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Bisphenol A and bisphenol S: environmental risk factors in health and disease

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In nature nothing exists alone.

INDEX

+ = positive
5-HT = serotonin
AGD = anogenital distance
ANOVA = analysis of variance
AR = androgen receptor
AREs = androgen response elements
Arc = arcuate nucleus
AVP = vasopressin
BPA = bisphenol A
BPs = bisphenols
BPS = bisphenol S
BS = balano-preputial separation
BST = bed nucleus of stria terminalis
BW = body weight
CBIs = coactivator-binding inhibitors
CD = cumulative duration
CNS = central nervous system
CRH = corticotropin-releasing hormone
CS = clinical score
DBD = DNA-binding domain
DR = dorsal raphe nucleus
DRD = dorsal region of dorsal raphe
DRV = ventral region of dorsal raphe
EAE = experimental autoimmune encephalomyelitis
EDCs = endocrine disrupting chemicals
EDSTAC = Endocrine Disruptor Screening and Testing Advisory Committee
EFSA = European Food Safety Authority
EPA = Environmental Protection Agency
EPM = elevated plus maze
ERα = estrogen receptor α
ERβ = estrogen receptor β
EREs = estrogen response elements
ERRα = estrogen-related receptor α
ERRγ = estrogen-related receptor γ
ERs = nuclear estrogen receptors
FA = fractional area
FAO = Food and Agriculture Organization of the United Nations
FDA = Food and Drug Administration
FI = food intake
GnRH = gonadotropin-releasing hormone
GD = gestational day
GPER1 = G-protein coupled estrogen membrane receptor
GR = glucocorticoid receptor

GREs = glucocorticoid response elements
HPA = hypothalamic-pituitary-axis
HPG = hypothalamic-pituitary-gonadal axis
ir = immunoreactivity
kiss = kisspeptin
kiss1r = kisspeptin receptor 1
LBD = COOH-terminal ligand-binding domain
MeA = medial amygdala
MePOA = medial preoptic area
mERs = membrane estrogen receptors
MnR = median raphe nucleus
MDCs = metabolism disrupting chemicals
MNs = motoneurons
MOG = myelin oligodendrocyte glycoprotein
MS = multiple sclerosis
NOAEL = no observed adverse effect level
NTD = NH_2-terminal domain
OF = open field
OXT = oxytocin
PaAP = anterior parvicellular subnucleus
PaDC/PaLM = dorsal cap/lateral magnocellular subnucleus
PaMM = the medial magnocellular subnucleus
PaMP = medial parvocellular subnucleus
PaV = ventral parvocellular subnucleus
PBS = phosphate buffer
PFA = paraformaldehyde
PND = postnatal day
POA = preoptic area
POPs = persistent organic pollutants
PPARγ = Peroxisome proliferator-activated receptor γ
PPREs = Peroxisome proliferator-activated receptor response elements
PR = progesterone receptor
PvIIs = perivascular inflammatory infiltrates
PVN = paraventricular nucleus of the hypothalamus
ROI = region of interest
RP3V = rostral periventricular area of the third ventricle
RXR = retinoid X receptor
SCh = suprachiasmatic nucleus
SEM = mean standard error
SERMs = selective estrogen receptor modulators
SML = specific migration limit
SON = supraoptic nucleus
TDI = tolerable daily intake
TH = thyroid hormone
THRs = thyroid hormone receptors
TPH = tryptophan hydroxylase

v = mean velocity
VO = vaginal opening
WHO = World Health Organization

GENERAL INTRODUCTION

1. Endocrine Disrupting Chemicals (EDCs)

Environmental concerns about the massive use of pollutants reached a wake-up call in early '60, when Rachel Carson firstly highlighted the terrible effects of pesticides indiscriminately used in American agriculture (Carson, 1962). However, the concept of "Endocrine Disruptor" was introduced only thirty years later. In 1991, Theo Colborn and colleagues during a workshop drafted the Wingspread Statement summarizing the effects of these compounds on human and environmental health (Colborn et al., 1992). Finally, in 1996, the USA Environmental Protection Agency (EPA) formally introduced the endocrine disruptors as "exogenous agents that interfere with the synthesis, secretion, transport, binding, action, or elimination of natural hormones in the body that are responsible for the maintenance of homeostasis, reproduction, development and/or behavior" (EDSTAC, 1998). More recently, the Endocrine Disrupting Chemicals (EDCs) have been defined by the Endocrine Society as "exogenous chemicals, or mixture of chemicals, that interfere with any aspect of hormone action and cause adverse effects at the level of the organism, its progeny, populations, or subpopulations of organisms" (Zoeller et al., 2012).

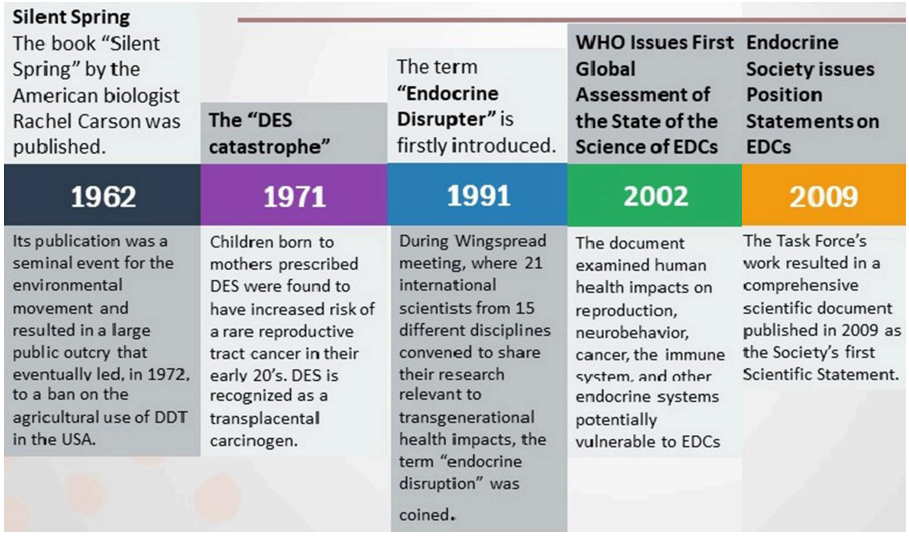

Figure 1. *Historical landmarks in EDCs research (adapted from Papalou et al., 2019).*

In 2020, an Expert Consensus Statement (La Merrill et al., 2020) identified ten key characteristics of EDCs (*Fig. 2*) which aimed to help recognition, classification, and hazard identification of these compounds:

1. Interaction with or activation of hormone receptors.
2. Antagonizes hormone receptors.
3. Altering hormone receptor expression.
4. Altering signal transduction in hormone-responsive cells.
5. Inducing epigenetic modifications in hormone-producing or hormone-responsive cells.
6. Altering hormone synthesis.

7. Altering hormone transport across cell membranes.
8. Altering hormone distribution or circulating levels of hormones.
9. Altering hormone metabolism or clearance.
10. Altering the fate of hormone-producing or hormone-responsive cells (La Merrill et al., 2020).

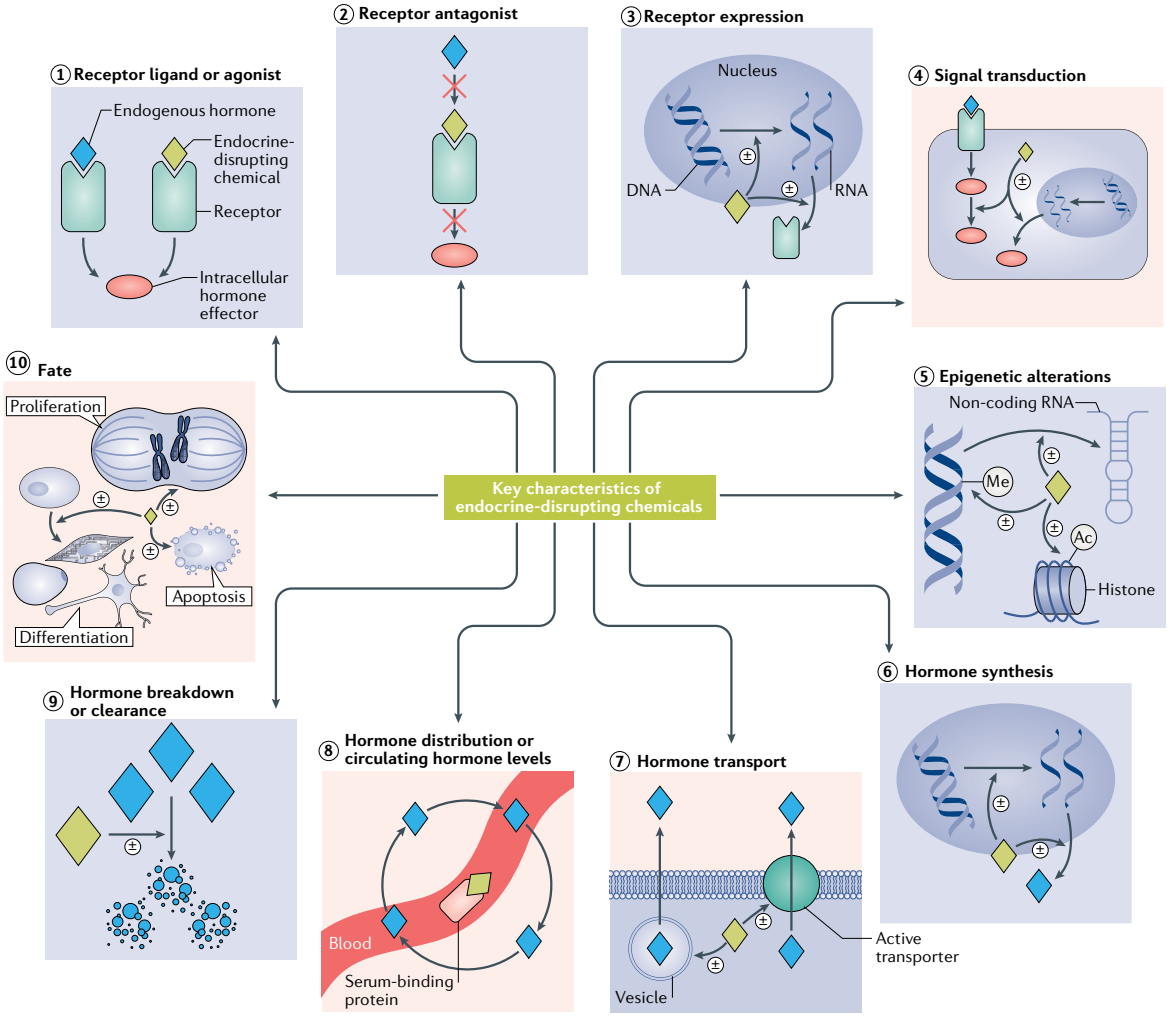

***Figure 2**. Key characteristics of EDCs (from La Merrill et al, 2020).*

At present, thousands of chemicals, some banned and some still in use, have been classified as EDCs. They represent a heterogeneous class of compounds of both natural and synthetic origin (Frye et al., 2012; Gore et al., 2015; Papalou et al., 2019). Natural EDCs are generally plant-derived dietary compounds (usually called phytoestrogens), which can be found in lots of foods, especially in leguminous plants (*e.g.*, soy) (Rietjens et al., 2017), or fungi-produced compounds (*i.e.*, mycoestrogens) (Jarosova et al., 2015). Also, some heavy metals, such as mercury, arsenic, or cadmium, have been classified as natural EDCs (Vuong et al., 2020).

Synthetic EDCs can be found in a wide variety of products, such as pesticides, food packaging, personal care products, detergents, household goods, fabrics, upholstery, electronics, and medical equipment (Gore et al., 2015; Kassotis et al., 2020). From these products they can contaminate food (Mantovani, 2016), water (Gonsioroski et al., 2020), soil (Ying & Kookana, 2005), and ambient air (Rudel & Perovich, 2009).

"Critical periods" of development, such as intrauterine, perinatal or puberty periods, when organisms are particularly sensitive to alterations of the hormonal environment, represent a peculiar time window during which exposure to EDCs are extremely dangerous (Frye et al., 2012). However, exposure during adulthood has been linked to worrying alterations (Frye et al., 2012; Rattan et al., 2017).

The mechanisms of action through which EDCs can exert their deleterious effects on the organisms have been partially unrevealed, and generally count on the involvement of one or more hormone receptors (Wuttke et al., 2010; Yilmaz et al., 2020). However, it is now known that also some non-endocrine mediated mechanisms contribute to the adverse effects displayed by EDCs exposure (Marty et al., 2018; Toporova & Balaguer, 2020).

Despite their increasing presence and persistence into the environment (Encarnacao et al., 2019) and the not insignificant evidence of their health adverse effects (Kahn et al., 2020; Yilmaz et al., 2020), EDCs have not been clearly codified into regulations as a hazard category yet (Kassotis et al., 2020).

2. Bisphenols (BPs)

Bisphenols (BPs) are organic synthetic compounds characterized by the presence of two phenols connected by an alkyl group, mainly used to produce polycarbonate plastics (Catenza et al., 2021). They represent an extremely abundant class of synthetic EDCs, as they are present in plastic-based consumer goods (Catenza et al., 2021). The concerns on BPs do not only regard their massive presence among a long list of products, but also their widespread and persistence into the environment (Catenza et al., 2021).
BPs are not classified as persistent organic pollutants (POPs, *i.e.*, carbon-based organic chemicals that are persistent, bioaccumulative and have long-range transport potential) (Guo et al., 2019), due to their low tendency to bioaccumulate in the body, as they are usually eliminated in urine in less than 24h for most (Collet et al., 2015; Thayer et al., 2015). However, they are cause of concerned because of the extensive exposure to them on a daily basis due to their wide use (Yilmaz et al., 2020).

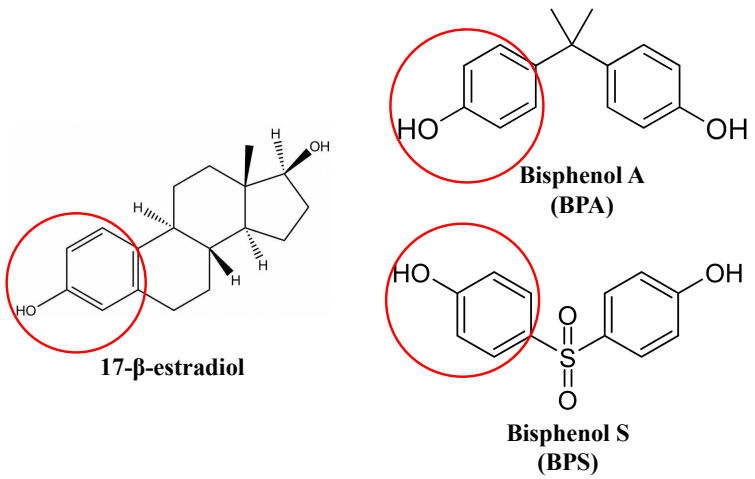

Figure 3. *Chemical structures of 17-β-estradiol, bisphenol A and bisphenol S. Red circles highlight the presence of the phenolic group, common to all the compounds.*

3. Bisphenol A (BPA)

The first and still the most globally produced BP is the bisphenol A (2,2-bis [4-hydroxyphenol] propane; BPA), obtained by condensation of phenol and acetone (Abraham & Chakraborty, 2020; Catenza et al., 2021). It has been synthetized in 1891 and starting from the Fifties it has been largely used for the production of polycarbonate plastics, epoxy resins, other polymers and thermal papers (Bousoumah et al., 2021; Catenza et al., 2021). It has been estimated that the worldwide consumption amounts up to 8 million metric tons and it is and is expected to overtake the 10 million metric tons by 2022 (Abraham & Chakraborty, 2020; Lehmler et al., 2018).

3.1 Structure and mechanisms of action

Thanks to the presence of two phenolic groups, its structure is similar to the one of the estradiol (*Fig.3*) (Murata & Kang, 2018): in fact, BPA has been initially proposed as synthetic estrogen for clinical use (Vogel, 2009) and firstly classified as xenoestrogen (*i.e.*, any exogenous natural or synthetic which mimics the effects of estrogens or promotes their production) (Wang et al., 2021).
Even if, the most known BPA mechanism of action (*Fig.4*) was through nuclear estrogen receptors (ERs) (Baker & Lathe, 2018), current knowledge supports the idea that BPA could lead to either receptor-mediated or non-receptor mediated effects (MacKay & Abizaid, 2018; Sonavane & Gassman, 2019).

