

## Effects of chronic exposure to bisphenol A in adult female mice on social behavior, vasopressin system, and estrogen membrane receptor (GPER1)

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### ABSTRACT

Bisphenol A (BPA), an organic synthetic compound found in some plastics and epoxy resins, is classified as an endocrine disrupting chemical. Exposure to BPA is especially dangerous if it occurs during specific "critical periods" of life, when organisms are more sensitive to hormonal changes (*i.e.*, intrauterine, perinatal, juvenile or puberty periods). In this study, we focused on the effects of chronic exposure to BPA in adult female mice starting during pregnancy. Three months old C57BL/6J females were orally exposed to BPA or to vehicle (corn oil). The treatment (4  $\mu$ g/kg body weight/day) started the day 0 of pregnancy and continued throughout pregnancy, lactation, and lasted for a total of 20 weeks. BPA-treated dams did not show differences in body weight or food intake, but they showed an altered estrous cycle compared to the controls. In order to evidence alterations in social and sociosexual behaviors, we performed the Three-Chamber test for sociability, and analyzed two hypothalamic circuits (well-known targets of endocrine disruption) particularly involved in the control of social behavior: the vasopressin and the oxytocin systems. The test revealed some alterations in the displaying of social behavior: BPA-treated dams have higher locomotor activity compared to the control dams, probably a signal of high level of anxiety. In addition, BPA-treated dams spent more time interacting with no-tester females than with no-tester males. In brain sections, we observed a decrease of vasopressin immunoreactivity (only in the paraventricular and suprachiasmatic nuclei) of BPA-treated females, while we did not find any alteration of the oxytocin system. In parallel, we have also observed, in the same hypothalamic nuclei, a significant reduction of the membrane estrogen receptor GPER1 expression.

**Key words:** Endocrine disrupting chemicals; BPA; Three-Chamber test; vasopressin, oxytocin; GPER1; paraventricular nucleus; suprachiasmatic nucleus.

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## Introduction

Thousands of chemicals, some banned and some still in use, have been classified as endocrine disruptor compounds (EDCs), *i.e.*, exogenous chemicals, or mixture of chemicals, that can interfere with any aspect of hormone action.<sup>1</sup> In particular, bisphenols (BPs), organic synthetic compounds largely used for the production of polycarbonate plastics and epoxy resins, are an extremely abundant class of EDCs. As reviewed by Catenza *et al.*,<sup>2</sup> the first synthesized BP, bisphenol A (BPA), has been utilized in the production of plastics since the 1950s. It is still the most highly produced BP: in 2018, about 7.2 million tons of BPA have been produced globally, and its consumption has been estimated to increase by 3.1 million tons by 2022.<sup>3,4</sup> Thanks to its structure, BPA can interact with a wide set of hormone receptors both nuclear and membrane-bound, including estrogen receptors (ER $\alpha$ , ER $\beta$ , GPER1, ERR $\gamma$ ), androgen receptor, peroxisome proliferator-activated receptor  $\gamma$ , glucocorticoid receptors and thyroid hormone receptors.<sup>5,6</sup> The capability of BPA to act through different types of receptors, differentially distributed in the tissues, is responsible for the wide range of effects it exerts on the organism.<sup>7</sup> BPA, as other EDCs, is known to have organizational effects during development, and/or activational effects in adulthood.<sup>1</sup> Exposure to EDCs is more dangerous if it occurs during specific “critical periods” of life, such as intrauterine, perinatal, juvenile or puberty periods, when organisms are more sensitive to hormonal action.<sup>8</sup> Early pregnancy seems to be a particular sensitive period to BPA exposure, linked to the development of some adverse effects, such as intrauterine growth restriction.<sup>8</sup> In addition, pregnancy, delivery and maternal care are highly regulated by hormonal actions. In fact, progesterone, prolactin and estradiol are involved in the organization and activation of brain area appointed to the control of these functions, such as the medial preoptic area (MePOA), the bed nucleus of stria terminalis (BST) and the medial amygdala (MeA) which are enriched in estrogen, vasopressin and oxytocin receptors.<sup>9,10</sup>

Vasopressin (AVP) and oxytocin (OXT) systems play a key role in the control of different type of behaviors, and in particular the maternal<sup>11</sup> and the social one.<sup>12-15</sup> They are two nonapeptides mainly synthesized in neurons of supraoptic (SON) and paraventricular (PVN) nuclei of the hypothalamus, and they, as well as their analogues in non-mammalian vertebrates, represent target systems underlying the alterations observed in social behavior after exposure to different types of EDCs.<sup>16-18</sup> There are many studies, performed not only in rodents, which demonstrated that BPA exposure, mainly during pre- or peri-natal periods, is linked to alterations in both AVP and OXT number of neurons and innervation, especially in sexually dimorphic regions associated with social and aggressive behaviors and to anxiogenic effects.<sup>19</sup>

In the present study, we proposed pregnancy as a critical period not only for the developing fetus but also for the mother. We investigated the long-term consequences of chronic exposure to low-dose BPA, starting at mating and continuing throughout pregnancy and lactation, reaching 20 weeks of treatment, directly on the exposed dams. Primarily we focused on social behavior<sup>18</sup> and on two of the circuits mainly involved in the control of this behavior, AVP and OXT,<sup>12-15</sup> which are well-known targets of endocrine disruption,<sup>11,16,19</sup> as well as the expression of the membrane estrogen receptor (GPER1) which is largely present in these nuclei.<sup>20</sup>

## Materials and Methods

### Animals

Adult C57BL/6J male and female mice from our outbred colony at the Neuroscience Institute Cavalieri Ottolenghi (originally purchased from Envigo, S. Pietro al Natisone, Udine, Italy) were housed in standard conditions in 45 × 25 × 15 cm polypropylene mouse cages at 22±2°C, under 12:12 light dark cycle (lights on at 8:00 am). Food and water were provided *ad libitum* (standard mouse chow 4RF21, Mucedola Srl, Settimo Milanese, Milan, Italy). One 3-months old male and two 3-months old female mice were caged together to achieve a successful mating, assessed by the evaluation of the presence of the vaginal plug (assumed as gestational day 0, GDO).

### Treatment

BPA (Sigma Aldrich, St. Louis, MO, USA; 239658, CAS 80-05-7) was prepared for oral administration by dissolving it in corn oil (Sigma-Aldrich, C8267). 20 pregnant dams were divided into two experimental groups: control dams (receiving only vehicle, corn oil; n=10) and treated dams (receiving 4 µg/kg BW/day of BPA, corresponding to the European TDI; n=10).

