAL BPA EXPOSURE
ADULT BPA EXPOSURE
(A)
Time from VO to 1st regular cycle (days)
CTL
BPA-25ng
BPA-5mg
Regular cycling females (%)
Age (days)
(B)
PRE
EXP
POST
Regular cycling females (%)
(C)
Proestrus (%)
(D)
Estrus (%)
(E)
Diestrus (%)
CTL
BPA-25ng
BPA-5mg

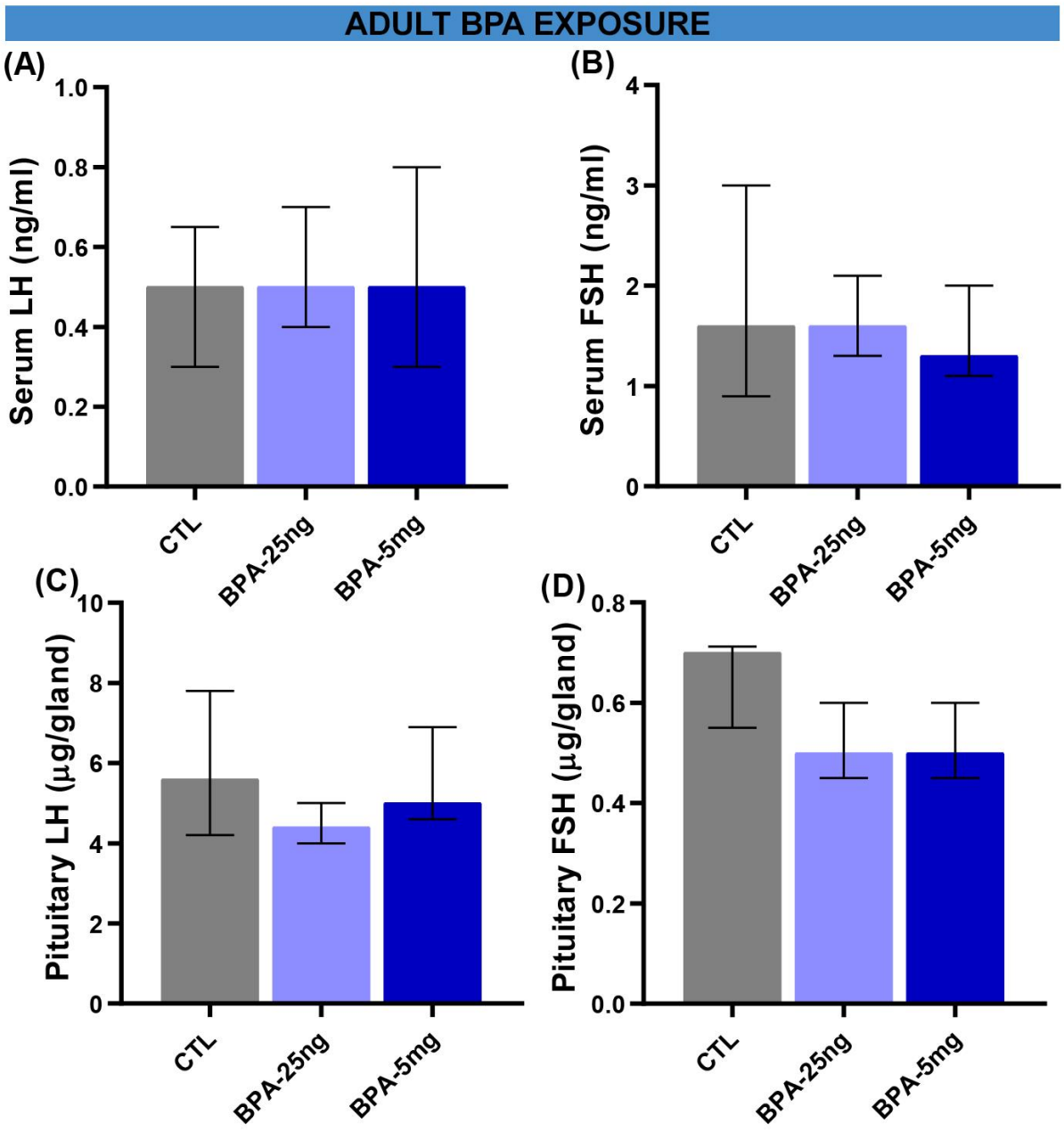
689 Figure 1


ADULT BPA EXPOSURE
(A)
GnRH interpulse interval (pg/7.5min)
CTL
BPA-25ng
BPA-5mg
(B)
Δ LH surge (ng/ml)
PRE
EXPO
POST
(C)
Time between lights off and LH surge (hours)
BPA 25ng
BPA 5mg
(D)
PRE
EXPO
POST
LH (ng/ml)
Time (hours)
BPA-5mg
BPA-25ng

690 Figure 2


NEONATAL BPA EXPOSURE
ADULT BPA EXPOSURE
(A)
Number of follicles/mm³
Number of cysts
Primordial
Primary
Secondary
Antral
Atretic
CL
Cysts
(B)
Ovarian weight (mg)
CTL
BPA-25ng
BPA-5mg
(C)
24h after exposure
(D)
BPA-25ng
BPA-5mg
(E)
30 days after exposure
CTL
BPA-25ng
BPA-5mg

691 Figure 3


ADULT BPA EXPOSURE
(A)
Serum LH (ng/ml)
1.0
0.8
0.6
0.4
0.2
0.0
CTL
BPA-25ng
BPA-5mg
(B)
Serum FSH (ng/ml)
4
3
2
1
0
CTL
BPA-25ng
BPA-5mg
(C)
Pituitary LH (µg/gland)
10
8
6
4
2
0
CTL
BPA-25ng
BPA-5mg
(D)
Pituitary FSH (µg/gland)
0.8
0.6
0.4
0.2
0.0
CTL
BPA-25ng
BPA-5mg

692 Figure 4