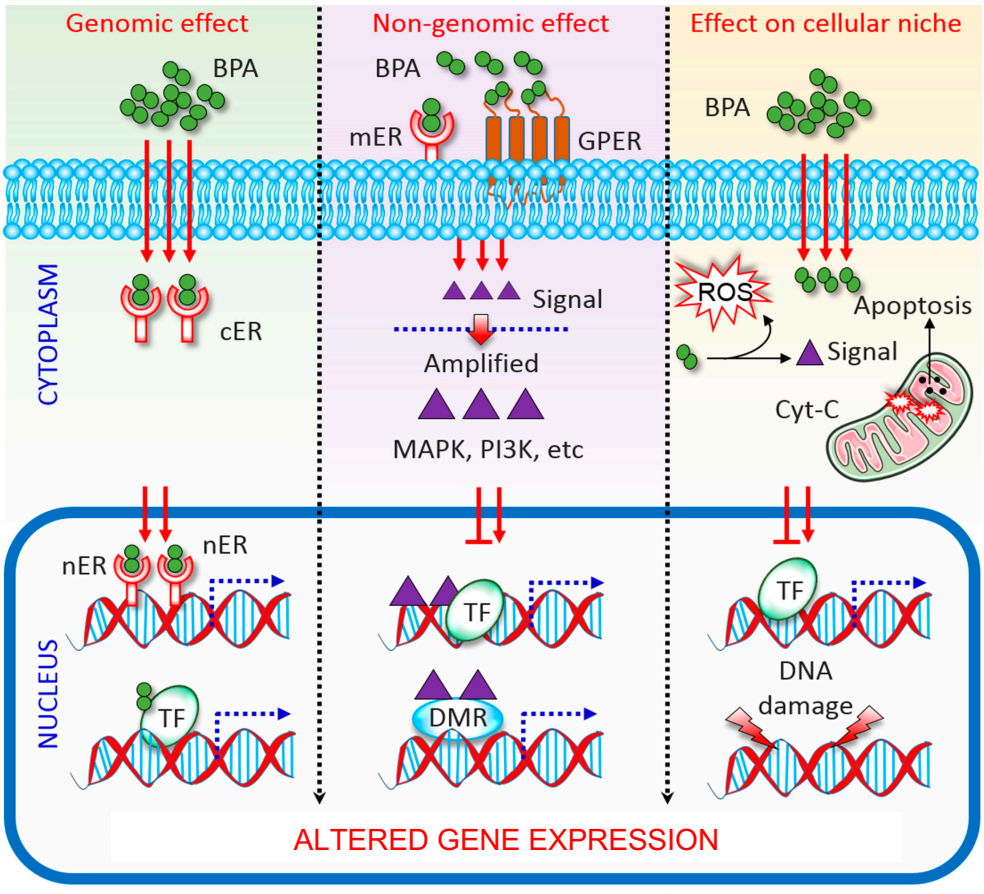

Figure 4. *Mechanisms of BPA activity (adapted from Amjad et al., 2020).*

3.1.1 Receptor-mediated mechanisms of action

BPA can bind a wide set of hormone receptors, both nuclear and membrane bound (MacKay & Abizaid, 2018; Murata & Kang, 2018; Yilmaz et al., 2020). The increasing number of targeted receptors allows to better explain some unclear aspects of BPA pharmacology, including its low-dose effects, non-monotonic dose-response curve, and estrogen-independent observed effects (MacKay & Abizaid, 2018; Sonavane & Gassman, 2019). Among the targeted receptors, here we reported the most studied ones.

Estrogen receptor α (ERα): it was the first described ER, expressed especially in reproductive tissues, breast, kidney, bone, white adipose tissue, and liver (Jia et al., 2015).
In general, ERs count three functional domains: the NH_2-terminal domain (NTD), the DNA-binding domain (DBD), and the COOH-terminal ligand-binding domain (LBD). Within the NTD, there is the ligand-independent activation function (AF1) domain involved in transcriptional activation of target genes, while in the LBD there is the ligand-dependent activation (AF2) (Jia et al., 2015). Particularly, three ERα isoforms (ERαΔ3, ERα36 and ERα46) have been identified, produced by the alternative splicing mechanism (Jia et al., 2015). Once the ligand-receptor bound occurs, ERs dimerize, thanks to a region present in the DBD, and bind specific DNA sequences called estrogen response elements (EREs) (Fuentes & Silveyra, 2019; Jia et al., 2015). However, it is now known that more than one third of human genes regulated by ERs do not contain EREs (O'Lone et al., 2004), and that they could mediate not only genomic but also non-genomic effects (Fuentes & Silveyra, 2019). BPA displays weak estrogenic properties, due to its binding to ERs hundred times weaker than 17-β-estradiol (Leonel et al., 2020).
The BPA-ERα bound involves 42 van der Waals interaction and depends on hydrogen binding between BPA's dual phenol rings and a series of three polar residues buried within the ligand binding domain (LBD) of the receptor (Delfosse et al., 2012). BPA can act on ERα in an agonistic and antagonistic manner (MacKay & Abizaid, 2018). Interestingly, it has been demonstrated that BPA can acts on ERα as SERM (selective estrogen receptor modulator), whose activity mainly involved the AF-1 domain (the ligand-independent transcriptional activation function domain) and depends on the cellular context: this could partially explain the antagonistic effects on ERα (Delfosse et al., 2012).
BPA bound to ERα activates numerous cell signaling pathways, including the ones linked to cell growth, migration and proliferation, but also to invasion, apoptosis and drug-resistance (Jia et al., 2015).

Estrogen receptor β (ERβ): this receptor was discovered ten years later than ERα, and it is expressed especially in the ovary and in male reproductive organs, in the central nervous system (CNS), in the cardiovascular system, in the immune system, in lungs, prostate, colon and kidneys (Jia et al., 2015). The DBD is highly (97%) conserved between ERα and ERβ, while the LBD shows only a 59% amino acid sequence identity, which, however, does not entail any significant difference in the structure of the ligand-binding pocket (Jia et al., 2015). Interestingly, the AF-1 domain displays only 16% of similarity between the two ERs (Jia et al., 2015).

BPA can bind ERα with higher affinity than ERβ (Iwamoto et al., 2021), on which it was firstly described to act only as agonist (Hiroi et al., 1999). However, it has been demonstrated that BPA can act on ERβ also as antagonist (Acconcia et al., 2015), and, more interestingly, as coactivator-binding inhibitor (CBI) (Iwamoto et al., 2021).
BPA bound to ERβ activates numerous cell signaling pathways that generally lead to opposite effects compared to ERα activation (Murata & Kang, 2018).
Indeed, the *ratio* between the two ERs is fundamental to ensure correct cellular functions (Fuentes & Silveyra, 2019), and BPA is known to unbalance their expression, usually increasing that ERα of and decreasing those of ERβ (MacKay & Abizaid, 2018; Murata & Kang, 2018).

Estrogen-related receptor γ (ERRγ): ERRγ is a more recently described orphan nuclear receptor (Hong et al., 1999), which is expressed very early during the development in both mouse (Hong et al., 1999) and humans (Heard et al., 2000) and is still expressed in adulthood especially in endocrine- and metabolic-relevant tissues (Misra et al., 2017). ERRγ does not bind estrogens and is a major player in the control of cellular energy metabolism (Toporova & Balaguer, 2020). Due to the conformation of its LBD, ERRγ is constitutively active, even in the absence of a ligand (Misra et al., 2017).
BPA seems to display good affinity to ERRγ (MacKay & Abizaid, 2018), and it has been proposed as a key mediator of low-dose effects (Toporova & Balaguer, 2020).

G-protein coupled estrogen receptor 1 (GPER-1): GPER-1 was firstly described as an orphan receptor, and it has then been demonstrated to bind estrogens (Filardo et al., 2000), even if with lower affinity compared to classic ERs, and especially ERα (MacKay & Abizaid, 2018). It is a mostly ubiquitous 7-transmembrane receptor (Thomas & Dong, 2006), coupled with G proteins that mediate its down-stream effects (Revankar et al., 2005). In particular, the induced rapid non-genomic effects include the increase of adenylyl cyclase activity, the mobilization of intracellular Ca2+ and the activation of the PI3K and MAPK/ERK signaling pathways. Through the activation of these pathways, it can indirectly also mediate some genomic effects which partially overlap with the ones activated by ERs (MacKay & Abizaid, 2018).
BPA displays higher affinity to GPER-1 compared to both classic ERs (MacKay & Abizaid, 2018), and, in particular, it has been demonstrated that BPA binds GPER-1 with 8 to 50 times greater affinity compared to ERα (Thomas & Dong, 2006). BPA seems to act most as an agonist on GPER-1, activating the adenylyl cyclase and the MAP-kinase activity (Dong et al., 2011; Thomas & Dong, 2006).

Androgen receptor (AR): more recently, AR has been recognized as a BPA target, even if BPA displays lower affinity to AR compared to estrogens receptors (MacKay & Abizaid, 2018). AR is a nuclear receptor which, after being bound to the ligand, forms homodimers and translocates to the nucleus, where it binds the androgen response elements (AREs) (Tan et al., 2015).
Most of available studies are performed *in vitro* and described an antagonistic effect of BPA on AR (Fang et al., 2003; Perera et al., 2017; Sun et al., 2006). However, more recent *in vivo* evidence suggested also a potential weak agonistic effect of BPA (Molina-Molina et al., 2013), which seems to be tissue-dependent (Kinch et al., 2015).

Progesterone receptor (PR): progesterone signaling through PR is fundamental in a lot of reproductive events (*e.g.*, establishment and maintenance of pregnancy, ovarian function, alveolar development in the mammary gland, sexual behavior) (Grimm et al., 2016; Taraborrelli, 2015). The ability of BPA of binding PR was well described in a 2015 study, through a molecular docking approach (Rehan et al., 2015). The relationship between BPA and PR is not totally clear, however, BPA seems to act mainly as an antagonist (Li et al., 2010).

Peroxisome proliferator-activated receptor γ (PPAR γ): PPARγ is nuclear receptors, which thus exerts mainly genomic effects (Janani & Ranjitha Kumari, 2015). After the binding to an agonist, it translocates to the nucleus, dimerizes with Retinoid X Receptor (RXR) and bind the PPAR response elements (PPREs) (Janani & Ranjitha Kumari, 2015). Among all PPARs, PPARγ seems to be the most susceptible to BPA, and in general EDCs, adverse effects (MacKay & Abizaid, 2018). BPA acts as an agonist on PPARγ, mediating metabolic alterations especially in the adipose tissue (Hoepner, 2019). *In silico* analysis suggests that BPA displays lower affinity for PPARγ compared to estrogen receptors, but, interestingly, its affinity for RXR seems to be quite similar to the one of ERα (Montes-Grajales & Olivero-Verbel, 2013). As RXR is necessary for PPARγ action, it may represent a new promising BPA-sensitive receptor which could unveiled some still unclear BPA effects (MacKay & Abizaid, 2018).

Glucocorticoid receptors (GR): as glucocorticoids regulated a wide range of cell functions, the GR nuclear receptor is practically ubiquitous, exerting their genomic effects binding to the glucocorticoid response elements (GREs) (Nicolaides et al., 2000). *In silico* and *in vitro* evidence suggest that BPA should act on GR, directly and indirectly, mediating mainly agonistic effects (Zhang et al., 2017). Considered the lack of data, the *in vivo* situation still remains quite unclear (MacKay & Abizaid, 2018).

Thyroid hormone receptors (THRs): the thyroid hormones play a key role in the control of a lot of fundamental functions, such as growth, development, and energy expenditure (Ortiga-Carvalho et al., 2014). THRs are produced in two splicing isoforms, α and β (Ortiga-Carvalho et al., 2014). It has been demonstrated that BPA can act on both as an antagonist, displaying higher affinity for the β isoform (Kim & Park, 2019). Even if BPA can interfere also with thyroid hormones synthesis, metabolism and transporter, current evidence suggests that the main mechanism of action in that receptor-mediated (Kim & Park, 2019).

3.1.2 Non-receptor-mediated mechanisms of action

Current literature supports the idea that BPA, as most of other EDCs, acts mainly in a receptor-dependent manner (MacKay & Abizaid, 2018; Toporova & Balaguer, 2020), mediating either genomic or non-genomic effects (MacKay & Abizaid, 2018). Even if distinguishing between receptor-mediated and non-receptor mediated mechanisms is quite arduous, considering the strict interplay between the different pathways (Ma et al., 2019), some receptor-independent effects of BPA have been described.
Receptor-independent effects of BPA are strictly related to cell cytotoxicity and oxidative stress (Amjad et al., 2020; Kose et al., 2020; Ma et al., 2019). In fact, BPA can interfere with the activity of different enzymes, especially affecting mitochondrial functions and decrease the activity of antioxidant enzymes (Ma et al., 2019), causing the increased production of reactive

oxygen species (ROS) (Gassman, 2017). *In vitro* evidence also proposed BPA as genotoxic (Kose et al., 2020). In fact, the generation of ROS, but also of phenoxyl radicals due to BPA metabolism, can lead to the damage of the DNA (Kose et al., 2020; Ma et al., 2019). Those mechanisms appear to be particularly worrying and suggest the need to reconsider the BPA classification among the carcinogen compound class (Jalal et al., 2018).

3.2 Replacing BPA: regulations and substitutes

As the impact of BPA in the organism became clearer (Abraham & Chakraborty, 2020), some international bodies (*e.g.*, EFSA, FAO/WHO, U.S. FDA) has extensively characterized its toxicity, drafting different risk assessments.
In particular, the 2015 EFSA BPA risk assessment (EFSA, 2015) established a Tolerable Daily Intake (TDI) of 4µg/kg body weight/day. This TDI is more than ten times lower compared to the previous one (50µg/kg body weight/day), set in 2006 (EFSA, 2006), and is as well temporary (EFSA, 2015).
In fact, the EFSA is committed to reevaluating the available literature concerning the effects of BPA in order to establish if the set TDI is consistent with the current data or if it is necessary to reassess it. Furthermore, to protect against exposure especially during neonatal life, BPA has already been banned from some specific products, such as the infant feeding bottles, in 2011 (European Commision, 2011). Finally, in 2016, BPA has been classified by the European Commission as reproductive toxicant cat.1B ("*presumed human reproductive toxicant*") (European Commision, 2016).

Stricter regulations together with the increasing concerns about the impact of BPA on human health (Abraham & Chakraborty, 2020), have led to extensive search for safe alternatives (Catenza et al., 2021).
However, to date, the main proposed substitutes are BPA analogues, and recent evidence suggest that they display the same, or even worse, endocrine disrupting properties as BPA (Catenza et al., 2021; den Braver-Sewradj et al., 2020).

4. Bisphenol S (BPS)

Bisphenol S (2,2-bis [4-hydroxyphenol] sulfone; BPS), obtained by condensation of phenol and sulfur trioxide, is, to date, one of the most famous proposed BPA substitutes (Catenza et al., 2021; den Braver-Sewradj et al., 2020). In fact, BPS was thought to leach fewer monomers into food and drink (Kuruto-Niwa et al., 2005) and thus it has been massively used in the production of the so-called "BPA-free" consumer goods (Bittner et al., 2014; Catenza et al., 2021; Thoene et al., 2020). However, BPS is more resistant to biodegradation and so it tends to accumulate and persist in the environment more easily compared to BPA (Qiu, Zhan, et al., 2019; Wu et al., 2018).
Considered its extensive use (Abraham & Chakraborty, 2020; Catenza et al., 2021), BPS has already been detected in the environment (*e.g.*, sediment, water, soil, indoor dust) (Catenza et al., 2021) and also in human samples (Bousoumah et al., 2021; Liao et al., 2012; H. Wang et al., 2020). Even if BPS is more heat- and photo-resistant than BPA (Kuruto-Niwa et al., 2005), it is now evident that these properties are not enough to contain the spread of the compound

among worldwide human population (Bousoumah et al., 2021; Liao et al., 2012; H. Wang et al., 2020).

Despite this, the use of BPS in not formally regulated. In his latest report, in April 2020, the EFSA declared that available data support a No Observed Adverse Effect Level (NOAEL) of 20 mg BPS/kg BW/day for developmental toxicity and developmental immunotoxicity, which, however, do not affect the current Specific Migration Limit (SML) of 0.05 mg/kg food (EFSA et al., 2020). Therefore, EFSA concluded that there is no need of further limitations, but, as the report did not take into consideration all the available toxicological dataset, it committed to re-evaluate BPS hazard in light of more complete evidence (EFSA et al., 2020). Furthermore, there is an ongoing proposal for classification of BPS as Reproductive Toxicant Cat.1B, for its adverse effects on development, sexual functions, and fertility (FPS, 2019).

4.1 Structure and mechanisms of action

BPS molecule presents two phenolic groups: thus, it shares the same similarities of BPA to estradiol's structure (*Fig.3*) (Rochester & Bolden, 2015).
Available data suggest that metabolism, potencies, and mechanisms of action of BPS are worryingly similar to the one of BPA, supporting the idea that the two compounds might display similar potential health hazards (Catenza et al., 2021; Rochester & Bolden, 2015).

As the presence of the two phenolic group give BPS the ability to bind to ERs, those receptors have been considered among the first mediators of BPS actions. However, as for BPA, it has been demonstrated that BPS can bound a wide set of receptors activating numerous cell signaling pathways (*Fig.6*) (Naderi & Kwong, 2020).

4.1.1 BPA and BPS: receptors in comparison

BPS has been demonstrated to act as an agonist on both ERα and ERβ (Naderi & Kwong, 2020), showing different affinity compared to BPA possibly due to different recruitment of coregulators (Li et al., 2018).
BPS displays lower affinity to ERα compared to BPA, but it was shown *in vitro* that BPS would act more specifically on ERα showing lower effects on ERβ (Kojima et al., 2019; Li et al., 2018). These data have not been confirmed by other *in vitro* and, especially, *in vivo* studies, that showed, conversely, that BPS displays higher affinity to ERβ compared to BPA (Le Fol et al., 2017; Marroqui et al., 2021; Molina-Molina et al., 2013).
There is also some evidence of BPS' activity on ERRs: in fact, it has been shown that it can activate and up-regulate ERRα (Jia et al., 2018) and down-regulate ERRγ (Helies-Toussaint et al., 2014). Furthermore, BPS can also act as an agonist on GPER-1 and on membrane ERs (mERs), leading to ERK and JKN phosphorylation (Vinas & Watson, 2013). On the AR, BPS acts as a weak agonist but, as opposed to BPA, does not display any antagonistic effects (Kojima et al., 2019; Molina-Molina et al., 2013).
The BPS activity on GRs is only partially understood. Previous evidence of lack of BPS's influence on GRs' activity (Roelofs et al., 2015) have been now rebutted by suggested weak antagonistic effects (Zenata et al., 2017).

BPS has been shown to have similar agonistic activity of BPA on PPARγ, not only in the adipocytes (Ahmed & Atlas, 2016; Boucher et al., 2016) but also in the macrophages (Gao et al., 2020) and in the liver (Qiu, Yang, et al., 2019).
The TH signaling appears to be a preferential target for the BPS neuroendocrine effects (Naderi & Kwong, 2020). In fact, it has been shown, by *in vitro* and *in vivo* studies, that BPS can interfere with TH homeostasis interacting with THRs (especially THRβ) (Lu et al., 2018) or with TH transporters (Zhang et al., 2016), but also altering the activity of the thyroid transcription factors or inducing changes in TH synthesis (Naderi & Kwong, 2020). On THRs, BPS displays either agonistic or antagonistic effects (Skledar et al., 2016; Zhang et al., 2018).