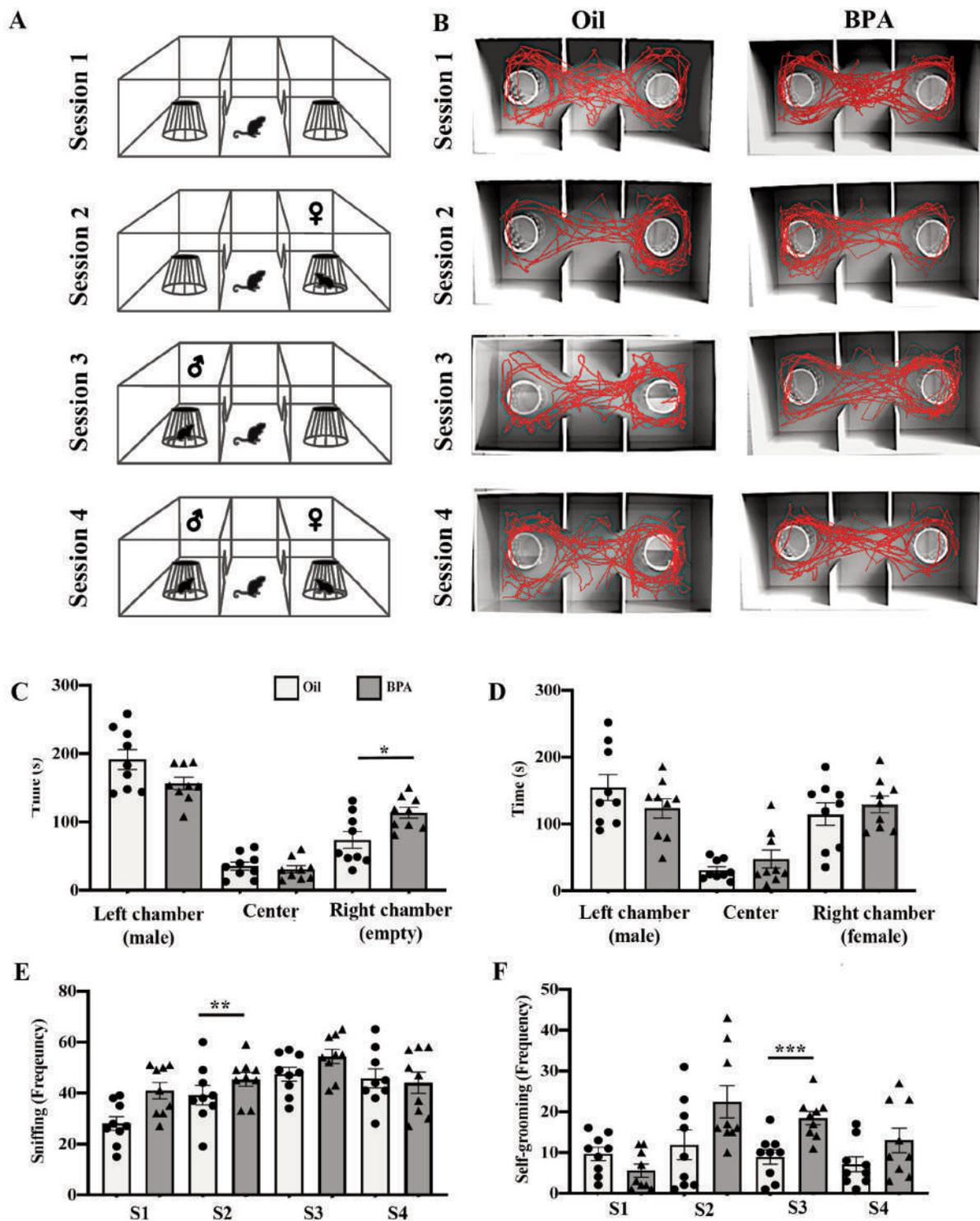
Dams were treated started at GDO, throughout pregnancy and lactation, and continuing after the weaning of the offspring, for a total of 20 weeks of treatment. To mimic human exposure conditions, the daily treatment or the vehicle was given orally to the dams, with a pipette, in order to minimize the discomfort and the stress provoked to the dams during the treatment.<sup>21,22</sup> The dose was calculated according to their body weight, recorded with an electronic precision balance (Mod. Kern-440-47N, resolution 0.1 g).

We monitored the dams, evaluating in particular: i) body weight (BW), recorded daily; ii) after the weaning, the food intake (g of food/animal/day) once a week; iii) the estrous cycle, evaluating the vaginal cytology smears,<sup>23</sup> after the 18<sup>th</sup> week of treatment, for at least 2 cycles.

### Three-Chamber test

Dams were tested after the 18<sup>th</sup> week of treatment, in estrus phase (evaluated by vaginal smear). The test was conducted using a Three-Chamber social approach apparatus: a rectangular plastic box consisting of three same-sized chambers (20 cm × 25 cm × 20 cm) with openings in the dividing walls that allowed the subject to access all three chambers without restriction. A plastic holding cylinder, for the novel and familiar mice, was placed in each of the side chambers. These cylinders were drilled to allow interactions between tester and no-tester mice.

Tester mice were placed in the room in which the test was performed at least 2 h before starting, to allow the habituation to room lighting. Before starting and between each session, the testing apparatus was cleaned with 70% ethanol, being sure to thoroughly dry the apparatus to avoid exposure of mice to alcohol. The testing procedure consisted of four chronological sessions: Habituation, two Sociability sessions, and Social preference.<sup>24</sup> Each session lasted 5 min (schematic representation in Figure 1A) and at the end of each session the tester mouse was temporarily moved to a clean housing cage while the investigator set up for the next session. As no-tester mice we selected unknown age-matched male or female C57BL/6J mice. In the habituation phase (Figure 1A, Session 1) the tester mouse was placed into the middle chamber and allowed to explore all three chambers freely. In the first sociability phase (Figure 1A, Session 2), an age- and gender-matched (female, in estrus phase) novel C57BL/6J mouse was placed into the holding cylinder placed in the right chamber. The same occurs in the second sociability phase (Figure 1A, Session 3), when an age- and



**Figure 1.** Analysis of social behavior of oil-treated and BPA-treated dams through Three-Chamber test. A) Experimental set-up and schematic representation of the apparatus used for the Three-Chamber test for the four experimental sessions: session 1 (habituation), session 2 (sociability with the gender-matched mouse), session 3 (sociability with the gender-mismatched mouse) and session 4 (social preference). B) Representative images for total distance traveled by Oil-treated dams (left column) or BPA-treated ones (right column) during the four sessions of the test. C,D) Time spent by the oil-treated (light gray) or BPA-treated (dark gray) dams within the three different chambers (left, center and right chamber) of the apparatus during (C) the third session (sociability with gender-mismatched mouse) and (D) the fourth session (social preference) of the test. E,F) Frequency in sniffing (E) and grooming (F) behavior during the four sessions of the test displayed by the oil-treated (light gray) or BPA-treated (dark gray) dams. Data are expressed as mean  $\pm$  SEM. One-way ANOVA revealed a significant effect of the treatment for  $p \leq 0.05$ .

gender-mismatched (male) novel C57BL/6J mouse was presented to tester mouse in the holding cylinder placed in the left chamber. In the fourth and last session (Figure 1A, Session 4), the social preference was assessed, presenting to the tester mice both known female (right chamber) and male (left chamber) in the holding cylinder.

Each session was recorded with a camera placed above the apparatus in order to subsequently perform the behavioral analysis through the Ethovision XT program (Noldus Information Technology, Wageningen, The Netherlands). For each session we measured different parameters (listed above) in order to evaluate sex-dependent sociability, anxiety-related and explorative behaviors of the tester mouse:

- *Distance*: the total distance traveled (cm) by the tester mice in each chamber and in the total arena.
- *Time*: the time (s) spent in the different chambers by the tester mice.
- *Sniffing*: the number of times and the time (s) spent by the tester mouse interacting with the no-tester mice in the holding cup or exploring the different chambers.
- *Self-grooming*: the number of times and the time (s) spent by the tester mouse grooming itself.
- *Rearing*: number of times and time (s) spent by the tester mouse rearing (*i.e.*, the mouse is standing only on its posterior legs).

### Fixation and tissue sampling

Dams were sacrificed after 20 weeks of treatment, in the estrus phase, assessed by vaginal smear, by deep irreversible anesthesia (intraperitoneal injection of zoletil 80 mg/kg/ rompum 10 mg/kg) and transcardially perfused with 4% paraformaldehyde (PFA) solution. Brains were removed and stored in a 4% PFA solution for 24 h, followed by several washings in 0.01 M saline phosphate buffer (PBS). Finally, they were stored in a 30% sucrose solution in PBS at 4°C, frozen in isopentane pre-cooled in dry ice at -35°C and stored in a deep freezer at -80°C until sectioning.<sup>25</sup>

Brains (5±1/group) were serially cut in the coronal plane at 40 µm thickness with a cryostat, in four series. The plane of sectioning was oriented to match the drawings corresponding to the coronal sections of the mouse brain atlas.<sup>26</sup> Sections were collected in a cryoprotectant solution<sup>27</sup> and stored at -20°C. Three series were processed, for AVP, OXT and GPER1 immunohistochemistry, using the free-floating technique.

### Immunohistochemistry

The presence of AVP, OXT, or GPER1 was detected by immunohistochemistry performed, according to our previous studies,<sup>20,25,28</sup> on free-floating sections. Briefly, the sections were washed overnight in PBS at pH 7.3. The following day, sections were first washed in PBS containing 0.2% Triton X-100 for 30 min and then treated to inhibit endogenous peroxidase activity with a solution of PBS containing methanol/hydrogen peroxide for 20 min. Sections were then incubated for 30 min with normal goat serum (Vector Laboratories, Burlingame, CA, USA) and incubated overnight at room temperature with anti-AVP antibody (gift of Dr. Michael Sofroniew, UCLA, Los Angeles, CA, USA, Rabbit, 1:20,000)<sup>29,30</sup> or anti-OXT antibody (EMD Millipore AB911, Rabbit, 1:10,000) diluted in PBS, pH 7.3-7.4, containing 0.2% Triton X-100. For GPER1 immunohistochemistry sections were washed for 30 min at room temperature in PBS containing 0.2% Triton X-100 and 0.2% BSA and then incubated 48 h at 4°C with anti-GPER-1 antibody (Abcam, Cambridge, UK, ab39742, Rabbit, 1:250) diluted in PBS containing 0.2% Triton X-100, 0.2% BSA and 3% normal serum goat (Vector Laboratories). A biotinylated goat anti-rabbit secondary antibody (Vector Laboratories) was then

employed at a dilution of 1:250 for 60 min at room temperature (dilution of 1:300 for 120 min at room temperature for GPER1). The antigen-antibody reaction was revealed by 60 (AVP and OXT) or 90 (for GPER1) min incubation with avidin-peroxidase complex (Vectastain ABC Kit Elite; Vector Laboratories). The peroxidase activity was visualized with a solution containing 0.400 mg/ml 3,3-diamino-benzidine (Sigma-Aldrich, Milan, Italy) and 0.004% hydrogen peroxide in 0.05 M Tris-HCl buffer at pH 7.6. Sections were mounted on chromallum-coated slides, air-dried, cleared in xylene and cover slipped with New-Entellan mounting medium (Merck, Milan, Italy). These antibodies were successfully used in previous studies.<sup>25,28,31-33</sup> The specificity of these antisera was previously assessed,<sup>34-36</sup> but, as a further control, we omitted the primary antiserum or the secondary biotinylated one, replaced with PBS. In both cases positive cell bodies and fibers were totally absent.

### Quantitative analysis

For quantitative analysis, selected standardized sections of comparable levels covering the paraventricular nucleus (PVN, Bregma -0.58 to -0.94 mm), the supraoptic nucleus (SON, Bregma -0.58 to -0.94 mm), the suprachiasmatic nucleus (SCH, Bregma -0.34 to -0.82 mm) and the medial amygdala (MeA, Bregma -1.06 to 1.22 mm) were chosen according to the mouse brain atlas.<sup>26</sup> Two sections for each nucleus were acquired with a NIKON DS-U1 digital camera (Software of acquisition: NIS-Element AR 2.10) connected to a NIKON Eclipse 90i microscope (Nikon Italia S.p.A., Florence, Italy). Images were digitized by using a 20x objective. Digital images were processed and analyzed by ImageJ (v. 2.10/1.53c; Wayne Rasband, NIH, Bethesda, MD, USA). Measurements were performed within predetermined fields (region of interest, ROI), boxes of fixed size and shape that are inserted inside each labeled considered nucleus (0.066 mm<sup>2</sup> for SON; 0.077 mm<sup>2</sup> for SCH; 0.104 mm<sup>2</sup> for MeA). The PVN was instead divided into subregions, following the different distribution within the nucleus of the two analyzed systems.<sup>26</sup> On one hand, for the AVP- immunoreactivity (ir) analysis, the PVN (total area 0.049 mm<sup>2</sup>) was divided into two sub-regions, the anterior parvicellular nucleus (PaAP, 0.013 mm<sup>2</sup>) and the ventral nucleus (PaV, 0.036 mm<sup>2</sup>). On the other hand, for the OXT-ir analysis, the PVN (total area 0.068 mm<sup>2</sup>) was divided in three subregions, the dorsal cap/lateral magnocellular part (PaDC/PaLM, 0.013 mm<sup>2</sup>), the medial parvicellular part (PaMP, 0.035 mm<sup>2</sup>) and the medial magnocellular part (PaMM, 0.02 mm<sup>2</sup>). Finally, the GPER1-ir was analyzed in the entire PVN.

We evaluated the extension of the immunoreactivity (cell bodies, dendrites, fibers) in all the selected nuclei as fractional area covered by immunopositive material.<sup>37</sup> In addition, we counted the number of AVP-positive cells in PVN, SON and SCH, while the OXT-positive cells were counted in PVN and SON.

### Statistical analysis

Quantitative data were examined with SPSS 26 statistic software (SPSS Inc., Chicago, USA) by one-way analysis of variance (ANOVA). Differences were considered statistically significant for values of  $p \leq 0.05$ . Data are shown as mean ± SEM (mean standard error).

## Results

### Effects of chronic adult exposure to BPA on physiological parameters of the dams

The performed treatment had not significant effects on body

weight or food intake of the dams (*data not shown*). However, the estrous cycle of the BPA-exposed dams seems to be altered compared to the one of the control dams. In fact, the percentage of time spent in the estrus phase was significantly increased ( $p=0.041$ ) in the BPA-treated dams ( $63.58\pm 5.62\%$ ) compared to the controls ( $44.34\pm 5.91\%$ ).

### Three-Chamber test

Results obtained from the analysis of the Three-Chamber test are summarized in Table 1 and Table 2, reporting all the values. Here we highlight the most interesting results (Figure 1), for each session:

Session 1 (Habituation). The total distance traveled by the BPA-treated dams was significantly higher compared to the controls ( $p=0.018$ ) (Figure 1B). Furthermore, BPA dams also showed higher total sniffing behavior than controls ( $p=0.007$ ) (Figure 1E).

Session 2 (Sociability with the gender-matched mouse). The total distance covered by the BPA-treated dams was, again, significantly higher compared to the controls ( $p=0.006$ ) (Figure 1B). Both groups preferred to spend time in the right chamber, where the female no-tester animal was placed, but, interestingly, BPA-treated dams did more sniffing compared to the controls ( $p=0.04$ ) (Figure 1E).

Session 3 (Sociability with the gender-mismatched mouse). In this session, the distance covered by BPA-treated dams was significantly higher compared to the controls ( $p=0.005$ ) only in the left chamber, where the male no-tester animal was placed (Figure 1B).

Both BPA- and oil-treated dams preferred to spend the time in the left chamber, but the BPA-treated groups demonstrated a tendency to spend less time in this chamber compared to the control ( $p=0.058$ ) (Figure 1C). Moreover, BPA-treated dams spent more time in the right chamber compared to the controls ( $p=0.014$ ) (Figure 1C). Besides, BPA-treated group did more grooming compared to the control ( $p=0.001$ ), spending more time doing it ( $p=0.006$ ) (Figure 1F).

Session 4 (Social preference). We did not find any significant differences in this last session. However, we noticed that difference of time spent in the two chambers was flattened in the BPA-treated group ( $p=0.759$ ), showing no preference (Figure 1D). Moreover, the BPA-treated dams had a tendency ( $p=0.074$ ) to spend less time sniffing the male no-tester mice compared to the controls (Figure 1E). Finally, the BPA-treated dams showed a tendency to do more rearing compared to the control ( $p=0.053$ ), spending more time doing it ( $p=0.064$ ).

### AVP-ir analysis

The analysis of the AVP-ir (summarized in Table 3) revealed that the PVN (Figure 2 A-C) and the SCh (Figure 3) were affected by the treatment, whereas there was no effect on SON and MeA (Table 3).