As for BPA, also for BPS some receptor-independent effects, mainly related to cell cytotoxicity and oxidative stress have been described (Naderi & Kwong, 2020; Pelch et al., 2019; Qiu, Zhan, et al., 2019): BPS exposure enhances oxidative stress (promoting the production of ROS, the lipid peroxidation and reducing the antioxidant activity), increases apoptosis, decreases cell viability, and alters enzymatic activity. The increase in oxidative stress caused by BPS has been linked, as for BPA, to DNA damage.

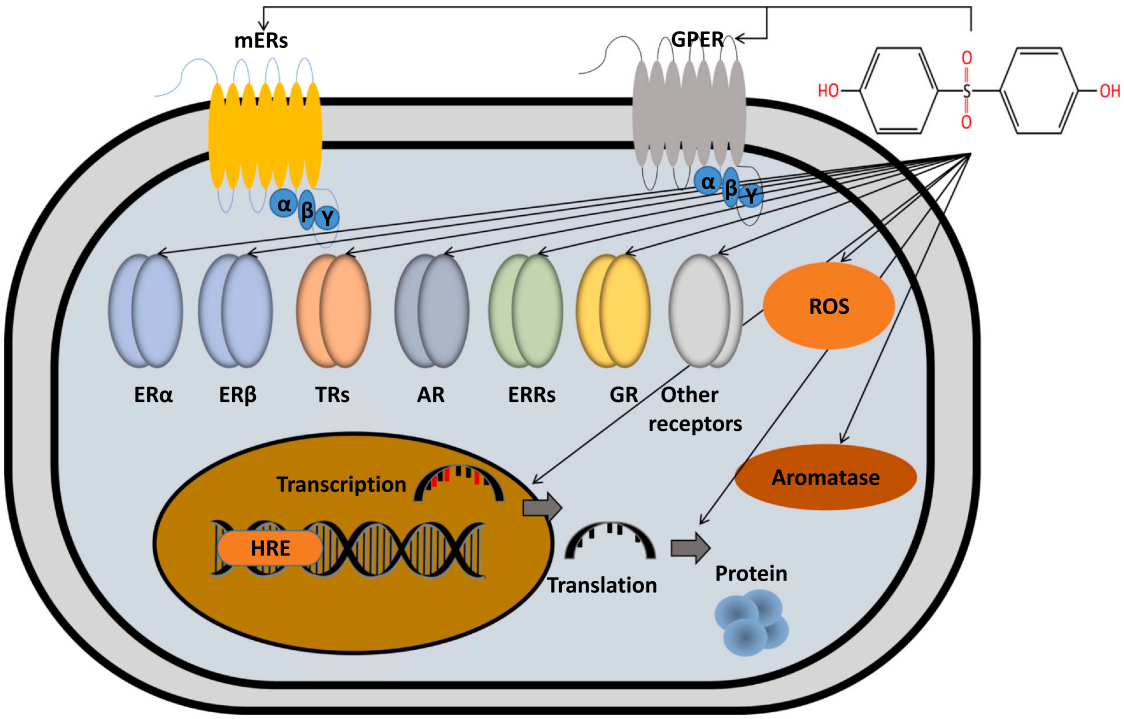

Figure 5. *Schematic representation of BPS mechanisms of action (Naderi and Kwong, 2020).*

5. Brain and behavior as targets of BPA and BPS

Considering the variety of molecular targets of BPA (MacKay & Abizaid, 2018), its action can affect different tissues in the organism (*Fig.5*) (Rochester, 2013; Rubin, 2011). Large literature describes the effects of BPA on reproductive and reproductive-related tissues, as primary sites of adverse outcomes (Tomza-Marciniak et al., 2018). However, it is now widely accepted that other tissues represent a target for BPA action (Rochester, 2013; Rubin, 2011). More recently, BPA has also been added to the list of the Metabolism Disrupting Chemicals (MDCs, *i.e.*, "EDCs that are able to promote metabolic changes that can result in obesity, Type 2 Diabetes

Mellitus (T2DM) or fatty liver in animals including humans") (Heindel et al., 2015), considered its huge impact on metabolic relevant tissues (Marraudino et al., 2019; Rubin et al., 2019).

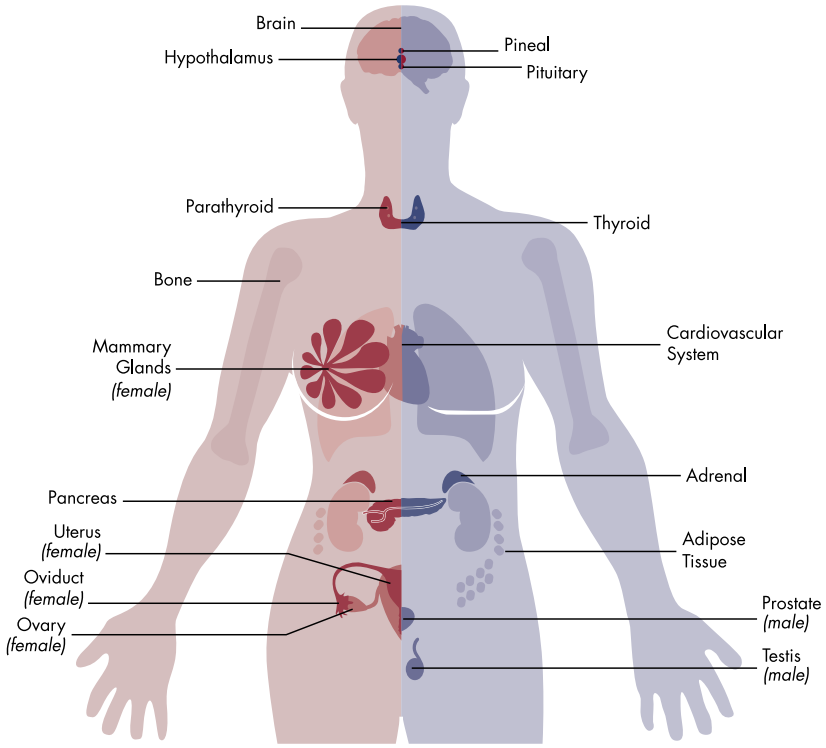

Figure 6. *The plurality of BPA's target tissues (Gore et al., 2015).*

Effects of BPA on brain and behavior are to date supported by a quite large number of experimental and epidemiological studies (Frye et al., 2012; Gore et al., 2019; Mustieles et al., 2015; Patisaul, 2020; Wolstenholme et al., 2011), which considered exposure during critical period of development particularly worrying (Mustieles et al., 2015; Z. Wang et al., 2020; Wolstenholme et al., 2011). However, exposure during adulthood is not without concerns (Bao et al., 2020; Frye et al., 2012; Gioiosa et al., 2013).

Brain has indeed been demonstrated to be targeted by BPA exposure, especially within the sexually dimorphic regions, which are highly sensitive to sex hormones (Frye et al., 2012; Patisaul, 2020).
Among regions which appear to be particularly sensitive to BPA effects have been enumerated different hypothalamic areas (*e.g.*, the anteroventral periventricular nucleus, the paraventricular nucleus, the ventromedial nucleus, the arcuate nucleus), often correlated with the hypothalamic-pituitary control of peripheral endocrine system (Frye et al., 2012; Goldsby et al., 2017; Patisaul, 2020), but also regions which are highly involved in the response to exogenous stimuli (*e.g.*, medial amygdala, bed nucleus of stria terminalis) (Goldsby et al., 2017) or are highly dynamic (*e.g.*, hippocampus and cortex) (Khadrawy et al., 2016; Tavakkoli et al., 2020).

Even if the effects on behaviors are quite difficult to assess, given their dependence on a wide range of parameters (Bakoyiannis et al., 2021; Gioiosa et al., 2013), BPA exposure has indeed been associated with altered behavioral outcome (Bakoyiannis et al., 2021; Patisaul, 2020; Rebolledo-Solleiro et al., 2021; Wolstenholme et al., 2011). An increasing number of

experimental studies show that BPA exposure, mainly during critical periods of development but also in adulthood (Gioiosa et al., 2013), is linked with behavioral disturbances (*i.e.*, disruption of "normal" behavioral patterns) (Rebolledo-Solleiro et al., 2021).

The main affected behaviors appear to be the one which are sexually dimorphic and differentiated by exposure to sex hormones during critical periods (Rebolledo-Solleiro et al., 2021; Wolstenholme et al., 2011). Among them, have been listed the social and socio-sexual behavior (Gore et al., 2019; Rosenfeld, 2015), the parental care and especially the mother-infant interaction (Keller et al., 2019; Rosenfeld, 2015), learning and memory (Mhaouty-Kodja et al., 2018), and stress and anxiety (Wiersielis et al., 2020).

BPS has comparable hazard profiles as BPA (den Braver-Sewradj et al., 2020), as it displays its effects on the organism in different tissues (den Braver-Sewradj et al., 2020; Rochester & Bolden, 2015). As for the BPA, the reproductive-relevant tissues have been initially considered as the primary site of adverse outcomes (den Braver-Sewradj et al., 2020; Rochester & Bolden, 2015).
However, as *Naderi and Kwong* recently reviewed, also BPS affects brain and behavior in many ways (Naderi & Kwong, 2020). In particular, hypothalamus (*e.g.*, the preoptic area and the arcuate nucleus) (Catanese & Vandenberg, 2017; John et al., 2019) has been described as particularly sensitive to BPS effects, as well as basolateral amygdala (Hu et al., 2022) and prefrontal and frontal cortex (Castro et al., 2015; Mornagui et al., 2019). Particularly worrying are the BPS interference with several processes fundamental for neuronal development, such as axon guidance, neurite length, serotonergic neurotransmission, glutamatergic synapses and long-term depression (Naderi & Kwong, 2020). Last, BPS has been shown to display neurotoxicity (Qiu, Zhan, et al., 2019), mainly in hippocampal cell line (Meng et al., 2021; Pang et al., 2019).

Affecting the brain, BPS also leads to behavioral alterations (Naderi & Kwong, 2020). In fact, in rodents, BPS alters anxiety response (mainly inducing anxiety-like behaviors, contextually altering locomotor activity and explorative behavior) (da Silva et al., 2019; Hu et al., 2022), sociability (Kim et al., 2015), and maternal care (Catanese & Vandenberg, 2017). Thus, as these effects often emerge even at low doses (Bakoyiannis et al., 2021; Naderi & Kwong, 2020; Rebolledo-Solleiro et al., 2021), the behavioral outcomes should be taken into consideration to reach a complete evaluation of the effects of BPA and BPS exposure (Bakoyiannis et al., 2021).

6. Health risk of exposure to bisphenols

The massive presence of BPA in the daily routine has led to numerous epidemiological studies which have highlighted that exposure to BPA, either in developmental or adult age, increases the risk of disease development or exacerbation (Cimmino et al., 2020; Rochester, 2013), mainly for the reproductive (*e.g.*, polycystic ovary syndrome, endometriosis) (Buck Louis et al., 2013; Kechagias et al., 2020), metabolic (*e.g.*, type-2 diabetes, cardiovascular diseases, and hypertension) (Sowlat et al., 2016; Wehbe et al., 2020) or immune-mediates ones (Kimber, 2017; Lazurova et al., 2021).
Furthermore, exposure during critical periods, such as *in utero* or before puberty exposure, is linked to altered behaviors in children, suggesting potential alteration in the brain in humans

(Ejaredar et al., 2017; Mesnil et al., 2020). In fact, exposure to BPA has also been linked to neurodevelopmental (*e.g.*, autism spectrum disorders, attention deficit and hyperactivity, schizophrenia) (Fujiwara et al., 2016; Tsai et al., 2020), neurobehavioral (*e.g.*, major depressive disorders) (Perera et al., 2016), and neurodegenerative diseases (*e.g.*, Alzheimer's diseases, Parkinson's diseases) (Landolfi et al., 2017; Manivannan et al., 2019; Masuo & Ishido, 2011). Considered the already reported presence of BPS in human samples (Bousoumah et al., 2021; Liao et al., 2012; H. Wang et al., 2020), and even if its impact on human health is still poorly investigated, emerging evidence support the idea that also the exposure to this compound could lead to increased risk of some diseases, such as endometriosis (Peinado et al., 2020), metabolic diseases (Jones et al., 2021; Ranciere et al., 2019), cardiovascular diseases (An et al., 2021; Zhang et al., 2020) or asthma (Mendy et al., 2020).

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AIM OF THE THESIS

Bisphenols (BPs), organic synthetic compounds mainly used to produce plastics, are an abundant class of synthetic Endocrine Disrupting Chemicals (EDCs; *i.e.*, exogenous chemicals, or mixture of chemicals, that interfere with any aspect of hormone action).
As it is reported in the introduction, the concerns on BPs regard their massive presence among a long list of consumer goods and their subsequent widespread into the environment. Bisphenol A (BPA) is the first and still the most highly produced BPs. Thanks to its structure, BPA can act through different types of nuclear and membrane-bound hormone receptors, exerting a wide range of effects on the organism. To stem the deleterious effects of BPA, the extensive search for safe alternatives has led to the production of a variety of substitutes. To date, one of the most used one is bisphenol S (BPS), which, unfortunately, seems to display the same, or even worse, endocrine disrupting properties as the BPA.

Thus, considering that:

- The worldwide consumption of BPA and BPS is rapidly increasing, leading to alarming environmental levels,
- Even if, the European regulation on BPA has become stricter, its use is still widely authorized, and, at present, there are almost no guidelines for the use of its analogues,
- The consequences of exposure to such compounds differ based on numerous parameters (*e.g.*, age, sex, health condition, dose, route and duration of exposure), making the evaluation of health hazard particularly tricky to assess,

The aim of this thesis is to evaluate the effects of oral exposure, either during particularly sensitive period of adulthood (*i.e.,* pregnancy and lactation) or during development (*i.e.,* perinatal period), to low dose (*i.e.*, 4 µg/kg BW/day, EFSA TDI for BPA) BPA or BPS in the murine model.

In particular, this thesis has five main goals:
1. Investigating, on the direct exposed dams, the long-term consequences of chronic exposure, covering pregnancy and lactation and reaching 20 weeks of treatment, to low-dose BPA on social behavior and related vasopressin and oxytocin systems;
2. Investigating, on the direct exposed dams, the consequences of exposure throughout pregnancy and lactation to low dose of either BPA or BPS on the spontaneous maternal behavior and related hypothalamic oxytocin system, and how this can affect pups' survival within the first postnatal week;
3. Evaluating the effects on sexual behaviors and on related hypothalamic kisspeptin system of adult male and female mice perinatally exposed to low-dose BPA or BPS;
4. Evaluating the effects on anxiety-related behaviors and on related serotonin system within the Raphe nucleus of adult male and female mice perinatally exposed to low-dose BPA or BPS;
5. Last, taking the advantage of the Experimental Autoimmune Encephalomyelitis mouse model, investigating, in both sexes, the consequences of perinatal exposure to BPA or BPS in this mouse model of multiple sclerosis (MS). Considering that MS is a sexual dimorphic and multifactorial disease and that environmental components have been implicated in the etiology

of MS, exposure to BPs could represent new environmental risk factors which can contribute to the different prevalence and clinical features of the disease observed in the two sexes.

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 μg/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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Contributions: BB, designed and performed experiments, analyzed data and wrote the draft; AC, MB, performed experiments and analyzed data; SG, GCP, revised the draft and wrote the paper; MM, designed the experiment, revised the draft and wrote the paper.

Ethics approval: Animal care and handling were according to the European Union Council Directive of 22nd September 2010 (2010/63/UE). All the procedures reported in the present study were approved by the Italian Ministry of Health (407/2018-PR) and by the Ethical Committee of the University of Turin (Project n° 360384).

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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.[1] 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*.,[2] 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.[3,4] Thanks to its structure, BPA can interact with a wide set of hormone receptors both nuclear and membrane-bound, including estrogen receptors (ERα, ERβ, GPER1, ERRγ), androgen receptor, peroxisome proliferator-activated receptor γ, glucocorticoid receptors and thyroid hormone receptors.[5,6] 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.[7] BPA, as other EDCs, is known to have organizational effects during development, and/or activational effects in adulthood.[1] 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.[8] 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.[8] 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.[9,10]

Vasopressin (AVP) and oxytocin (OXT) systems play a key role in the control of different type of behaviors, and in particular the maternal[11] and the social one.[12-15] 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.[16-18] 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.[19]

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[18] and on two of the circuits mainly involved in the control of this behavior, AVP and OXT,[12-15] which are well-known targets of endocrine disruption,[11,16,19] as well as the expression of the membrane estrogen receptor (GPER1) which is largely present in these nuclei.[20]

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, GD0).

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 GD0, 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.[21,22] 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,[23] after the 18th week of treatment, for at least 2 cycles.

Three-Chamber test

Dams were tested after the 18th 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.[24] Each session lasted 5 min (schematic representation in Figure 1A) and at the end of each session the tester mouse was temporally 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

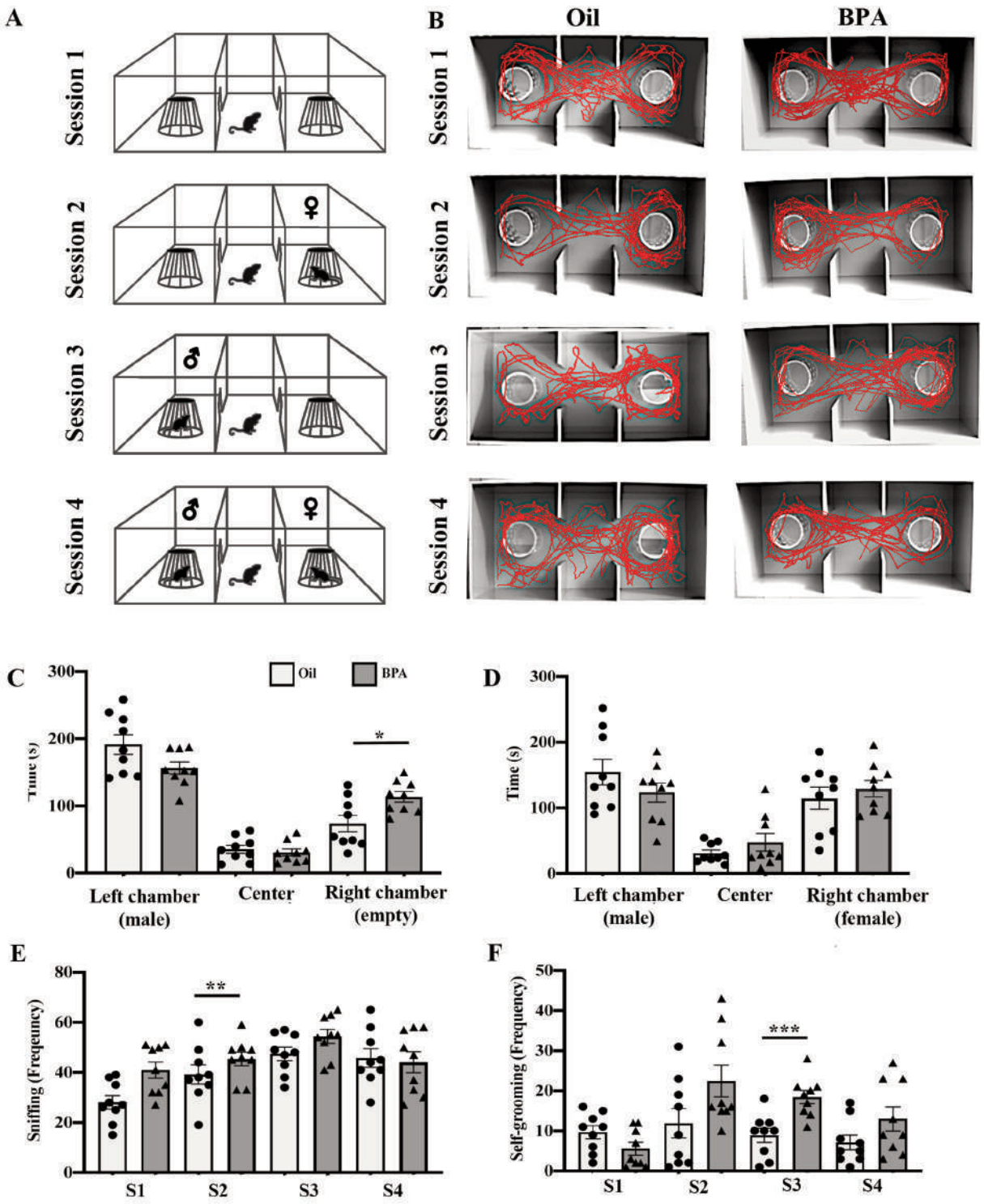

Figure 1. Analysis of social behavior of oil-treated and BPA-treated dams through Three-Chamber test. A) Experimental set-up and schematic representation of the apparatus used for the Three-Chamber test for the four experimental sessions: session 1 (habituation), session 2 (sociability with the gender-matched 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 ± 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 evaluated 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.[25]

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.[26] Sections were collected in a cryoprotectant solution[27] 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,[20,25,28] 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)[29,30] 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.[25,28,31-33] The specificity of these antisera was previously assessed,[34-36] 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.[26] 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^2 for SON; 0.077 mm^2 for SCh; 0.104 mm^2 for MeA). The PVN was instead divided into subregions, following the different distribution within the nucleus of the two analyzed systems.[26] On one hand, for the AVP- immunoreactivity (ir) analysis, the PVN (total area 0.049 mm^2) was divided into two sub-regions, the anterior parvicellular nucleus (PaAP, 0.013 mm^2) and the ventral nucleus (PaV, 0.036 mm^2). On the other hand, for the OXT-ir analysis, the PVN (total area 0.068 mm^2) was divided in three subregions, the dorsal cap/lateral magnocellular part (PaDC/PaLM, 0.013 mm^2), the medial parvicellular part (PaMP, 0.035 mm^2) and the medial magnocellular part (PaMM, 0.02 mm^2). 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.[37] 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±5.62%) compared to the controls (44.34±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 1 B). 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 ± SEM. One-way ANOVA revealed a significant effect of the treatment for p≤0.05.

Chamber Parameter	Arena	Left chamber	Center	Right chamber
		Session 1 (Habituation)		
Distance traveled (cm)	Oil: 86408.452±10979.28 BPA: 126892.944±10764.76 (p=0.018)	Oil: 32590.944±4441.891 BPA: 51877.389±5830.23 (p=0.018)	Oil: 19823.03±2448.964 BPA: 31452.311±3025.188 (p=0.009)	Oil: 3394.478±4713.435 BPA: 43563.244±3842.471 (p=0.135)
Time (s)	Oil: 300.181±0.035 BPA: 300.11±0.03 (p=0.153)	Oil: 112.626±7.141 BPA: 112.076±7.272 (p=0.958)	Oil: 78.492±5.561 BPA: 62.311±5.031 (p=0.046)	Oil: 109.063±6.56 BPA: 125.724±10.482 (p=0.197)
		Session 2 (Sociability with no-tester female)		
Distance traveled (cm)	Oil: 83438. 054±12399.336 BPA: 137443.87±11607.57 (p=0.006)	Oil: 41711.411±6090.7754 BPA: 66523±7443.3324 (p=0.02)	Oil: 21088.391±3296.322 BPA: 36700.822±3511.7 (p=0.005)	Oil: 20638.252±4315.31 BPA: 34220.044±3806.443 (p=0.031)
Time (s)	Oil: 300.197±0.041 BPA: 300.111±0.031 (p=0.116)	Oil: 71.936±8.824 BPA: 92.209±12.174 (p=0.196)	Oil: 52.725±5.93 BPA: 37.272±4.22 (p=0.049)	Oil: 175.509±12.648 BPA: 170.63±12.226 (p=0.785)
		Session 3 (Sociability with no-tester male)		
Distance traveled (cm)	Oil: 111206.4±17506.127 BPA: 124788.84±15582.89 (p=0.57)	Oil: 19509.378±2115.5327 BPA: 40075.422±6023.7911 (p=0.05)	Oil: 30649.682±5578.267 BPA: 33305.933±4539.924 (p=0.717)	Oil: 61047.344±12048.584 BPA: 51407.489±6711.153 (p=0.495)
Time (s)	Oil: 300.158±0.04 BPA: 300.138±0.038 (p=0.722)	Oil: 191.422±14.775 BPA: 156.247±8.873 (p=0.058)*	Oil: 35.453±5.911 BPA: 30.607±5.213 (p=0.547)	Oil: 73.281±12.224 BPA: 113.283±7.809 (p=0.014)
		Session 4 (Social preference)		
Distance traveled (cm)	Oil: 110284.94±15934.653 BPA: 124198.21±17361.655 (p=0.563)	Oil: 33876.808±6436.024 BPA: 47583.567±8235.806 (p=0.208)	Oil: 31700.611±5075.1257 BPA: 33132.369±5619.05 (p=0.852)	Oil: 44707.522±8985.061 BPA: 43482.278±7476.7306 (p=0.918)
Time (s)	Oil: 300.181±0.035 BPA: 300.137±0.038 (p=0.413)	Oil: 154.534±19.698 BPA: 123.32±14.641 (p=0.222)	Oil: 30.981±4.9 BPA: 47.487±13.349 (p=0.263)	Oil: 114.666±16.898 BPA: 129.33±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 significative 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 ± SEM, both as frequency (F) and cumulative duration (CD). One-way ANOVA revealed a significant effect of the treatment for p≤0.05.

Parameter	Session 1	Session 2	Session 3	Session 4
Sniffing in left chamber (F)	Oil: 11.556±2.304 BPA: 20.889±3.442 (p=0.039)	Oil: 23±4.69 BPA: 19.222±2.645 (p=0.493)	Oil: 23.778±2.666 BPA: 24.667±1.763 (p=0.784)	Oil: 19.444±2.982 BPA: 24±3.742 (p=0.355)
Sniffing in left chamber (CD)	Oil: 14.406±6.052 BPA: 24.374±7.767 (p=0.326)	Oil: 28.382±9.533 BPA: 20.507±4.379 (p=0.464)	Oil: 28.139±5.012 BPA: 25.218±5.88 (p=0.71)	Oil: 22.754±5.103 BPA: 28.824±6.332 (p=0.446)
Sniffing in right chamber (F)	Oil: 16.444±2.9 BPA: 20±3.202 (p=0.422)	Oil: 16.222±3.122 BPA: 26.222±3.205 (p=0.04)	Oil: 23.667±2.744 BPA: 29.778±3.403 (p=0.181)	Oil: 26.333±3.693 BPA: 20.111±1.495 (p=0.138)
Sniffing in right chamber (CD)	Oil: 15.296±3.934 BPA: 17.696±4.013 (p=0.675)	Oil: 15.013±4.427 BPA: 28.142±5.49 (p=0.081)	Oil: 25.64±4.322 BPA: 28.64±4.442 (p=0.635)	Oil: 30.39±6.766 BPA: 16.886±2.062 (p=0.074)
Total sniffing (F)	Oil: 28±2.724 BPA: 40.889±3.203 (p=0.007)	Oil: 39.222±3.792 B PA: 45.444±2.739 (p=0.202)	Oil: 47.444±2.672 BPA: 54.444±3.096 (p=0.089)	Oil: 45.778±3.696 xBPA: 44.111±4.185 (p=0.769)
Total sniffing (CD)	Oil: 29.702±6.396 BPA: 42.072±7.248 (p=0.219)	Oil: 43.396±8.201 BPA: 48.649±5.579 (p=0.604)	Oil: 53.779±4.394 BPA: 53.858±5.868 (p=0.992)	Oil: 53.144±6.394 BPA: 45.710±6.386 (p=0.423)
Grooming (F)	Oil: 9.667±1.616 BPA: 5.556±1.573 (p=0.087)	Oil: 11.889±3.615 BPA: 22.444±3.969 (p=0.067)	Oil: 8.889±1.791 BPA: 18.444±1.651 (p=0.001)	Oil: 7.111±1.829 BPA: 13±3.023 (p=0.115)
Grooming (CD)	Oil: 18.911±2.250 BPA: 11.991±4.758 (p=0.207)	Oil: 17.102±5.858 BPA: 32.649±5.845 (p=0.079)	Oil: 9.131±2.402 BPA: 20.64±2.787 (p=0.006)	Oil: 11.697±2.935 BPA: 21.738±9.423 (p=0.324)
Rearing (F)	Oil: 13.889±1.798 BPA: 17.667±4.794 (p=0.408)	Oil: 4.333±1.75 BPA: 7.556±3.72 (p=0.421)	Oil: 10.333±2.635 BPA: 11±2.217 (p=0.849)	Oil: 5.222±1.176 BPA: 9.111±1.448 (p=0.053)*
Rearing (CD)	Oil: 14.74±2.692 BPA: 17.908±4.794 (p=0.573)	Oil: 3.92±1.75 BPA: 5.742±2.678 (p=0.577)	Oil: 9.584±3.055 BPA: 8.284±1.488 (p=0.707)	Oil: 3.06±0.993 BPA: 6.283±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 suprachiasmatic 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 significative 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.[18] 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.[18] 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.[18,38] 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.[39-41] 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 ± SEM, both, when possible, as number of positive cells and fractional area. One-way ANOVA revealed a significant effect of the treatment for p≤0.05.

Marker	Nucleus	Number of positive cells	Fractional area (%)
AVP	PVN	Oil: 84.8±10.841 BPA: 43.2±6.262 (p=0.005)	Oil: 116.762±19.091 BPA: 66.548±5.709 (p=0.036)
	PaAP	Oil: 29.7±5.178 BPA: 25±6.569 (p=0.053)*	Oil: 45.412±7.021 BPA: 30.089±5.368 (p=0.121)
	PAV	Oil: 55.1±5.932 BPA: 28±3.943 (p=0.005)	Oil: 71.35±13.803 BPA: 36.459±4.653 (p=0.043)
	SCh	Oil: 72±4.41 BPA: 33.2±8.032 (p=0.002)	Oil: 37.293±1.659 BPA: 27.607±1.852 (p=0.004)
	SON	Oil: 22.286±1.345 BPA: 21.917±1.65 (p=0.864)	Oil: 26.462±1.254 BPA: 25.356±1.342 (p=0.828)
	MeA	-	Oil: 2.235±0.246 BPA: 2.219±0.372 (p=0.971)
OXT	PVN	Oil: 28.6±1.958 BPA: 29.375±2.366 (p=0.806)	Oil: 48.921±5.08 BPA: 43.667±6.848 (p=0.548)
	PaDC/PaLM	Oil: 4.1±0.4 BPA: 5.125±1.82 (p=0.557)	Oil: 11.331±2.414 BPA: 8.189±1.729 (p=0.349)
	PaMM	Oil: 11.331±2.414 BPA: 8.189±1.729 (p=0.349)	Oil: 21.898±0.781 BPA: 22.937±2.532 (p=0.678)
	PaMP	Oil: 6.3±1.102 BPA: 5.875±2.989 (p=0.888)	Oil: 15.692±3.425 BPA: 12.541±4.515 (p=0.588)
	SON	Oil: 23.7±1.991 BPA: 20.4±1.089 (p=0.184)	Oil: 19.903±0.983 BPA: 20.022±1.009 (p=0.935)
	MeA	-	Oil: 2.029±0.358 BPA: 1.956±0.316 (p=0.883)
GPER1	PVN	-	Oil: 21.253±0.489 BPA: 12.921±0.489 (p<0.001)
	SCh	-	Oil: 31.735±1.467 BPA: 23.139±1.114 (p=0.003)
	SON	-	Oil: 20.27±1.341 BPA: 19.787±1.862 (p=0.84)
	MeA	-	Oil: 9.482±0.647 BPA: 9.201±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.[39] In particular, BPA exposure is associated to increased anxiety-like behavior in rodents,[19] not

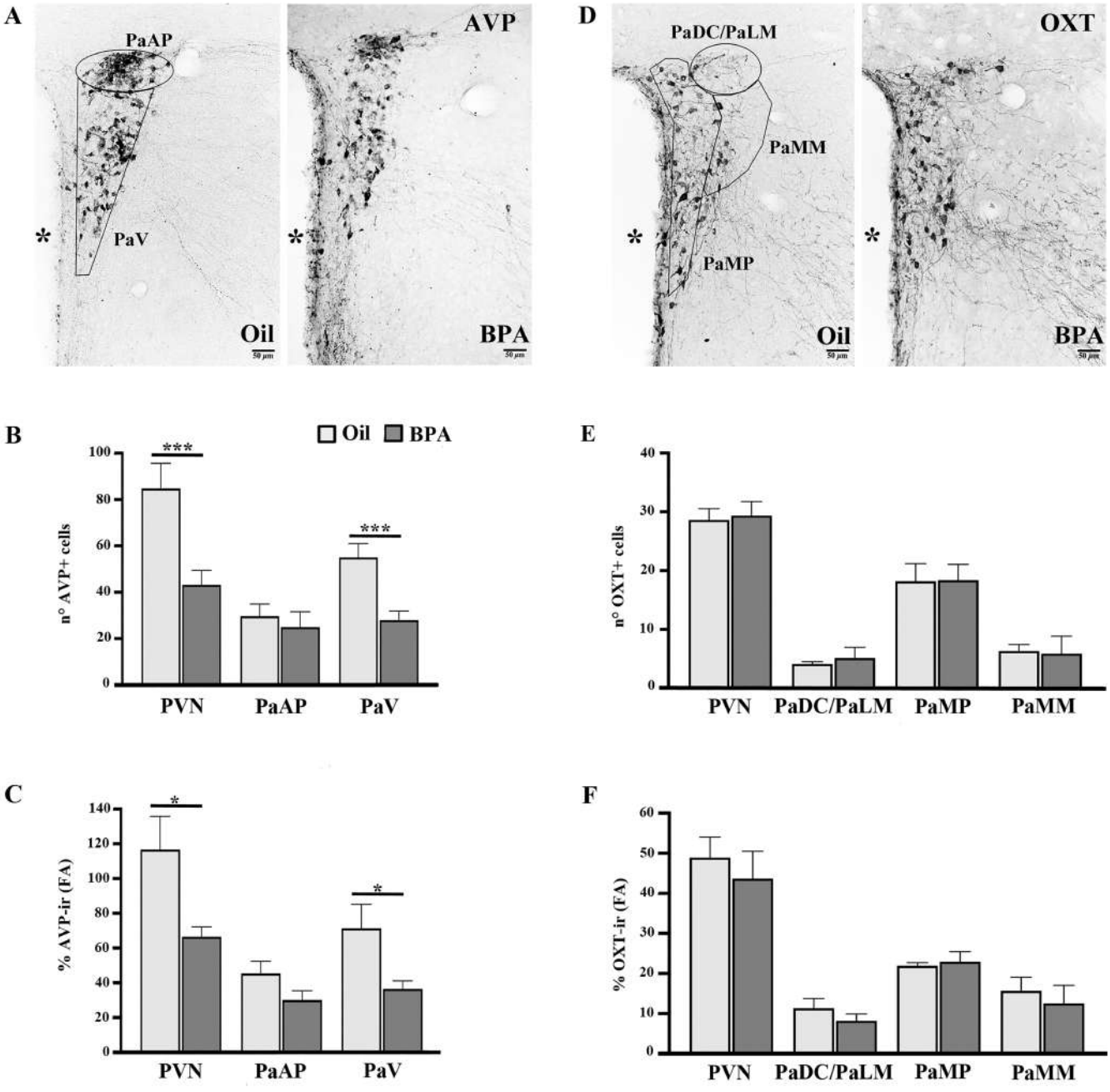

Figure 2. AVP-ir and OXT-ir in the PVN of oil-treated and BPA-treated dams. A) Representative image of AVP-ir in a coronal section of PVN of oil-treated (left images) or BPA-treated (right images) dams. Analysis of AVP-ir in PVN, expressed both as (B) number of AVP positive cells and (C) fractional area (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 ± SEM. One-way ANOVA revealed a significant effect of the treatment for p≤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,[42] but also in adulthood.[43] Long-term oral exposure to BPA during adulthood is associated with alterations of anxiety-related behaviors mainly in male mice.[43] 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 sociability 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