In particular, we observed a significative reduction (Figure 2A) in both number of cells ( $p=0.005$ ) (Figure 2B), and fractional area ( $p=0.036$ ) (Figure 2C) in the total PVN. This reduction is mainly due to the reduction of AVP-ir in the PaV of the BPA-treated group

**Table 1. Results obtained from the analysis of the distance traveled and of the time spent in the total arena or different chambers of the Three-Chamber test. Data are reported as mean  $\pm$  SEM. One-way ANOVA revealed a significant effect of the treatment for  $p\leq 0.05$ .**

Chamber Parameter	Arena	Left chamber Session 1 (Habituation)	Center	Right chamber
Distance traveled (cm)	Oil: 86408.452 $\pm$ 10979.28 BPA: 126892.944 $\pm$ 10764.76 ( $p=0.018$ )	Oil: 32590.944 $\pm$ 4441.891 BPA: 51877.389 $\pm$ 5830.23 ( $p=0.018$ )	Oil: 19823.03 $\pm$ 2448.964 BPA: 31452.311 $\pm$ 3025.188 ( $p=0.009$ )	Oil: 3394.478 $\pm$ 4713.435 BPA: 43563.244 $\pm$ 3842.471 ( $p=0.135$ )
Time (s)	Oil: 300.181 $\pm$ 0.035 BPA: 300.11 $\pm$ 0.03 ( $p=0.153$ )	Oil: 112.626 $\pm$ 7.141 BPA: 112.076 $\pm$ 7.272 ( $p=0.958$ )	Oil: 78.492 $\pm$ 5.561 BPA: 62.311 $\pm$ 5.031 ( $p=0.046$ )	Oil: 109.063 $\pm$ 6.56 BPA: 125.724 $\pm$ 10.482 ( $p=0.197$ )
<b>Session 2 (Sociability with no-tester female)</b>				
Distance traveled (cm)	Oil: 83438.054 $\pm$ 12399.336 BPA: 137443.87 $\pm$ 11607.57 ( $p=0.006$ )	Oil: 41711.411 $\pm$ 6090.7754 BPA: 66523 $\pm$ 7443.3324 ( $p=0.02$ )	Oil: 21088.391 $\pm$ 3296.322 BPA: 36700.822 $\pm$ 3511.7 ( $p=0.005$ )	Oil: 20638.252 $\pm$ 4315.31 BPA: 34220.044 $\pm$ 3806.443 ( $p=0.031$ )
Time (s)	Oil: 300.197 $\pm$ 0.041 BPA: 300.111 $\pm$ 0.031 ( $p=0.116$ )	Oil: 71.936 $\pm$ 8.824 BPA: 92.209 $\pm$ 12.174 ( $p=0.196$ )	Oil: 52.725 $\pm$ 5.93 BPA: 37.272 $\pm$ 4.22 ( $p=0.049$ )	Oil: 175.509 $\pm$ 12.648 BPA: 170.63 $\pm$ 12.226 ( $p=0.785$ )
<b>Session 3 (Sociability with no-tester male)</b>				
Distance traveled (cm)	Oil: 111206.4 $\pm$ 17506.127 BPA: 124788.84 $\pm$ 15582.89 ( $p=0.57$ )	Oil: 19509.378 $\pm$ 2115.5327 BPA: 40075.422 $\pm$ 6023.7911 ( $p=0.05$ )	Oil: 30649.682 $\pm$ 5578.267 BPA: 33305.933 $\pm$ 4539.924 ( $p=0.717$ )	Oil: 61047.344 $\pm$ 12048.584 BPA: 51407.489 $\pm$ 6711.153 ( $p=0.495$ )
Time (s)	Oil: 300.158 $\pm$ 0.04 BPA: 300.138 $\pm$ 0.038 ( $p=0.722$ )	Oil: 191.422 $\pm$ 14.775 BPA: 156.247 $\pm$ 8.873 ( $p=0.058$ )*	Oil: 35.453 $\pm$ 5.911 BPA: 30.607 $\pm$ 5.213 ( $p=0.547$ )	Oil: 73.281 $\pm$ 12.224 BPA: 113.283 $\pm$ 7.809 ( $p=0.014$ )
<b>Session 4 (Social preference)</b>				
Distance traveled (cm)	Oil: 110284.94 $\pm$ 15934.653 BPA: 124198.21 $\pm$ 17361.655 ( $p=0.563$ )	Oil: 33876.808 $\pm$ 6436.024 BPA: 47583.567 $\pm$ 8235.806 ( $p=0.208$ )	Oil: 31700.611 $\pm$ 5075.1257 BPA: 33132.369 $\pm$ 5619.05 ( $p=0.852$ )	Oil: 44707.522 $\pm$ 8985.061 BPA: 43482.278 $\pm$ 7476.7306 ( $p=0.918$ )
Time (s)	Oil: 300.181 $\pm$ 0.035 BPA: 300.137 $\pm$ 0.038 ( $p=0.413$ )	Oil: 154.534 $\pm$ 19.698 BPA: 123.32 $\pm$ 14.641 ( $p=0.222$ )	Oil: 30.981 $\pm$ 4.9 BPA: 47.487 $\pm$ 13.349 ( $p=0.263$ )	Oil: 114.666 $\pm$ 16.898 BPA: 129.33 $\pm$ 12.524 ( $p=0.496$ )

\*Tendency towards significance ( $0.05 < p < 0.06$ ).

compared to the controls (cell number,  $p < 0.005$ , fractional area,  $p < 0.043$ ). Also, in the SCh we observed a significant reduction in the BPA-treated group (Figure 3A) of AVP-ir in both number of cells ( $p = 0.002$ ) (Figure 3B) and fractional area ( $p = 0.004$ ) (Figure 3C).

The analysis of both number of cells and fractional area revealed no effects of the treatment in the SON, as well as in the MeA.

### OXT-ir analysis

The analysis of the OXT-ir did not show any significant difference between groups in the analyzed nuclei (Table 3).

In particular, we did not observe any difference in the total PVN (Figure 2D), both in number of cells ( $p = 0.806$ ) (Figure 2E) and fractional area ( $p = 0.548$ ) (Figure 2F). Moreover, the further analysis of the PVN subnuclei (PaDC/PaLM, PaMP, PaMM) confirmed the absence of effects of treatment on OXT-ir: in fact, the two experimental groups also maintained the same distribution of the OXT-ir within the subnuclei, both for the number of cells (PaDC/PALM,  $p = 0.557$ , PaMP,  $p = 0.967$ , PaMM,  $p = 0.888$ ) (Figure 2E) and the fractional area (PaDC/PALM,  $p = 0.349$ , PaMP,  $p = 0.678$ , PaMM,  $p = 0.588$ ) (Figure 2F).

The treatment did not affect the OXT-ir in the SON and in the MeA (Table 3).

### GPER1-ir analysis

We performed the quantitative analysis for GPER1-ir in all analyzed nuclei (Table 3). This analysis revealed a significant effect of the treatment only in PVN and SCh, the nuclei in which we observed also significant changes in AVP-ir. In particular, we observed a significant reduction (Figure 4) in GPER1-ir in terms of fractional area both in PVN (Figure 4 A,B) ( $p < 0.001$ ) and in SCh (Figure 4C) ( $p = 0.003$ ) in the BPA-treated animals compared to the control ones.

### Discussion

The results of this study support the idea that pregnancy represents a particularly sensitive period of adult life for endocrine disruption and that the continued exposure to BPA could lead to behavioral and neuroendocrine circuits alterations not only in the offspring but also in the exposed dams. In fact, we observed some alterations in the displaying of social behavior, although BPA-treated dams did not lose the sociability skills. Interestingly BPA-treated dams demonstrated higher interactions towards no tester female and lower interactions toward the male one compared to the control dams. The analysis of two systems strongly correlated to the control of social behavior, vasopressin and oxytocin hypothal-

**Table 2.** Results obtained from the analysis of different behaviors (sniffing, grooming, rearing, escape) during the four sessions of the Three-chamber test. Data are reported as mean  $\pm$  SEM, both as frequency (F) and cumulative duration (CD). One-way ANOVA revealed a significant effect of the treatment for  $p \leq 0.05$ .