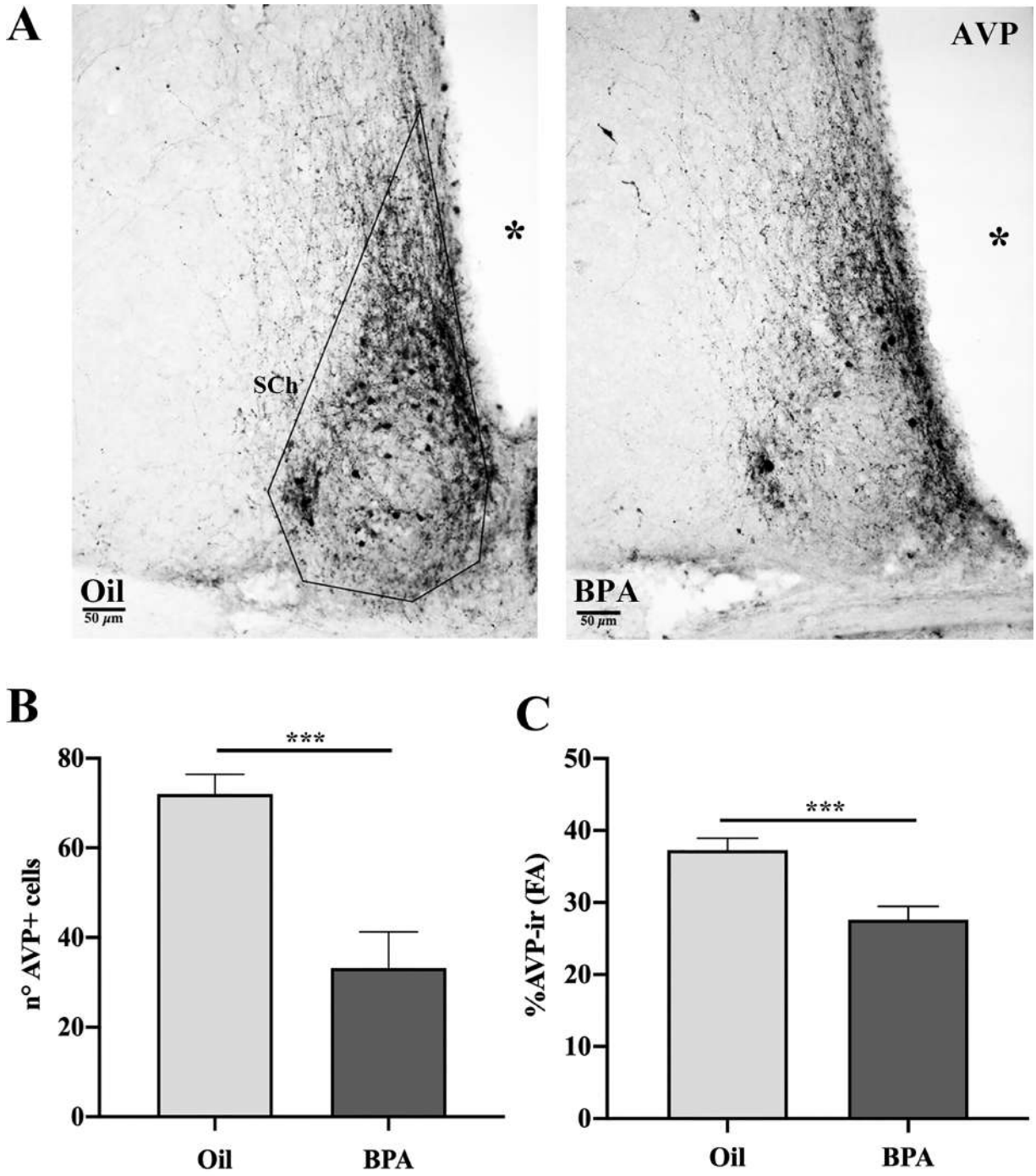

Figure 3. AVP-ir in the SCh of oil-treated and BPA-treated dams. A) Representative image of AVP-ir in a coronal section of SCh of oil-treated (left images) or BPA-treated (right images) dams. Analysis of AVP-ir in SCh, expressed both as (B) number of AVP positive cells and (C) fractional area (FA), revealed a significant reduction in BPA-treated dams (dark gray) compared to the control ones (light gray). Data are expressed as mean ± SEM. One-way ANOVA revealed a significant effect of the treatment for p≤0.05. AVP, vasopressin; OXT, oxytocin; SCh, suprachiasmatic 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.[18,19] 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.[2,44,45] Furthermore, it is known that olfactory discrimination, which is fundamental in both social and sexual behavior, can be altered by BPA exposure.[45,46]

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

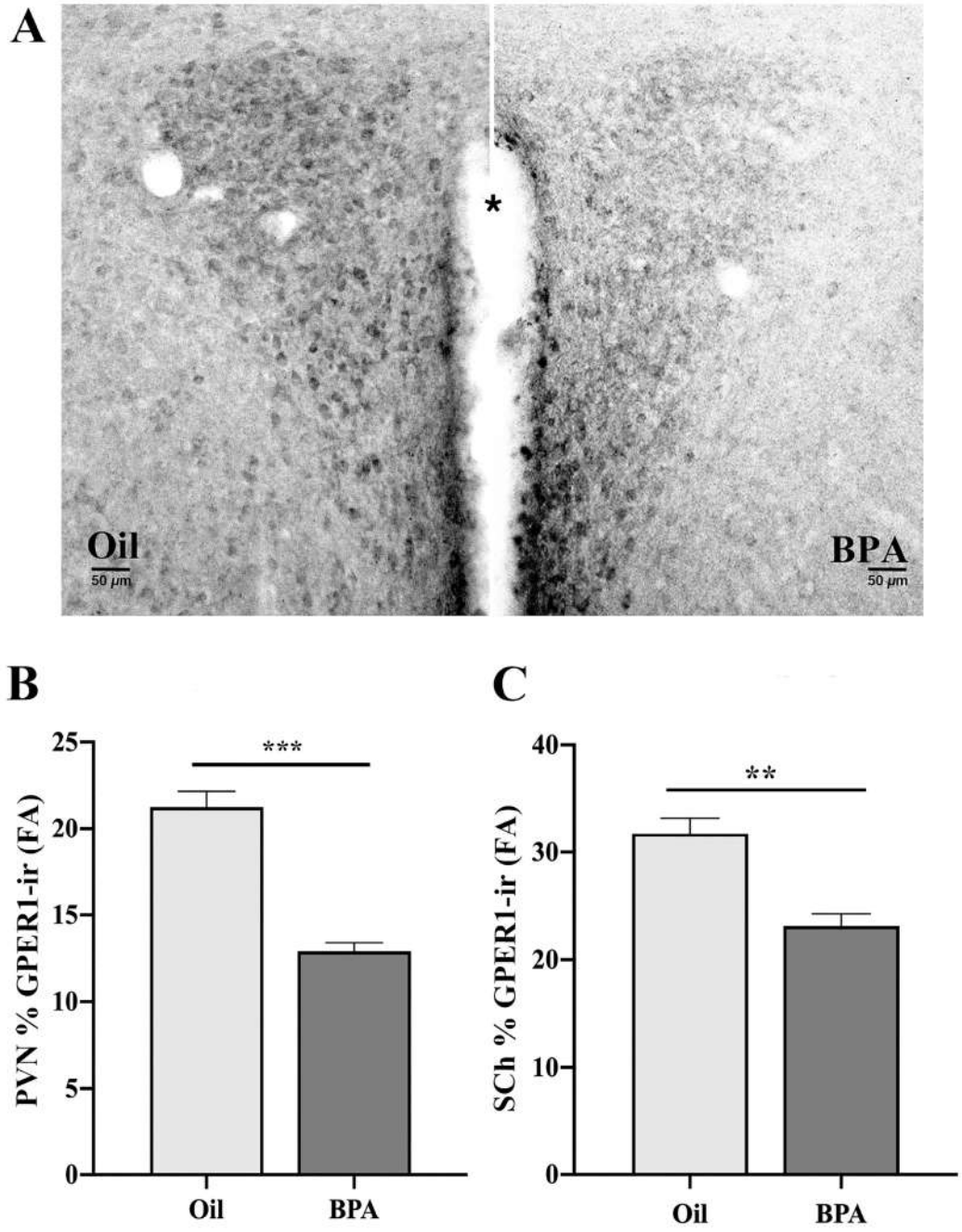

Figure 4. **GPER1-ir in the PVN and SCh of oil-treated and BPA-treated dams. A) Representative images of GPER1-ir in a coronal section of PVN of oil-treated (left image) or BPA-treated (right image) dams. Analysis of GPER1-ir, expressed as fractional area (FA), revealed a significant reduction in BPA-treated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

main target systems underlying the alterations observed in social behavior after exposure to different types of EDCs;[19,47] 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.[10,48,49] 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.[50] 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.[51] AVP positive cells located in the SCh and in the PVN express estrogen receptors.[52,53] *In vitro* studies showed that estradiol, acting through ERβ and GPER1, induces a downregulation of AVP expression.[54-56] 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.[57,58] 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.[59] In the literature, numerous studies show how prolonged exposure to BPA affects the homeostasis of this system.[60] 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.[61] 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.[62] Stress responses are integrated and regulated at PVN level involving AVP and corticotropin-releasing hormone (CRH) neurons.[63] 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).[64] 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.[65]

In rodents, GPER1 is strongly expressed in PVN and SCh,[66] with a high co-expression with vasopressin neurons,[56,66] and is involved in the control of a variety of behaviors, including the social one.[67,68] Interestingly, it is known that BPA displays low affinity for ERβ and high affinity for GPER1.[69] 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,[56,67] 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.[70] 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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Exposure to either bisphenol A or S represents a risk for crucial behaviors for pup survival, such as spontaneous maternal behavior and related oxytocinergic circuits in mice.

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ABSTRACT

Background: Maternal behavior depends on a multitude of factors, including the environmental ones, such as Endocrine Disrupting Chemicals (EDCs), which are increasingly attracting attention. Bisphenol A (BPA), an EDC present in plastic, is known to exert negative effects on maternal behavior. Bisphenol S (BPS), a BPA-substitute, seems to share some endocrine disrupting properties.
Objectives: In this study we focused on the analysis of the effects of low-dose (*i.e.*, 4µg/kg body weight/day, EFSA TDI for BPA) BPA or BPS exposure throughout pregnancy and lactation in mice.
Methods: We administered adult C57BL/6J females orally BPA, BPS, or vehicle from mating to offspring weaning. We assessed the number of pups at birth, the sex *ratio* and the percentage of dead pups in each litter and during the first postnatal week we observed the spontaneous maternal behavior. Finally, we analyzed the oxytocin system, known to be involved in the control of the maternal care, in the hypothalamic magnocellular nuclei.
Results: At birth, pups from BPA-treated dams tended to have lower male-to-female *ratio* compared to controls, while the opposite was observed among BPS-treated dams' litters. During the first postnatal week, offspring mortality impacted differentially BPA and BPS litters, with more female dead pups among the BPA litters, while more male dead pups in the BPS litters, sharpening the difference in the sex *ratio*. BPA- and BPS- treated dams spent

significantly less time in pup-related behaviors than controls. Oxytocin immunoreactivity in the paraventricular and supraoptic nuclei was increased only in the BPA-treated dams.
Discussion: Alterations in maternal care, along with the treatment itself, may affect, later in life, the offspring physiology and behavior. The exposure to BPs during sensitive developmental periods represents a risk for both dams and offspring, even at low environmentally-relevant doses, through the functional alteration of neural circuits controlling fundamental behaviors for pup survival, such as maternal behaviors.

Keywords: endocrine disrupting chemicals, environmentally low doses, BPA, BPS, OXT, paraventricular nucleus, supraoptic nucleus.

Author's contributions
BB designed and performed experiments, analyzed data, and wrote the draft. LM analyzed data, revised the draft, and wrote the paper. GCP and MM revised the draft and wrote the paper.

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All authors have no conflicts of interest to declare with respect to the research, authorship, and/or publication of this article.

Introduction

Endocrine Disrupting Chemicals (EDCs) are defined as "exogenous chemical(s), or mixtures of chemicals, which can interfere with any aspect of hormone action", leading to deleterious effects not only on individuals, which are directly exposed, but also on their progeny (Gore et al., 2015). Current knowledge strongly supports the idea that exposure to EDCs represent a real risk for both wildlife and human health (Ribeiro et al., 2017; Street et al., 2018). Increasing concerns are linked to the effects of some EDCs on brain and behavior (Bakoyiannis et al., 2021).

Bisphenols (BPs) belong to the class of EDCs capable of perturbing different aspects not only of brain development or neurochemistry (Itoh et al., 2012), but also of behavior (Bakoyiannis et al., 2021; Gioiosa et al., 2013). In particular, most of the available studies, performed in rodents, are focused on the effects of bisphenol A (BPA) on behavior (Wolstenholme et al., 2011). BPA is still the most highly produced BP (Catenza et al., 2021; Lehmler et al., 2018). Exposure to BPA during well-known critical period of development, such as the pre- or peri-natal one (Street et al., 2018), leads later in life to consequences on a wide set of behaviors, including the exploratory, the anxiety-like, the social, the sexual and the parental behavior (Bakoyiannis et al., 2021; Keller et al., 2019; Rosenfeld, 2015). However, few data are available regarding the effects of exposures to other BPs during adulthood (Bakoyiannis et al., 2021). As the deleterious effects of BPA become clearer, some substitutes have been proposed, such as bisphenol S (BPS). BPS was thought to leach fewer monomers into food and drink (Kuruto-Niwa et al., 2005), therefore is one of the BPA analogs mainly used to produce the BPA-free goods (Thoene et al., 2020). Even though BPS is more heat- and photo-resistant than BPA (Kuruto-Niwa et al., 2005), it is now evident that these properties are not enough to contain the spread of the compound among worldwide human population (Bousoumah et al., 2021; Liao et al., 2012). Furthermore, an increasing number of studies are highlighting that the BPS endocrine

disrupting properties are comparable to those of BPA (Catenza et al., 2021; Chen et al., 2016; Eladak et al., 2015; Li et al., 2018; Thoene et al., 2020). Still, little concern has been raised on the behavioral outcomes linked to BPS exposure.
Maternal behavior is crucial for pup survival and represents a complex variety of behaviors (Kohl et al., 2017), which can be modulated by a multitude of factors. For instance, the oxytocin (OXT) secretion at delivery and/or pup behavior (*e.g.*, suckling, callings *etc.*) facilitate the occurrence of some aspects of maternal behavior (Bealer et al., 2010; Panaro et al., 2020). OXT magnocellular system in the hypothalamus, within the paraventricular (PVN) and supraoptic nuclei (SON), is known to be the source of intracerebral OXT, by means of its huge number of projections within in the cerebral ventricles (Althammer et al., 2021). Indeed, the OXT system is a key regulator of maternal behavior (Caldwell et al., 2017; Kohl et al., 2017; Yoshihara et al., 2018).

Nowadays, there is growing scientific evidence that the environmental exposure to EDCs, in particular to BPs, is capable of impacting on maternal behavior and of inducing long lasting effect on the offspring (Keller et al., 2019). Specifically, BPA is known to affect the OXT system (Keller et al., 2019; Patisaul, 2020), which is highly involved in the regulation of maternal care (Caldwell et al., 2017; Kohl et al., 2017; Yoshihara et al., 2018). Also, BPA exposure during the pre- or peri-natal period has already been linked to alterations in the maternal behavior (Keller et al., 2019; Palanza et al., 2002). However, very few studies focused on the BPS effects on the OXT system and behavior of directly exposed dams (Catanese & Vandenberg, 2017; da Silva et al., 2019; Naderi et al., 2021) and on their offspring (Catanese & Vandenberg, 2017).

In the present study, we propose pregnancy and lactation as a "critical period" for the adult dams (Alonso-Magdalena et al., 2010; Alonso-Magdalena et al., 2015) directly exposed to BPs. Therefore, we investigate the consequences of exposure throughout pregnancy and lactation to low dose (4µg/kg BW/day, assessed by the European Food Safety Authority as the Tolerable Daily Intake, TDI, for BPA) ((EFSA), 2015) of either BPA or BPS. We monitored both dams and offspring, observing the spontaneous maternal behavior of the dams during the first post-natal weeks of the pups and assessing the number of pups at birth, the sex *ratio* and the percentage of dead pups in each litter. Finally, we focused on OXT system within the PVN and SON, a well-known target of EDCs (Keller et al., 2019; Patisaul, 2020), which is highly involved in the control of this behavior (Caldwell et al., 2017; Kohl et al., 2017; Yoshihara et al., 2018).

Methods

Animals

Adult C57BL/6J mice from our *vivarium* 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 10:00 AM). Food (standard mouse chow 4RF21, Mucedola srl, Settimo Milanese, Italy) and water were provided *ad libitum*. One male and two female mice (3-month-old) were housed together to achieve a successful mating, assessed by the evaluation of the presence of the vaginal plug (assumed as gestational day 0, GD0) (Hasegawa et al., 2017).

Animal care and handling were according to the European Union Council Directive of 22nd September 2010 (2010/63/UE); all the procedures reported in the present study were approved by the Italian Ministry of Health (407/2018-PR) and by the Ethical Committee of the University of Torino (Project n° 360384). The experimental design conforms to the ARRIVE guidelines originally published by Kilkenny *et al.* in 2010 (Kilkenny et al., 2010).

Chemical administration

BPA (Sigma Aldrich, 239658, CAS 80-05-7) or BPS (Sigma Aldrich, 103039, CAS 80-09-1) were prepared for oral administration, dissolving them in corn oil (Sigma-Aldrich, C8267). 35 pregnant dams were assigned randomly to three experimental groups: control dams (vehicle, corn oil; n=10), BPA-treated dams (4 µg/kg BW/day of BPA, corresponding to the European TDI; n=10) and BPS- treated dams (4 µg/kg BW/day of BPS; n=15). We decided to test the same dose for both BPA and BPS to allow a precise comparison of the effects of the two BPs. Besides, although BPS is one of the most used BPA substitutes and it has already been detected in environmental and human samples (Catenza et al., 2021), at present no user guidelines are available.

From GD0, throughout both pregnancy and lactation, until weaning of the offspring at postnatal day 28 (PND28), accordingly to their experimental groups, dams were administered either BPA or BPS or corn oil. To resemble human exposure conditions, the daily administration was *via os*, by means of a pipette, in order to minimize dams' stress (Bo et al., 2016; Palanza et al., 2002). The dose was calculated according to their body weight, measured with an electronic precision balance (*Mod. Kern-440-47N, resolution 0.1g*).

Observation of spontaneous maternal behavior

Mice are mostly active during the dark phase of the light/dark daily cycle. Moreover, possible alterations due to exposure to hormonally active agents are detectable only during such active (dark) phase (Palanza et al., 2002). Therefore, the spontaneous maternal behavior was assessed observing lactating dams in their own home cages, from 08.00 AM to 10.00 AM (*i.e.*, the last two hours of the dark phase), throughout the first postnatal week (PND1-7, considering PND1 as the day after the pup delivery, which is PND0). Dams were monitored by means of instantaneous sampling procedure. Briefly, each dam was observed every 4 min for a total of 30 observations. The observation period lasted 120 min and it was conducted with the aid of 25-W red lights, which mice cannot see (Palanza et al., 2002). During each observation, the experimenter recorded which behavior the lactating female was displaying. First, the experimenter defined whether the dam was either inside or outside the nest. Afterwards, the exhibited behaviors were as follows:

1) Nursing, the dam was nursing the pups and was not nursing with her body arched over the pups.
2) Arched-back nursing, the female was nursing with her body arched all over the pups (arched back posture).
3) Licking pups, the dam was licking or grooming her pups.
4) Nest building: the dam was engaged in some aspect of nest building.
5) Eating, the dam was nibbling at a food pellet.
6) Drinking, the dam was drinking from the water bottle.
7) Self-grooming, the dam was grooming her own body.

8) Active, the dam was moving around the cage in general activity, not engaged in the above behaviors.
9) Resting, the dam was lying motionless outside the nest, with no pup attached to her nipples. By grouping the observed data, two additional dependent variables were produced and analyzed: the category of the pup-related behaviors and that of the pup-unrelated behaviors. The pup-related behavior category was the sum of the observations for nursing, arched-back posture, licking pups and nest-building behaviors. The pup-unrelated behavior category was the sum of the observations for eating, drinking, self-grooming, active and resting behaviors (Bertocchi et al., 2011; Palanza et al., 2002).

Observations on dams' physiological parameters and offspring measures
We monitored the dams, recording the body weight (BW), assessed daily until the sacrifice (at weaning of the pups, PND28), and the food intake (g of food/animal/day) once a week starting from the delivery until PND28.
In the offspring we measured the body weight at birth (PND0) and at weaning (PND28). We assessed the sex *ratio* (n°males/n°females) (Grech, 2020) in each litter at birth (PND0) and at PND7. From PND0 to PND7 we monitored the number of dead pups in each litter, assessing it once a day in the morning.

Fixation and tissue sampling
According to the standard procedures of our laboratory (Bonaldo et al., 2021), dams were sacrificed at PND28, after the offspring's weaning. Dams were sacrificed, 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 hours, followed by several washings in 0.01 M saline phosphate buffer (PBS, pH 7.3-7.4). 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. Brains (n=4/group) were serially cut in the coronal plane at 30µm 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 (Paxinos et al., 2001). Sections were collected in a cryoprotectant solution (Watson Jr. et al., 1986) and stored at -20 °C.

Oxytocin immunohistochemistry
The OXT presence was detected by immunohistochemistry performed on free-floating sections from one series. Briefly, the sections were washed overnight in phosphate buffer (PBS) at pH 7.3. The following day, sections were first incubated with citrate buffer (citric acid 10 mM, 0.05% Tween, pH 6.0) previously heated at 95°C for antigen retrieval and then washed three times in PBS. Next, the sections were washed in PBS containing 0.5% 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) and incubated overnight at room temperature with anti-OXT antibody (EMD Millipore AB911, Rabbit, 1:5.000) diluted in PBS, pH 7.3–7.4, containing 0.5% Triton X-100. A biotinylated goat anti-rabbit secondary antibody (Vector Laboratories, Burlingame, CA, USA) diluted in PBS, pH 7.3–7.4, containing 0.2% Triton X-100 was then employed at a dilution of 1:200 for 60 min at room temperature. The antigen-antibody reaction was revealed by 60 min incubation with avidin–peroxidase complex (Vectastain ABC Kit Elite,

Vector Laboratories, Burlingame, CA, USA). 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, Milano, Italy). This antibody was successfully used in previous studies (Bonaldo et al., 2021; Villanueva et al., 2012). The specificity of this antiserum was previously assessed (Sawyer et al., 1986) 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) and the supraoptic nucleus (SON, Bregma -0.58 to -0.94 mm), were chosen according to the mouse brain atlas (Paxinos et al., 2001). Three 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.S., Firenze, Italy). Images were digitized by using a 20x objective for the PVN acquisition and a 40x objective for the SON acquisition. Digital images were processed and analyzed by ImageJ (version 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 (0.101 mm^2 for SON). The PVN was instead divided into subregions, following the different distribution within the nucleus of the analyzed system (Bonaldo et al., 2021). The PVN (total area 0.068 mm^2) was divided in three subregions, the dorsal cap/lateral magnocellular part (PaDC/PaLM, 0.013 mm^2), the medial parvicellular part (PaMP, 0.035 mm^2) and the medial magnocellular part (PaMM, 0.02 mm^2).

We evaluated the extension of the immunoreactivity (cell bodies, dendrites, fibers) in all the selected nuclei as fractional area (FA) covered by immunopositive material (Marraudino, Ponti, et al., 2021). In addition, we also counted the number of OXT-positive cells in the two analyzed nuclei.

Statistical analysis

Data obtained from observation of spontaneous maternal behavior were analyzed by two-way analysis of variance (ANOVA) for repeated measures (time and treatment as independent variables) for the day-by-day evaluation and by one-way ANOVA (treatment as independent variable) when the weekly mean of each behavior was taken into consideration. Dams' data (body weight and food intake) were analyzed by two-way ANOVA for repeated measures (time and treatment as independent variables) to evaluate whether the data changed during the days of experimentation. Whereas the litters' data were examined by two-way ANOVA (sex and treatment as independent variables), with SPSS 27 statistic software (SPSS Inc., Chicago, USA). Immunohistochemical data from dams' brain were analyzed by one-way ANOVA (treatment as independent variable). If the ANOVA was significant, the *post-hoc* analysis was performed using the Tuckey's HSD test. Differences were considered statistically significant for values of $p \leq 0.05$. Data are shown as mean ± SEM (mean standard error).

Results

Effects of exposure to BPA or BPS on spontaneous maternal behavior

Direct observation of mother-pup interactions highlighted the impact of BPs exposure on different aspects of spontaneous maternal behavior (*Fig.1*). Remarkably, the analysis of the weekly means of dams' behaviors yielded that both BPA and BPS dams spent more time on average outside the nest compared to control dams (*Fig.1A*), even if the difference was only close to significance ($F_{(2,27)}=3.107$, $p=0.061$). In line with these observation, exposure to BPs significantly decreased the average time spent by dams in pup-related behavior ($F_{(2,27)}=4.814$, $p=0.016$; *Fig.1B*), especially the average time spent by the BPS dams ($p=0.016$; *Fig.1B*), compared to controls; whereas, both BPs significantly increased the average time spent in pup-unrelated behaviors by dams ($F_{(2,27)}=6.358$, $p=0.005$; *Fig.1C*), compared to controls (BPA, $p=0.04$; BPS, $p=0.006$; *Fig.1C*).

The analysis of each specific behavior of the lactating dams showed that both BPs decreased significantly the average time spent in licking-pup behavior by dams ($F_{(2,27)}=4.566$; $p=0.02$; *Fig.1D*), in particular BPS compared to controls ($p=0.02$; *Fig.1D*). BPs dams tended to decrease significantly the weekly average time spent in arched-back posture ($F_{(2,27)}=3.316$, $p=0.052$; *Fig.1D*) and increased the average time spent in general activity ($F_{(2,27)}=3.476$, $p=0.045$; *Fig.1D*).

When running the day by day analysis, we observed that there was a significant effect of the treatment by time interaction on time spent by dams in pup-related behaviors ($F_{(12,162)}=2.072$, $p=0.021$; *Fig.1E*), in particular on PND1 BPA dams spent significantly less time in behaviors related to the care of the pups compared to controls ($p=0.01$). On PND1 BPA dams spent significantly more time in general activity when compared to control dams ($p=0.01$, *Fig.1F; treatment by time interaction just missed significance:* $F_{(12,162)}=1.649$, $p=0.083$).

Effects of exposure to BPA or BPS on dams' body weight and food intake throughout both pregnancy and lactation

Neither BPA nor BPS had significant effects on either body weight or food intake of the dams (respectively, $F_{(2,\ 8)}=0.481$, $p=0.955$; $F_{(2,\ 6)}=0.408$, $p=0.873$; *data not shown*). Moreover, BPs did not affect body weight increase during pregnancy ($F_{(2,\ 27)}=0.633$, $p=0.539$; Oil:14.99±0.677g; BPA:15.18±0.845g; BPS:16.1±0.706g) or body weight decrease after the delivery ($F_{(2,\ 27)}=0.015$, $p=0.985$; Oil:-11.74±0.415g; BPA:-11.78±0.547g; BPS:-11.87±0.628g).

Effects of exposure to BPA or BPS on litters

The effects of BPs administered to dams on their offspring are summarized in *Table 1*.

Both BPA and BPS had not significant effects on pup total number per litter at birth ($F_{(2,\ 32)}=1.825$, $p=0.178$; *Fig.2A*). However, exposure was almost significant on the *sex ratio* (n°males/n°females) at birth ($F_{(2,32)}=2.642$, $p=0.087$; *Fig.2B*), but it was significant after the first postnatal week of pups ($F_{(2,\ 27)}=3.904$, $p=0.032$; *Fig.2C*). There were more male pups than females among the BPA litters as opposed to BPS litters in which there were more female pups than males (*Table 1*), compared to control litters. Interestingly, the sharpening of this sex difference at PND7 was linked to the difference in pups' death, which was differential between litters (*Fig.2D*). Remarkably, when the two sexes were analyzed separately, the effect of exposure on pup death was significant (respectively, for females: $F_{(2,\ 32)}=2.844$, $p=0.073$; for

males: $F_{(2, 32)}=3.689$; $p=0.036$; *Fig.2D*). In particular, female pups from BPA-litters tended to die more ($p=0.056$; *Fig.2D*) compared to controls, as opposed to BOS litters in which there were significantly more male dead pups ($p=0.037$; *Fig.2D*). We also noticed that BPA pups tended to die during the first postnatal week, while BPS pups tended to die during or right after the birth (PND0). Moreover, in the 33.3% of the cases (5 out of 15), BPS dams lost the entire litter, and were thus excluded from the analysis of the spontaneous maternal behavior.
We observed a significant difference in body weight of female pups at birth (*Table 1*), due to the differences between the BPA and the BPS female pups (BPA females pups weighted less than BPS ones; $p=0.001$; *Fig.2E*), while no significant difference due to the treatment was highlighted on pups' body weight at weaning (*Fig.2F*).

Table 1

	Oil	**BPA**	**BPS**	**One-way ANOVA**	
	Mean ± SEM	*Mean ± SEM*	*Mean ± SEM*	*F*	*p*
Total number of pups per litter	7±0.333	8.61±0.658	8.61±0.47	*1.825*	*0.718*
Total number of male pups per litter	3.9±0.504	4.7±0.3	2.8±0.368	*6.057*	***0.006***
Total number of female pups per litter	3.1±0.407	3.4±0.636	4±0.39	*1.013*	*0.375*
Percentage of male pups at PND0	57.5±7.068	60.791±4.554	40.069±4.1	*4.962*	***0.013***
Percentage of female pups at PND0	45.83±5.439	39.209±4.954	58.598±4.731	*4.070*	***0.027***
Sex ratio at PND0	1.75±0.53	2.091±0.531	0,891±0.193	*2.642*	*0.087*
Sex ratio at PND7	1.64±0.52	2.517±0.541	0.747±0.196	*3.904*	***0.032***
Percentage of dead pups	5.04±2.071	25.792±6.953	37.407±12.09	*2.844*	*0.073*
Percentage of dead male pups	5.833±3.056	17.5±3.056	43.333±12.786	*3.689*	***0.036***
Percentage of dead female pups	1.667±1.667	41.31±10.856	34.286±12.451	*3.412*	***0.045***
Body weight of male pups at PND0	1.33±0.022	1.284±0.014	1.341±0.022	*2.408*	*0.095*
Body weight of female pups at PND0	1.293±0.021	1.234±0.022	1.335±0.016	*6.931*	***0.002***
Body weight of male pups at PND28	13.975±0.229	14.533±0.309	14.2±0.475	*0.819*	*0.444*
Body weight of female pups at PND28	13.267±0.015	12.853±0.387	13.171±0.208	*0.633*	*0.533*

Table 1. *Data collected from the litters of Oil-, BPA- or BPS-treated dams.* Data are reported as Mean ± SEM. In bold, significant effect of treatments ($p \leq 0.05$) as revealed by one-way ANOVA.

Effects of exposure to BPA or BPS on oxytocin system

The analysis of the immunoreactivity for OXT (summarized in *Table 2*) showed a differential effect of BPs in the analyzed nuclei of exposed dams. We observed a significant difference in the total PVN, both in number of cells ($F_{(2, 9)}=6.858$, $p=0.016$; *Fig.3B*) and fractional area ($F_{(2, 9)}=7.186$, $p=0.014$ *Fig.3C*). This difference was due to a significant increase in OXT-ir, both in terms of OXT+ cell number ($p=0.014$) and fractional area ($p=0.012$) in the BPA-treated dams compared to the control ones (*Fig.3*). Moreover, the further analysis of the PVN subnuclei (PaDC/PaLM, PaMP, PaMM) confirmed the effects of BPA treatment on OXT-ir. Interestingly, both OXT+ cell number (Fig.3B; $p<0.001$) and fractional area ($p=0.015$; *Fig.3C*) were increase

in PAMP, but only fractional area was affected in PAMM (*p=0.006*; *Fig.3C*). No significant effects due to BPA treatment were found in PaDC/PaLM (*Fig.3*).
Analogously, we observed a significant effect of exposure in OXT-ir in SON (*Fig.4*), both in number of cells ($F_{(2, 9)}=6.956$, *p=0.015*; *Fig.4B*) and fractional area ($F_{(2, 9)}=4.322$, *p=0.048*; *Fig.4C)*. Once again, this difference was due to a significant increase in OXT-ir, both in terms of OXT+ cell number (*p=0.012*; *Fig.4B*) and fractional area (*p=0.048*; *Fig.4C*) in the BPA-treated dams when compared to the controls.
Remarkably, the analysis on the OXT-ir in PVN (*Fig.3*) and SON (*Fig.4*) of the BPS-treated dams did not highlight any significant effect of exposure in these nuclei compared to the control dams.

Table 2					
n° OXT+ cells	**Oil**	**BPA**	**BPS**	**One-way ANOVA**	
	Mean ± SEM	*Mean ± SEM*	*Mean ± SEM*	$F_{(2, 9)}$	*p*
PVN	30.333±4.503	49.417±3.794	36.417±2.626	*6.858*	***0.016***
PaDC/PaLM	6.5±1.323	7.75±0.832	7.583±0.956	*0.412*	*0.674*
PaMP	16.25±2.149	30.167±0.986	21.083±1.848	*16.633*	***0.001***
PaMM	7.583±1.511	11.25±2.016	7.75±1.343	*1.578*	*0.259*
SON	15.833±0.995	20±0.527	17.833±1.280	6.946	***0.015***
% FA	**Oil**	**BPA**	**BPS**	**One-way ANOVA**	
	Mean ± SEM	*Mean ± SEM*	*Mean ± SEM*	$F_{(2, 9)}$	*p*
PVN	37.157±4.474	57.757±3.143	43.843±4.026	*7.186*	***0.014***
PaDC/PaLM	13.804±2.091	16.752±1.314	13.432±1.207	*1.313*	*0.316*
PaMP	17.879±1.372	26.223±1.419	20.494±2.083	*6.639*	***0.017***
PaMM	6.616±1.661	14.844±1.066	9.927±1.388	*8.830*	***0.008***
SON	9.889±0.568	12.221±0.651	10.445±0.531	*4.322*	***0.048***

Table 2. *Results obtained from the analysis of OXT-ir in PVN and SON.* Data are reported as Mean ± SEM, both as number of positive cells and fractional area (% FA). In bold, significant effect of treatments ($p \leq 0.05$) as revealed by one-way ANOVA.

Discussion

Overall, the present results support the idea that in adulthood, as well as during development, there are some critical periods, which are sensitive to EDC perturbation of the hormonal environment. Under present conditions, the direct exposure to either BPA or BPS during both pregnancy and lactation led to some alterations in dams' brain and behavior. In both BPA- and BPS-treated dams, we observed a decrease in pup-related behaviors, along with an increase in pup-unrelated behaviors. Interestingly, only the BPA dams showed a significant increase in the OXT-ir in the PVN and SON.
During the first postnatal week, offspring mortality impacted differentially BPA and BPS litters, with more female dead pups among the BPA litters, while more male dead pups in the BPS litters, sharpening the difference in the sex *ratio*.

Maternal behavior consists of a set of behaviors, and it is known to be "activated" at or close to the delivery, under the influence of several different factors, such as the drop in circulating levels of progesterone together with an increase in circulating estradiol, and the intracerebral release of OXT due to vagino-cervical stimulation (Keller et al., 2019). Considering that maternal behavior mainly depends on the action of specific hormones on hormone receptors, it is a well-known target of endocrine disrupting chemicals (Keller et al., 2019). BPA exposure

during critical period of development is known to cause some alterations in the maternal behavior of the adult females (Keller et al., 2019), however, little is known about the effects on the dams directly exposed to EDCs (Keller et al., 2019; Kundakovic et al., 2013; Palanza et al., 2002). Furthermore, the effects of BPS have been poorly investigated (Catanese & Vandenberg, 2017). Our results showed that exposure to low dose of both BPs caused alterations in the displaying of the maternal behaviors on the directly exposed dams, particularly the BPS dams (less time in pup-related behavior). This suggests more alarming outcomes compared to previous studies (Catanese & Vandenberg, 2017), in which, however, different dose, administration and mouse strain were used, highlighting the need of further investigation on BPS effects. We also confirmed some, partially previously described (Kundakovic et al., 2013; Palanza et al., 2002), effects of BPA treatment on maternal behavior, underlying the changes at PND1, when we also observed the highest mortality rate among these litters. This seems to suggest that in the BPA-treated dams some impairments, or at least a delay, in the activation of the maternal care and in the recognition of the pups can occur.