Parameter	Session 1	Session 2	Session 3	Session 4
Sniffing in left chamber (F)	Oil: 11.556 $\pm$ 2.304 BPA: 20.889 $\pm$ 3.442 ( $p = 0.039$ )	Oil: 23 $\pm$ 4.69 BPA: 19.222 $\pm$ 2.645 ( $p = 0.493$ )	Oil: 23.778 $\pm$ 2.666 BPA: 24.667 $\pm$ 1.763 ( $p = 0.784$ )	Oil: 19.444 $\pm$ 2.982 BPA: 24 $\pm$ 3.742 ( $p = 0.355$ )
Sniffing in left chamber (CD)	Oil: 14.406 $\pm$ 6.052 BPA: 24.374 $\pm$ 7.767 ( $p = 0.326$ )	Oil: 28.382 $\pm$ 9.533 BPA: 20.507 $\pm$ 4.379 ( $p = 0.464$ )	Oil: 28.139 $\pm$ 5.012 BPA: 25.218 $\pm$ 5.88 ( $p = 0.71$ )	Oil: 22.754 $\pm$ 5.103 BPA: 28.824 $\pm$ 6.332 ( $p = 0.446$ )
Sniffing in right chamber (F)	Oil: 16.444 $\pm$ 2.9 BPA: 20 $\pm$ 3.202 ( $p = 0.422$ )	Oil: 16.222 $\pm$ 3.122 BPA: 26.222 $\pm$ 3.205 ( $p = 0.04$ )	Oil: 23.667 $\pm$ 2.744 BPA: 29.778 $\pm$ 3.403 ( $p = 0.181$ )	Oil: 26.333 $\pm$ 3.693 BPA: 20.111 $\pm$ 1.495 ( $p = 0.138$ )
Sniffing in right chamber (CD)	Oil: 15.296 $\pm$ 3.934 BPA: 17.696 $\pm$ 4.013 ( $p = 0.675$ )	Oil: 15.013 $\pm$ 4.427 BPA: 28.142 $\pm$ 5.49 ( $p = 0.081$ )	Oil: 25.64 $\pm$ 4.322 BPA: 28.64 $\pm$ 4.442 ( $p = 0.635$ )	Oil: 30.39 $\pm$ 6.766 BPA: 16.886 $\pm$ 2.062 ( $p = 0.074$ )
Total sniffing (F)	Oil: 28 $\pm$ 2.724 BPA: 40.889 $\pm$ 3.203 ( $p = 0.007$ )	Oil: 39.222 $\pm$ 3.792 B PA: 45.444 $\pm$ 2.739 ( $p = 0.202$ )	Oil: 47.444 $\pm$ 2.672 BPA: 54.444 $\pm$ 3.096 ( $p = 0.089$ )	Oil: 45.778 $\pm$ 3.696 xBPA: 44.111 $\pm$ 4.185 ( $p = 0.769$ )
Total sniffing (CD)	Oil: 29.702 $\pm$ 6.396 BPA: 42.072 $\pm$ 7.248 ( $p = 0.219$ )	Oil: 43.396 $\pm$ 8.201 BPA: 48.649 $\pm$ 5.579 ( $p = 0.604$ )	Oil: 53.779 $\pm$ 4.394 BPA: 53.858 $\pm$ 5.868 ( $p = 0.992$ )	Oil: 53.144 $\pm$ 6.394 BPA: 45.710 $\pm$ 6.386 ( $p = 0.423$ )
Grooming (F)	Oil: 9.667 $\pm$ 1.616 BPA: 5.556 $\pm$ 1.573 ( $p = 0.087$ )	Oil: 11.889 $\pm$ 3.615 BPA: 22.444 $\pm$ 3.969 ( $p = 0.067$ )	Oil: 8.889 $\pm$ 1.791 BPA: 18.444 $\pm$ 1.651 ( $p = 0.001$ )	Oil: 7.111 $\pm$ 1.829 BPA: 13 $\pm$ 3.023 ( $p = 0.115$ )
Grooming (CD)	Oil: 18.911 $\pm$ 2.250 BPA: 11.991 $\pm$ 4.758 ( $p = 0.207$ )	Oil: 17.102 $\pm$ 5.858 BPA: 32.649 $\pm$ 5.845 ( $p = 0.079$ )	Oil: 9.131 $\pm$ 2.402 BPA: 20.64 $\pm$ 2.787 ( $p = 0.006$ )	Oil: 11.697 $\pm$ 2.935 BPA: 21.738 $\pm$ 9.423 ( $p = 0.324$ )
Rearing (F)	Oil: 13.889 $\pm$ 1.798 BPA: 17.667 $\pm$ 4.794 ( $p = 0.408$ )	Oil: 4.333 $\pm$ 1.75 BPA: 7.556 $\pm$ 3.72 ( $p = 0.421$ )	Oil: 10.333 $\pm$ 2.635 BPA: 11 $\pm$ 2.217 ( $p = 0.849$ )	Oil: 5.222 $\pm$ 1.176 BPA: 9.111 $\pm$ 1.448 ( $p = 0.053$ )*
Rearing (CD)	Oil: 14.74 $\pm$ 2.692 BPA: 17.908 $\pm$ 4.794 ( $p = 0.573$ )	Oil: 3.92 $\pm$ 1.75 BPA: 5.742 $\pm$ 2.678 ( $p = 0.577$ )	Oil: 9.584 $\pm$ 3.055 BPA: 8.284 $\pm$ 1.488 ( $p = 0.707$ )	Oil: 3.06 $\pm$ 0.993 BPA: 6.283 $\pm$ 1.282 ( $p = 0.064$ )

\*Tendency towards significance ( $0.05 < p < 0.06$ ).

amic systems, highlighted alterations in the AVP-ir in the hypothalamic paraventricular and supra-chiasmatic nuclei, while we did not find any alteration in the oxytocin system. In addition, the subsequent analysis of the GPER1-ir in PVN and SCh, revealed a significant reduction of the signal in the BPA-treated dams compared to the control ones.

Rodents are social animals, they not only live in groups, perceiving isolation as a great stress, but they are also engaged in a wide variety of social behaviors throughout life.<sup>18</sup> Therefore, alterations in the social skills due to EDCs exposure may have very

serious implications on the quality of life of those animals, impacting different aspects of their social lives and altering their response to other animals and to the environment.<sup>18</sup> Social behavior is a well-known target of endocrine disruption and specifically of BPA exposure, in particular during pre- and peri-natal periods, led to alterations in adult social and sociosexual behaviors in several species.<sup>18,38</sup> However, in the literature, there are few works highlighting the behavioral effects of chronic exposure to BPA during adulthood in female mice, as most of them focus on different exposure and targets, and are mainly performed in rats.<sup>39-41</sup> Our results