Considering the observed behavioral alterations, the analysis of the OXT-ir in PVN and SON, highlighted some alterations due to BPA exposure. In the two nuclei, BPA exposure increased both the number of OXT-positive cells and the fractional area covered by immunoreactive structures, whereas BPS exposure did not. Previous studies evidenced organizational effects of neonatally administered BPA on the OXT system in the PVN (Patisaul, 2020; Witchey et al., 2019), probably acting through estrogen receptor β (ERβ) (Patisaul et al., 2003; Witchey et al., 2019). No data are available for the BPS, but BPS seems to impact less on ERβ, which is fundamental for estrogen pathway regulation in magnocellular neurons of the hypothalamus (Mitra et al., 2003), and more to estrogen receptor α (ERα) compared to BPA (Catanese & Vandenberg, 2017; Nourian et al., 2020). Thus, BPA and BPS may be involved in different mechanisms in order to induce the outcomes. In the present study, we observed the effects of a continuous exposure to BPs from the gestational to the lactating period, showing that only BPA, probably acting through ERβ, impacted altering the OXT system. On the one hand, BPS could involve other receptors, possibly the G protein-coupled estrogen receptor (GPER) largely present in the PVN (Grassi et al., 2016; Marraudino, Carrillo, et al., 2021), which mediates rapid estrogen signaling (Naderi & Kwong, 2020), and so the alterations of the circuits controlling the maternal behavior could be more dynamic and restricted to the observational period, on the other one, it could impact on other neural circuits (Naderi & Kwong, 2020). It is also interesting to note that when the BPA exposure is prolonged after the lactating period no alterations of the OXT system in both PVN and SON were observed (Bonaldo et al., 2021), thus the changes observed in the present study seems directly related to the alteration of the maternal behavior that was no longer expressed in long-term exposed dams.

At PND0, BPA litters showed a tendency towards higher male-to-female *ratio* compared to controls, while we observed the opposite among the BPS pups (lower male-to-female sex *ratio* compared to controls). Besides, the percentage of dead pups was higher in the BPs-treated dams' litters, compared to the controls' ones. Remarkably, the offspring mortality impacted differentially BPA and BPS litters, with more female pups found dead in the BPA litters, while more male pups found dead in the BPS litters, sharpening the difference in the male-to-female *ratio* at PND7.
Even though BPs did not affect the total number of pups per litter at birth, they tended to alter

the sex *ratio*, with more female pups among BPA litters and more male pups among the BPS groups, compared to the controls. After the first postnatal week, this difference was sharpened, due to pups' mortality, which affected differentially the two sexes among BPA and BPS litters. If on one hand female pups from BPA litters seemed to be more affected compared to male ones, on the other hand male pups from BPS litters, which are overall the ones displaying the highest mortality, died significantly more frequently compared to controls. It is known that prenatal exposure of female mice to either BPA or BPS cause fertility problems, such as reduced pregnancy rates, delivery, and nursing issues, and increased pups' mortality (Shi, Sekulovski, et al., 2019; Shi, Whorton, et al., 2019). In our experiments we showed that also the direct exposed dams could be affected by similar impairments. Furthermore, to the best of our knowledge, we described for the first time the different sex *ratio* among litters of directly exposed dams. This peculiar effect of both treatments is particularly tricking to understand, and it could be linked to *in utero* altered conditions which can promote the implant or the selective loss of fetus of one sex compared the other one (Kobayashi et al., 2010). For example, BPs exposure could have led to alterations not only in hormonal levels, but also in metabolic/energy state of the mother. In fact, it is known that male fetuses are more sensitive to unbalanced energy state, or stress in general, compared to females (Kobayashi et al., 2010). This could be the case in the BPS litters, in which we also observed an increased male pups' mortality, particularly at the delivery or right after birth, suggesting some prior problems. In the BPA-litters, the underlying mechanism could be different. We observed only a tendency towards lower female-to-male *ratio* at birth, together with a tendency towards an increased pups' mortality within the first postnatal week, especially at PND1. This suggests a relevant impact on pups' survival of the first postnatal day care, which appeared to be the most impacted by the treatment, as we discuss below.

Exposure to BPs, particularly BPA, is known to affect different aspects of reproduction (Chianese et al., 2018; Rubin, 2011; Vom Saal, 2016), and pregnancy itself (Filardi et al., 2020; Pergialiotis et al., 2018) . Known effects of BPA and the emerging ones of BPS on reproduction in female mice mostly followed pre- or peri-natal exposure (Shi, Whorton, et al., 2019). However, under present conditions we showed that, not only the offspring, but also the exposed dams could be affected by EDCs.

One of the limits of our study is that we performed the experiment in an inbred mouse model. Despite this, our results on the spontaneous maternal behavior of BPs-treated dams are in line with those performed on CD-1 strain (Catanese & Vandenberg, 2017; Palanza et al., 2002). In fact, the study published by Palanza *et al.* in 2002 highlighted a decrease in pup-related behavior in CD-1 dams exposed to BPA (10 µg/kg BW/day) in adulthood (Palanza et al., 2002). Furthermore, a more recent study by Catanese *et al.* showed some alterations in spontaneous maternal behavior in CD-1 dams exposed during pregnancy and lactation to two different doses of BPS (*i.e.*, 2 µg/kg BW/day or 200 µg/kg/BW/day), highlighting major effects on those exposed to the higher dose (Catanese & Vandenberg, 2017). Another limit for the present study is that for each BPs we tested only one dose*, i.e.*, 4µg/kg BW/day which is indicated by the EFSA as the TDI for BPA. Even if a further evaluation of different doses is needed to achieve a full risk assessment of exposure to those EDCs, nonetheless our results showed some significant impairments at the tested dose which is considered "tolerable".

In conclusion, exposure to BPs, even at low, but environmentally relevant, doses, particularly when it occurs during sensitive periods of adulthood, such as pregnancy and lactation, represents a risk not only for the developing offspring, but also for the dams themselves, through the functional alteration of neural circuits controlling fundamental behaviors, such as maternal behavior. Present results support the idea that new and more specific strategies are necessary to reduce and to contain the impact of environmental BPs on public health.

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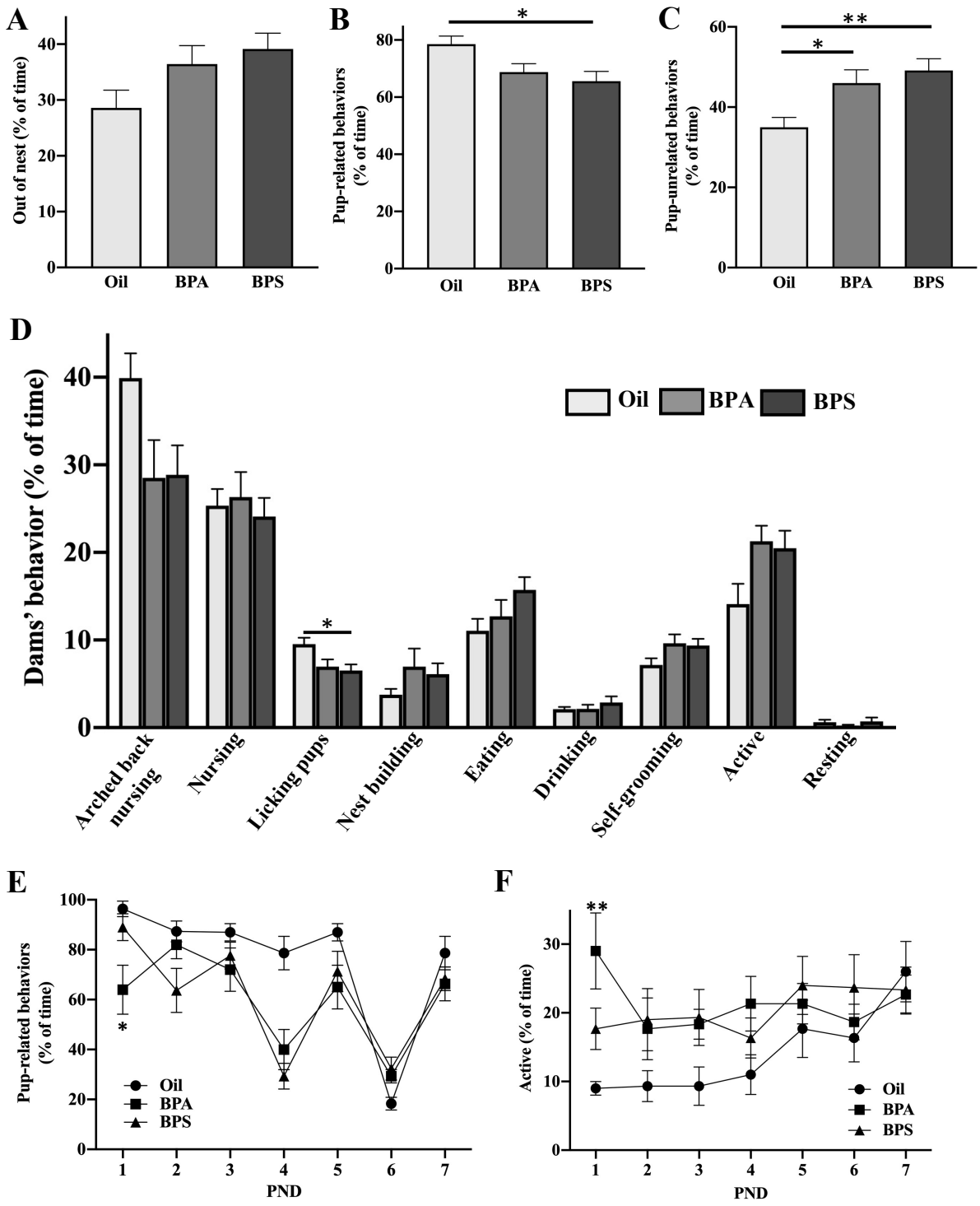

Figure 1*. Analysis of spontaneous maternal behavior of Oil-, BPA and BPS-treated dams.** Overall percentage of time spent out of nest ***(A), performing pup-related ***(B)*** or unrelated ***(C)*** behaviors by oil- (*light gray*), BPA- (*gray*) or BPS-treated (*dark gray*) dams within the seven days of observation of the spontaneous maternal behavior. ***(D)*** Overall percentage of time spent in the different analyzed behaviors by oil- (*light gray*), BPA- (*gray*) or BPS-treated (*dark gray*) dams. The analysis of daily percentage of time spent ***(E)*** performing pup-related behaviors or ***(F)*** active highlighted significant effects of BPA treatment on these parameters especially at postnatal day 1 (PND1). Data are expressed as mean ± SEM. One-way ANOVA ***(A, B, C)*** or two-way ANOVA for repeated measures ***(E, F, G)*** followed by Tuckey's HSD test revealed a significant effect of the treatments for $p \leq 0.05$. PND = postnatal day.

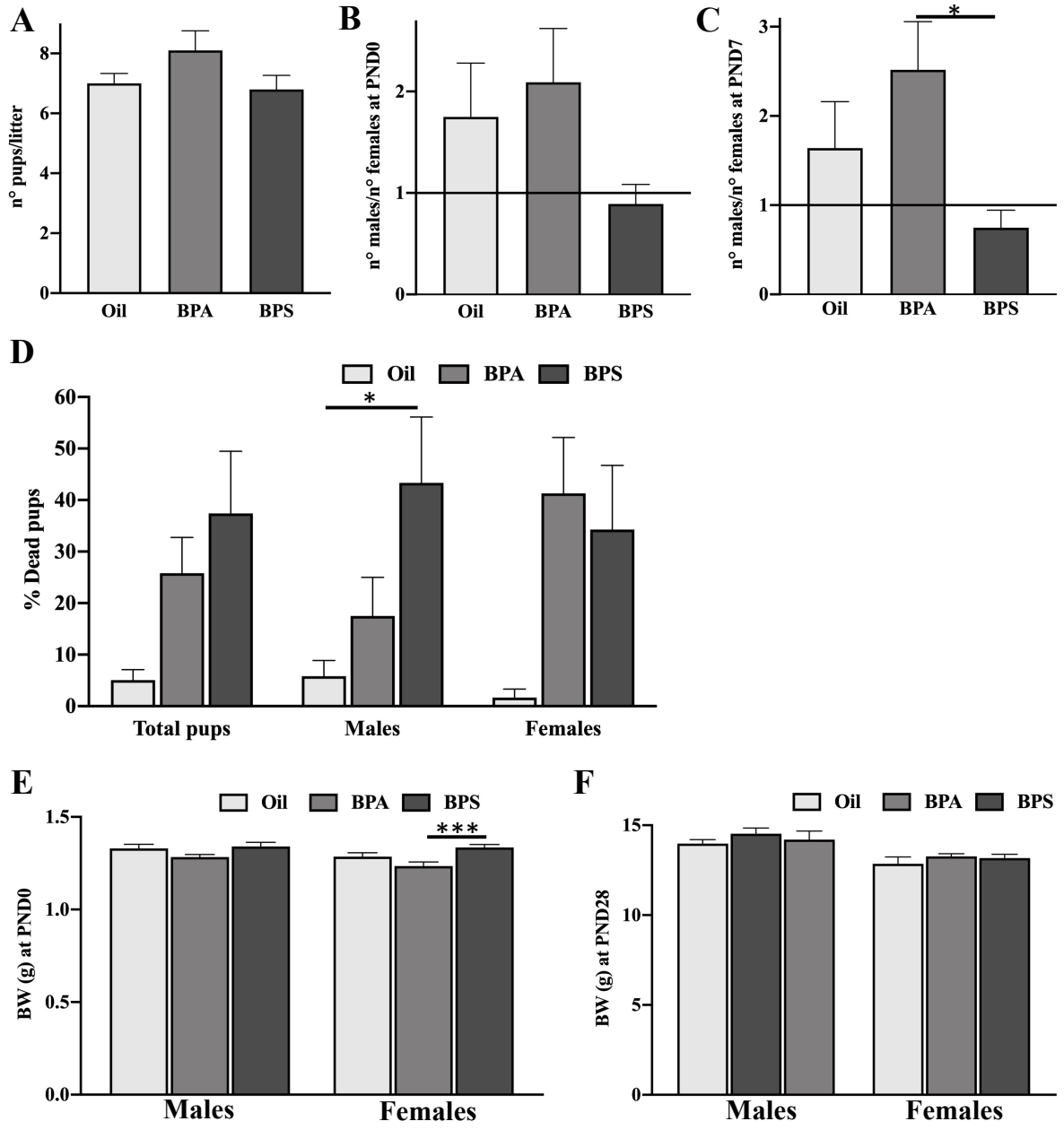

Figure 2*. Effects of BPA and BPS exposure to the litters.** ***(A) Total number of pups per litter at birth. Sex *ratio* (n° females/n° males) among litters at birth ***(B)*** and after the first postnatal week ***(C)***. ***(D)*** Percentage of total dead pups (*left*), male dead pups (*center*) and female dead pups (*right*) among litters obtained from oil (*light gray*), BPA- (*gray*) and BPS- (*dark gray*) treated dams. Body weight of male (*left side of the graph*) or female (*right side of the graph*) pups obtained from oil (*light gray*), BPA- (*gray*) and BPS- (*dark gray*) treated dams at birth ***(E)*** and after the first postnatal week ***(F)***. Data are expressed as mean ± SEM. One-way ANOVA followed by Tuckey's HSD test revealed a significant effect of the treatments for $p \leq 0.05$. PND = postnatal day.