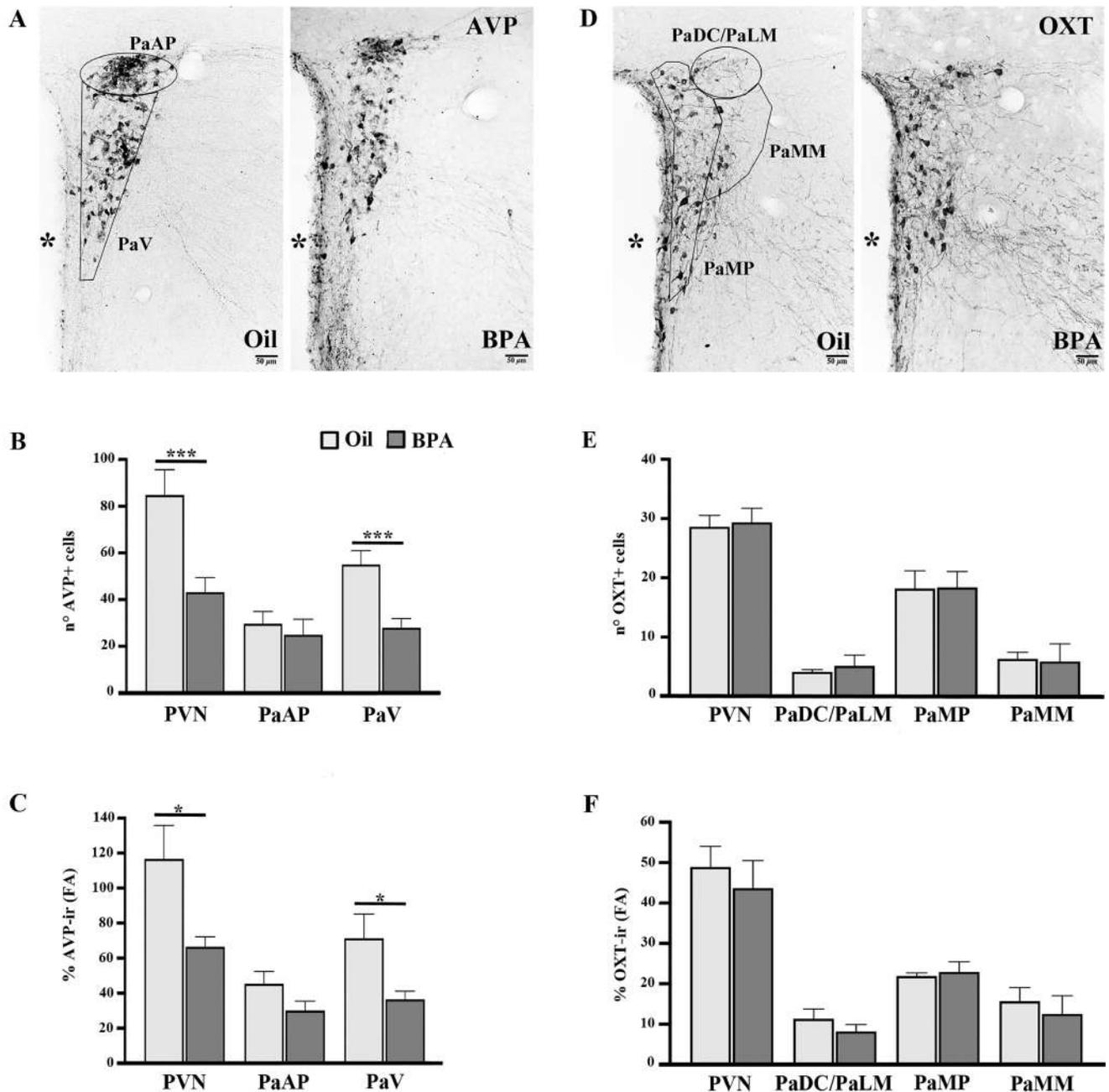
**Table 3.** Results obtained from the analysis of AVP-ir, OXT-ir and GPER1-ir in all the selected nuclei. Data are reported as mean  $\pm$  SEM, both, when possible, as number of positive cells and fractional area. One-way ANOVA revealed a significant effect of the treatment for  $p \leq 0.05$ .

Marker	Nucleus	Number of positive cells	Fractional area (%)
AVP	PVN	Oil: 84.8 $\pm$ 10.841	Oil: 116.762 $\pm$ 19.091
		BPA: 43.2 $\pm$ 6.262 (p=0.005)	BPA: 66.548 $\pm$ 5.709 (p=0.036)
	PaAP	Oil: 29.7 $\pm$ 5.178	Oil: 45.412 $\pm$ 7.021
		BPA: 25 $\pm$ 6.569 (p=0.053)*	BPA: 30.089 $\pm$ 5.368 (p=0.121)
	PAV	Oil: 55.1 $\pm$ 5.932	Oil: 71.35 $\pm$ 13.803
		BPA: 28 $\pm$ 3.943 (p=0.005)	BPA: 36.459 $\pm$ 4.653 (p=0.043)
	SCh	Oil: 72 $\pm$ 4.41	Oil: 37.293 $\pm$ 1.659
BPA: 33.2 $\pm$ 8.032 (p=0.002)		BPA: 27.607 $\pm$ 1.852 (p=0.004)	
SON	Oil: 22.286 $\pm$ 1.345	Oil: 26.462 $\pm$ 1.254	
	BPA: 21.917 $\pm$ 1.65 (p=0.864)	BPA: 25.356 $\pm$ 1.342 (p=0.828)	
MeA	-	Oil: 2.235 $\pm$ 0.246 BPA: 2.219 $\pm$ 0.372 (p=0.971)	
OXT	PVN	Oil: 28.6 $\pm$ 1.958	Oil: 48.921 $\pm$ 5.08
		BPA: 29.375 $\pm$ 2.366 (p=0.806)	BPA: 43.667 $\pm$ 6.848 (p=0.548)
	PaDC/PaLM	Oil: 4.1 $\pm$ 0.4	Oil: 11.331 $\pm$ 2.414
		BPA: 5.125 $\pm$ 1.82 (p=0.557)	BPA: 8.189 $\pm$ 1.729 (p=0.349)
	PaMM	Oil: 11.331 $\pm$ 2.414	Oil: 21.898 $\pm$ 0.781
		BPA: 8.189 $\pm$ 1.729 (p=0.349)	BPA: 22.937 $\pm$ 2.532 (p=0.678)
	PaMP	Oil: 6.3 $\pm$ 1.102	Oil: 15.692 $\pm$ 3.425
BPA: 5.875 $\pm$ 2.989 (p=0.888)		BPA: 12.541 $\pm$ 4.515 (p=0.588)	
SON	Oil: 23.7 $\pm$ 1.991	Oil: 19.903 $\pm$ 0.983	
	BPA: 20.4 $\pm$ 1.089 (p=0.184)	BPA: 20.022 $\pm$ 1.009 (p=0.935)	
MeA	-	Oil: 2.029 $\pm$ 0.358 BPA: 1.956 $\pm$ 0.316 (p=0.883)	
GPER1	PVN	-	Oil: 21.253 $\pm$ 0.489 BPA: 12.921 $\pm$ 0.489 (p<0.001)
	SCh	-	Oil: 31.735 $\pm$ 1.467 BPA: 23.139 $\pm$ 1.114 (p=0.003)
	SON	-	Oil: 20.27 $\pm$ 1.341 BPA: 19.787 $\pm$ 1.862 (p=0.84)
	MeA	-	Oil: 9.482 $\pm$ 0.647 BPA: 9.201 $\pm$ 0.248 (p=0.699)

\*Tendency towards significance (0.05<p<0.06).

support the idea that BPA exposure led to alterations in the displaying of social behavior, even when it occurs in adult life. The analysis of sex-driven social behavior through the Three-Chamber test highlighted some alterations in the BPA-treated dams. The higher distance traveled displayed by the BPA-treated dams during the

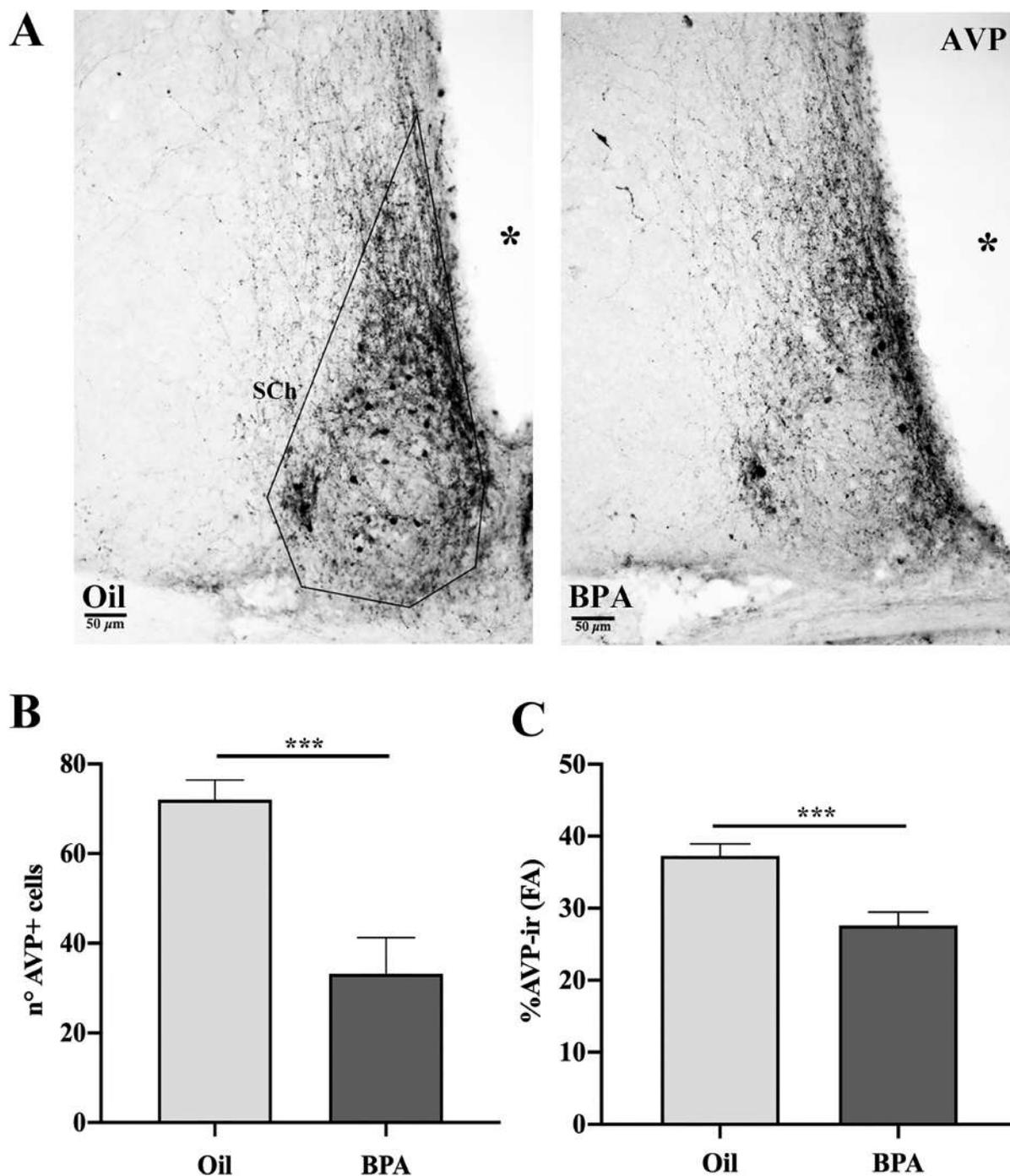
test indicates a higher locomotor activity compared to the control dams that could be due to a higher level of anxiety. In the literature, it has been demonstrated that exposure to BPA can cause alterations in the anxiety state in rodents.<sup>39</sup> In particular, BPA exposure is associated to increased anxiety-like behavior in rodents,<sup>19</sup> not



**Figure 2.** AVP-ir and OXT-ir in the PVN of oil-treated and BPA-treated dams. A) Representative image of AVP-ir in a coronal section of PVN of oil-treated (left images) or BPA-treated (right images) dams. Analysis of AVP-ir in PVN, expressed both as (B) number of AVP positive cells and (C) fractional area (FA), revealed a significant reduction in BPA-treated dams (dark gray) compared to the control ones (light gray), mainly due to the ventral component of the nucleus (PaV). D) Representative image of oxytocin signal in a coronal section of PVN of oil-treated (left images) or BPA-treated (right images) dams. Analysis of OXT-ir in PVN, expressed both as (E) number of OXT positive cells and (F) fractional area (FA), in oil-treated (light gray) and BPA-treated (dark gray) dams did not show any effect link to the treatment. Data are expressed as mean  $\pm$  SEM. One-way ANOVA revealed a significant effect of the treatment for  $p \leq 0.05$ . AVP, vasopressin; OXT, oxytocin; PVN, paraventricular nucleus; PaAP, anterior parvicellular nucleus; PaV, ventral nucleus; PaDC/PaLM, dorsal cap/lateral magnocellular part; PaMP, medial parvicellular part; PaMM, medial magnocellular part; FA, fractional area; \*third ventricle.

only when the exposure occurred during the perinatal period,<sup>42</sup> but also in adulthood.<sup>43</sup> Long-term oral exposure to BPA during adulthood is associated with alterations of anxiety-related behaviors mainly in male mice.<sup>43</sup> Interestingly, our treatment highlighted the fact that also female mice can be affected by anxiety-related behavioral changes when the chronic exposure involved the sensitive periods of pregnancy and lactation. Moreover, the two social

bility sessions showed that BPA-treated dams did not lose the sociability skills, spending in both sessions more time in the chamber with the no-tester animals. However, BPA-treated dams have shown a tendency ( $p=0.058$ ) to interact (higher sniffing behavior) more with the no-tester female compared to the controls, suggesting that they have no interest in interacting with the male. In the last session, the lack of sex-dependent social preference was more

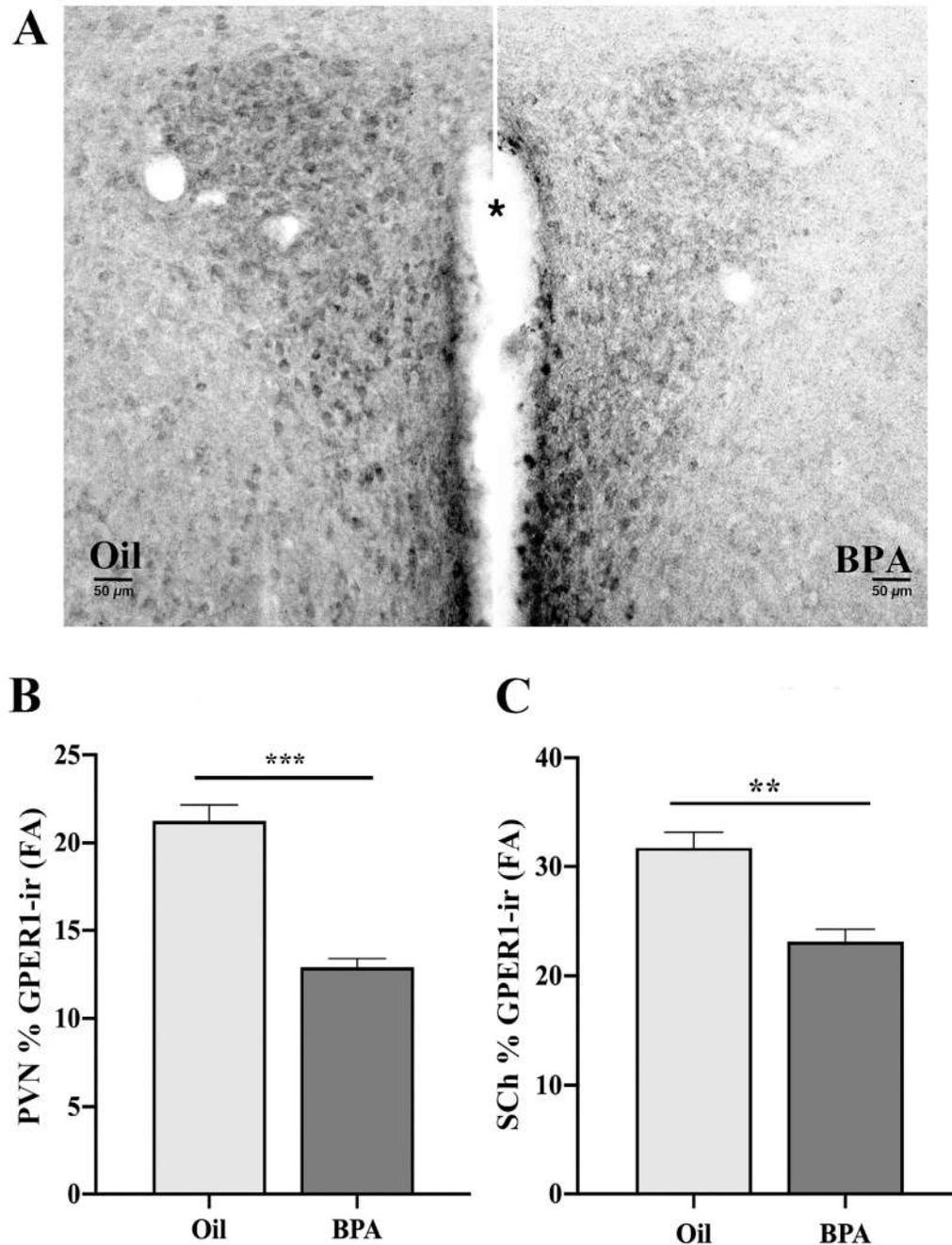


**Figure 3.** AVP-ir in the SCh of oil-treated and BPA-treated dams. A) Representative image of AVP-ir in a coronal section of SCh of oil-treated (left images) or BPA-treated (right images) dams. Analysis of AVP-ir in SCh, expressed both as (B) number of AVP positive cells and (C) fractional area (FA), revealed a significant reduction in BPA-treated dams (dark gray) compared to the control ones (light gray). Data are expressed as mean  $\pm$  SEM. One-way ANOVA revealed a significant effect of the treatment for  $p \leq 0.05$ . AVP, vasopressin; OXT, oxytocin; SCh, supra-chiasmatic nucleus; FA, fractional area; \*third ventricle.

evident in the BPA-treated group, and it seems to be even more evident towards the male no-tester mouse. Previous studies performed in rodents have demonstrated that different kinds of exposure to BPA cause different alterations in the social and socio-sexual behavior: in particular, the interactions between same-sex and opposite-sex no-tester animal seems to be differentially affected.<sup>18,19</sup> These studies suggest that the alterations in social behavior

linked to BPA exposure and alterations in mechanisms which are involved also in sexual preference and behavior could come together.<sup>2,44,45</sup> Furthermore, it is known that olfactory discrimination, which is fundamental in both social and sexual behavior, can be altered by BPA exposure.<sup>45,46</sup>

Vasopressin and oxytocin systems play a key role in the control of social behavior.<sup>13,15</sup> They have been therefore recognized as the



**Figure 4.** GPER1-ir in the PVN and SCh of oil-treated and BPA-treated dams. A) Representative images of GPER1-ir in a coronal section of PVN of oil-treated (left image) or BPA-treated (right image) dams. Analysis of GPER1-ir, expressed as fractional area (FA), revealed a significant reduction in BPA-treated dams (dark gray) compared to the control ones (light gray), both in PVN (B) and in SCh (C). Data are expressed as mean  $\pm$  SEM. One-way ANOVA revealed a significant effect of the treatment for  $p \leq 0.05$ . GPER1, G protein-coupled estrogen receptor 1; PVN, paraventricular nucleus; SCh, supra-chiasmatic nucleus; FA, fractional area; \*third ventricle.

main target systems underlying the alterations observed in social behavior after exposure to different types of EDCs;<sup>19,47</sup> nevertheless these abilities of environmental chemicals, including BPA, to alter nonapeptide signaling is poorly documented. Our analysis highlighted a decrease of AVP-ir in some of the analyzed hypothalamic nuclei of BPA-treated dams, but not for OXT-ir.

OXT plays a central role in the control of aggression, anxiety, pair and social bonding especially in females.<sup>10,48,49</sup> Nevertheless, we did not find any alterations in all the analyzed nuclei. Although the potential for BPA to disrupt the OXT-OXT receptor systems has previously been shown, the performed treatments were different from ours, in terms of dose, animal model, period of exposure and way of administration.<sup>50</sup> Moreover, the OXT system is highly dynamic, it is therefore possible that the effects of BPA could be different depending on brain region, gender and age.

We detected significant alterations of the AVP expression in the Sch and in the PVN. In fact, in both nuclei, BPA-treated dams showed a significant decrease of AVP-ir in terms of both number of cells and fractional area. Although it is not yet fully clear how exposure to BPA can directly influence the reduction in the number of cells expressing AVP in the Sch and in the PVN, it is conceivable that chronic exposure to BPA induces a chronic, direct or indirect, modulation of the AVP system. BPA is a xenoestrogen and thanks to its structure can pass the blood brain barrier and bind estrogen receptors exerting multiple effects.<sup>51</sup> AVP positive cells located in the Sch and in the PVN express estrogen receptors.<sup>52,53</sup> *In vitro* studies showed that estradiol, acting through ER $\beta$  and GPER1, induces a downregulation of AVP expression.<sup>54-56</sup> Thus, BPA can possibly mediate a direct downregulation of AVP expression through this pathway. Furthermore, the ability of BPA to alter estrous cycle is well known in the literature.<sup>57,58</sup> In fact, we also have observed a significant increase in the time spent in estrus phase in our BPA-treated females. Longer time spent in estrus, together with altered level of circulating estradiol, could be partially responsible to indirect modulation of BPA through estrogen receptor on AVP expression in Sch and PVN.

The Sch is involved in the regulation and maintenance of circadian rhythms.<sup>59</sup> In the literature, numerous studies show how prolonged exposure to BPA affects the homeostasis of this system.<sup>60</sup> Levels of circulating sex steroid hormones are responsible to the modulation of circadian rhythms and particularly of circadian locomotor rhythms and estrus phase is associated with higher locomotor activity in rodents.<sup>61</sup> Therefore, the persistence of estrus could be responsible of the higher distance traveled by our BPA-treated mice. Nevertheless, alterations in locomotor activity are considered as marker of altered stress response.<sup>62</sup> Stress responses are integrated and regulated at PVN level involving AVP and corticotropin-releasing hormone (CRH) neurons.<sup>63</sup> In physiological conditions, following exposure to stress stimuli, there is an increase in the AVP mRNA levels in the PVN with consequent excitation of the entire hypothalamic-pituitary axis (HPA).<sup>64</sup> Chronic exposures to different type of stress stimuli, including exposure to BPA, alter the homeostasis of the stress axis by influencing its inactivation, for example through changes in the stability of the AVP mRNA, causing its anticipated degradation and an incorrect signaling mechanism.<sup>65</sup>

In rodents, GPER1 is strongly expressed in PVN and Sch,<sup>66</sup> with a high co-expression with vasopressin neurons,<sup>56,66</sup> and is involved in the control of a variety of behaviors, including the social one.<sup>67,68</sup> Interestingly, it is known that BPA displays low affinity for ER $\beta$  and high affinity for GPER1.<sup>69</sup> The analysis within these nuclei, which displayed significant AVP-ir alteration, highlighted significant effect also on GPER1-ir. In fact, it shown a significant reduction in GPER1-ir in BPA-treated dams compared to the control ones. Although GPER1 seems to be involved in rapid

change of both AVP-ir and social behavior,<sup>56,67</sup> in our study we showed effects of long-term exposure to BPA on its expression. This response can be due to down-regulation mechanisms, which often follow the desensitization of the receptor caused by repeated or chronic administrations.<sup>70</sup> GPER1 could be involved at first in the down regulation of AVP-ir and then could go through down-regulation mechanisms itself.

In conclusion, our results support the idea that pregnancy represents a critical period in adulthood for endocrine disruption. In fact, the exposure to BPA may pose a risk even in adulthood (given the long-term exposure period, the persistence of these compounds in the environment and the ability of bisphenols to accumulate in certain compartments of the body). In particular, we showed that chronic exposure to low-dose BPA in adult female mice led to long-term alterations in both social behavior and a decrease of the vasopressin system in PVN and Sch, along with decreased expression of GPER1 within the same nuclei. These findings could be explained as BPA direct and indirect effects at central level, which could, finally, be partially linked to alterations in the behavioral outcome.

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