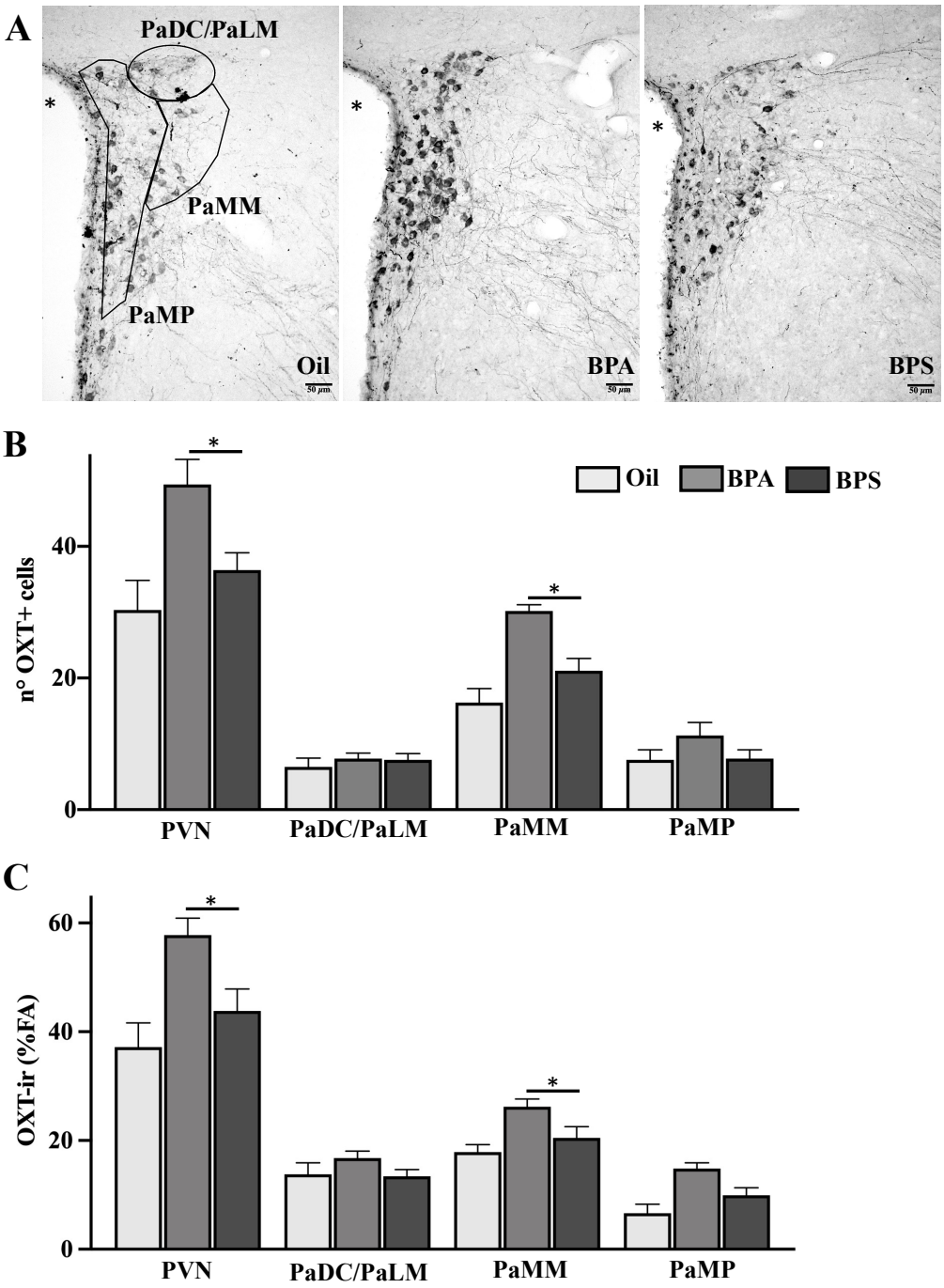

Figure 3. **Oxytocin immunoreactivity in the PVN of oil-, BPA- and BPS- treated dams.** ***(A)*** Representative image of oxytocin immunoreactivity in a coronal section of PVN of oil-treated (*left image*), BPA-treated (*central image*) and BPS-treated (*right image*) dams. Analysis of OXT-ir in PVN, expressed both as ***(B)*** number of OXT+ cells and ***(C)*** fractional area (FA), revealed a significant increase in BPA-treated dams (*gray*) compared to the control ones (*light gray*), mainly due to the medial magnocellular component of the nucleus (PaMM), while no significant effects were detected among the BPS-treated dams (*dark gray*). Data are expressed as mean ± SEM. One-way ANOVA followed by Tuckey's HSD test revealed a significant effect of the treatments for $p \leq 0.05$. Scale bar = 50µm. 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.

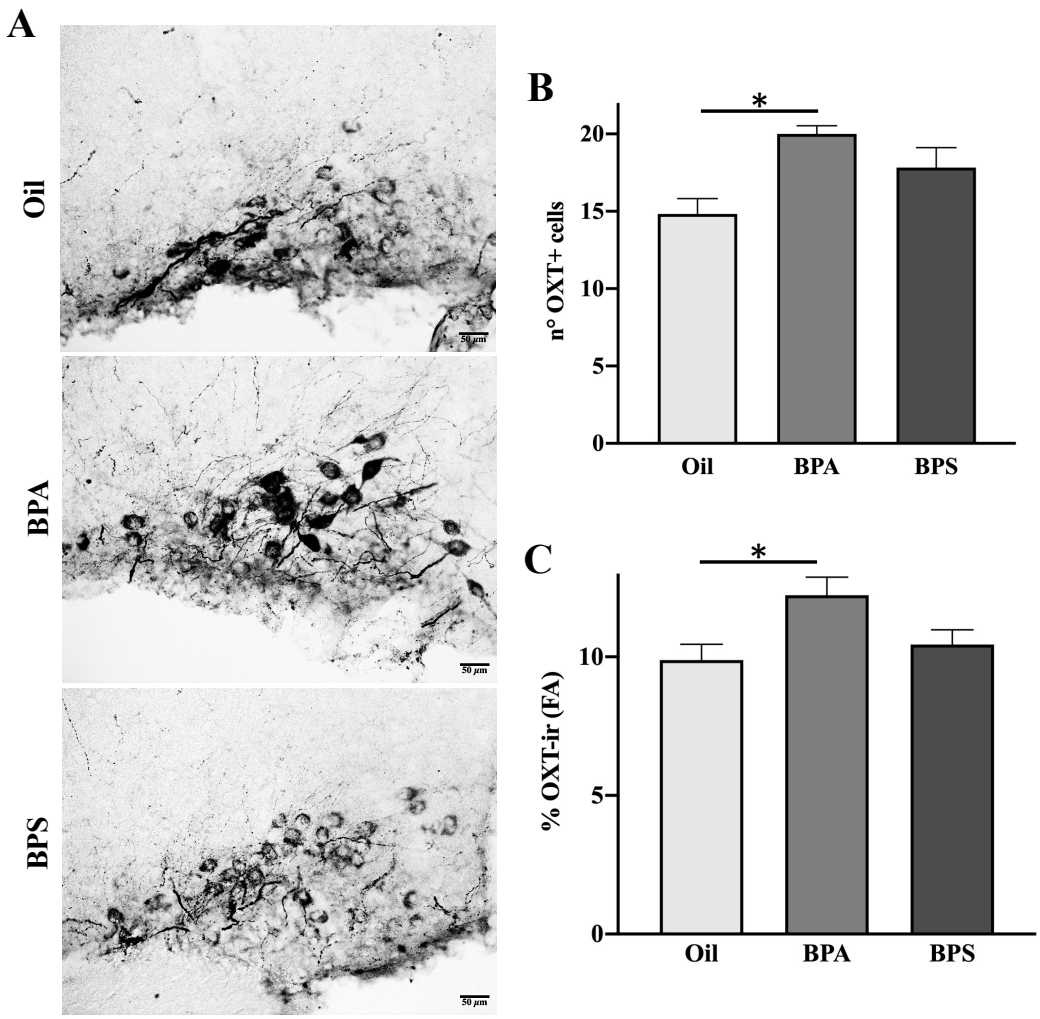

Figure 4. **Oxytocin immunoreactivity in the SON of oil-, BPA- and BPS- treated dams.** ***(A)*** Representative image of oxytocin immunoreactivity in a coronal section of SON of oil-treated (*left image*), BPA-treated (*central image*) and BPS-treated (*right image*) dams. Analysis of OXT-ir in SON, expressed both as ***(B)*** number of OXT+ cells and ***(C)*** fractional area (FA), revealed a significant increase in BPA-treated dams (*gray*) compared to the control ones (*light gray*), while no significant effects were detected among the BPS-treated dams (*dark gray*). Data are expressed as mean ± SEM. One-way ANOVA followed by Tuckey's HSD test revealed a significant effect of the treatments for $p \leq 0.05$. Scale bar = 50μm. OXT = oxytocin; SON = supraoptic nucleus; FA = fractional area.

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Perinatal exposure to bisphenol A or S alters differently sexual behavior and kisspeptin system in mice

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ABSTRACT

The effects of bisphenol A (BPA), a highly diffused endocrine disrupting chemical found mainly in plastics, on neural circuits and behaviors are well-known. However, the effects of its substitutes have not been fully investigated. For this reason, we have performed in the present study a comparison of the effects of perinatal exposure to bisphenol A (BPA) or S (BPS) on the kisspeptin system and reproductive behaviors in mice.
C57BL/6 dams were orally treated with a dose of 4 μg/kg body weight/day of BPA or BPS or with vehicle alone, from mating until the weaning of the offspring. We monitored the development of the offspring until postnatal day 90, when we analyzed the reproductive behavior (two-bedding T-Maze test and sexual behavior).
BPA caused a delay of the puberty in females, while BPS caused an anticipation in males, and, both BPs altered the estrous cycle in females. BPA-exposed males showed fewer mounts and intromissions and less time spent in the arm with the female bedding, while BPS-exposed males showed an increased number of mounts and intromissions and anogenital sniffing. Control males showed fewer mounts and intromissions towards BPS-exposed females.
The immunohistochemical analysis of the hypothalamic kisspeptin system highlighted some alterations in treated groups. In BPA- or BPS-treated females we observed an increase of kisspeptin within the rostral periventricular area, while BPA led to an increase in the paraventricular nucleus and BPS induced a reduction compared to control females. Among males, we observed a significant increase in the arcuate nucleus of BPA-treated males and a significant decrease in the paraventricular nucleus of BPS-treated ones. These results support the idea that perinatal exposure to low-dose of both BPA or BPS is altering, in a sexually differentiated way, some reproductive-relevant parameters, sexual behaviors and kisspeptin hypothalamic nuclei.

Keywords: endocrine disrupting chemicals, EDCs, BPA, BPS, kiss, hypothalamus, rostral periventricular area of the third ventricle, RP3V, paraventricular nucleus, PVN, arcuate nucleus, Arc

Author's contributions
BB designed and performed experiments, analyzed data, and wrote the draft. AC and MB

performed experiments and analyzed data. SG revised the draft. GCP and MM revised the draft and wrote the paper.

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All authors have no conflicts of interest to declare with respect to the research, authorship, and/or publication of this article.

Introduction

Bisphenols (BPs) are organic synthetic compounds mainly used to produce polycarbonate plastics (Catenza et al., 2021). They are an extremely abundant class of synthetic Endocrine Disrupting Chemicals (EDCs, *i.e.*, exogenous chemical, or mixtures of chemicals, that interfere with any aspect of hormone action) (Gore et al., 2015). The exposure to BPs is known to be responsible of a variety of adverse reproductive outcomes in the two sexes (Frye et al., 2012), impacting both on physiological (Siracusa et al., 2018) and behavioral aspects (Brehm & Flaws, 2019; Frye et al., 2012; Rebolledo-Solleiro et al., 2021) of reproduction.

Bisphenol A (BPA), the first synthetized BP, is known to be a reproductive toxicant in humans, affecting the oocyte and sperm production and quality, the uterine and ovary's health (*e.g.*, association with increased risk of endometriosis and polycystic ovary syndrome, PCOS), and the hormones' production in Sertoli and Leydig cells (Siracusa et al., 2018). Deleterious effects on male and female reproductive systems have been demonstrated in rodents (Rubin, 2011; You & Song, 2021), in which BPA exposure has been associated also with alterations of the sexual behavior (Palanza et al., 2021; Rebolledo-Solleiro et al., 2021), especially when the exposure occurs during critical period of development, such as the pre- or peri-natal one (Bakoyiannis et al., 2021; Rebolledo-Solleiro et al., 2021). However, given the complexity of sexual and socio/sexual behaviors, current literature mainly reported not obvious and often not consistent alterations (Arambula & Patisaul, 2019; Palanza et al., 2016, 2021). Despite this, previous studies generally highlighted a reduction in the sex-differences which are usually observed in the socio-sexual responses (Palanza et al., 2021).

Public health concerns persuaded the European Food Safety Authority (EFSA), after completing a full risk assessment, to establish a tolerable daily intake (TDI) for BPA, which is now set at 4 μg/kg of body weight (BW)/day (EFSA, 2015). Afterwards, BPA has been banned from the production of some consumer products (European Commision, 2011), and several structural analogues have been proposed (Catenza et al., 2021; Liu et al., 2021). Among these, bisphenol S (BPS), is, at present, one of the most used (Liao et al., 2012; Liao & Kannan, 2013, 2014), and it has already been detected in environmental and human samples (Wu et al., 2018). Increasing evidence suggest that BPS is not a safe alternative to BPA (den Braver-Sewradj et al., 2020; Mustieles et al., 2020; Naderi & Kwong, 2020; Thoene et al., 2020), as they share not only some structural similarities but also the endocrine disrupting properties (den Braver-Sewradj et al., 2020; Eladak et al., 2015; Gramec Skledar & Peterlin Masic, 2016; Naderi & Kwong, 2020; Rochester & Bolden, 2015). Therefore, the European Chemical Agency (ECHA) had classified as toxic for reproduction both BPA (Repr. 1B, H360F, *i.e.*, may damage fertility) and temporarily BPS (Repr. 2, H361f), for which the evaluation is still ongoing (European Chemicals Agency, n.d.).

BPS, as well as BPA, is known to have some effects on reproductive-relevant parameters in rodents, such as alterations of estrous cycle, folliculogenesis, plasma hormone levels, decreased implantation index, and decreased fertility in females, and decreased sperm counts and motility in males (den Braver-Sewradj et al., 2020).
If, on one hand, effects on sexual behavior of BPA exposure appear not to be consistent (Palanza et al., 2021), on the other one, potential effects of BPS still remain unexplored.

Even though behavioral outcomes of BPs exposure are still poorly addressed, alarming data come from the fact that different neural systems engaged in the control of sexual behavior are known to be affected by, at least, BPA exposure (Gore et al., 2019; Patisaul, 2020).
Among them, the kisspeptin (kiss) system is highly involved in the control of key aspects of reproductive functions, such as regulation of puberty onset and estrous cycle, and also peculiar behaviors like mate-preference and lordosis (Harter et al., 2018; Hellier et al., 2019; Navarro & Tena-Sempere, 2011).
In rodents, two major populations of kiss neurons, both in the hypothalamus, have been described: the one located in the rostral preoptic area of the third ventricle (RP3V) (Herbison, 2008), which responds to estrogens increasing kiss synthesis, and the other located in the arcuate nucleus (Arc), which responds to estrogens inhibiting of kiss production (Gottsch et al., 2004; Smith, Cunningham, et al., 2005; Smith, Dungan, et al., 2005). The kiss neurons from RP3V and Arc project primarily to the GnRH neurons as well as to other hypothalamic and extrahypothalamic areas (Yeo & Herbison, 2011), among which the paraventricular nucleus (PVN) arises as major target of the system (Marraudino et al., 2017; Yeo & Herbison, 2011). Within the whole kiss system (cells and projections), females display a much higher number of cells and fibers compared to males (Clarkson & Herbison, 2011; Kauffman, 2009; Knoll et al., 2013; Marraudino et al., 2017; Overgaard et al., 2013).
Kiss plays a fundamental role in the control of the activity of hypothalamic-pituitary-gonadal (HPG) axis. In fact, the gonadotropin-releasing hormone neurons (GnRH), the key regulators of gonadotropin pulsatile secretion, which is critical for the regulation puberty and fertility (Herbison, 2016), express the kisspeptin receptor 1 (kiss1r) (Novaira et al., 2014). GnRH neurons have the cell body mainly located in the rostral preoptic area (POA) and send their projections to the median eminence, where gonadotropins are release into the portal vasculature (Roa & Tena-Sempere, 2018). Their activity is regulated by estrogens via estrogen receptor α (ERα), which is not expressed in those neurons but is present in the kiss ones (Herbison, 2016). Thus, it has been demonstrated that the kiss/kiss1r signaling controls the estrogen-mediated regulation of GnRH, regulating the generation of the surge in the POA, and of the pulse in the Arc (Herbison, 2020).

The hypothalamic kiss system is known to be a target of endocrine disruption (Marraudino et al., 2021; Patisaul, 2013; Tena-Sempere, 2010), although few study directly investigated the effects of BPA, showing, in female rats, mainly a decreased expression of kiss in some hypothalamic nuclei (Cao et al., 2012; Navarro et al., 2009; Patisaul et al., 2009). In female mice, adult oral exposure to BPA (20µk/kg BW) results in an increased expression of both Kiss mRNA and protein in the RP3V, while the intracerebroventricular injection of BPA into the right lateral ventricle caused an increase as well in kiss mRNA within the RP3V, depending in both cases on the phase of the estrous cycle (X. Wang et al., 2014). More recently, it has been demonstrated in female mice that oral treatment with low-doses (5, 10, or 40 µg/kg BW/day)

BPA during the perinatal period caused an impairment of the developmental maturation Kiss system, with a consequently higher number of kisspeptin cells in RP3V and a fewer one in the Arc (Ruiz-Pino et al., 2019). If, on one hand, available data concerning the effects of BPA on Kiss system are mainly limited to females, on the other one, the possible effects of BPS are still not investigated at all. Still, the level of exposure to both BPs is increasing, due to their persistence in the environment (Chen et al., 2016; Vasiljevic & Harner, 2021; Wu et al., 2018).

Considering the low consistence of data regarding the BPA effects on sexual behavior and the limited ones available on kiss system, and the complete lack of data regarding possible effects of BPS exposure, in this study we aimed to evaluate the potential effects on sexual behaviors of adult male and female mice perinatally exposed to low-dose BPA or BPS (EFSA TDI for BPA, 4 µg/kg BW/day), performing the Two-bedding T-maze test and observing their spontaneous sexual behavior. Last, thanks to immunohistochemical techniques, we investigated the possible alterations of the kiss systems within the RP3V, Arc and PVN hypothalamic nuclei, which are known to be involved in the control of different aspect of reproductive-relevant parameters and behaviors (Harter et al., 2018; Hellier et al., 2019; Navarro & Tena-Sempere, 2011) and to be targeted at least by BPA exposure (Patisaul, 2013; Ruiz-Pino et al., 2019).

Materials and methods

Animals

Adult C57BL/6J mice from our 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 10:00 AM). Food (standard mouse chow 4RF21, Mucedola srl, Settimo Milanese, Italy) and water were provided *ad libitum*. One male and two female mice (3-month-old) were housed together to achieve a successful mating, assessed by the evaluation of the presence of the vaginal plug (assumed as gestational day 0, GD0) (Hasegawa et al., 2017).

Animal care and handling were according to the European Union Council Directive of 22nd September 2010 (2010/63/UE); all the procedures reported in the present study were approved by the Italian Ministry of Health (407/2018-PR) and by the Ethical Committee of the University of Torino (Project n° 360384).

Treatments

Experimental procedures are summarized in *Figure 1*. BPA (Sigma Aldrich, 239658, CAS 80-05-7) or BPS (Sigma Aldrich, 103039, CAS 80-09-1) were prepared for oral administration by dissolving them in corn oil (Sigma-Aldrich, C8267). 12 pregnant dams were assigned randomly to three experimental groups: control dams (receiving only vehicle, corn oil; n=4), BPA-treated dams (receiving 4 µg/kg BW/day of BPA, corresponding to the European TDI; n=4) and BPS-treated dams (receiving 4 µg/kg BW/day of BPS; n=4). The dose was calculated daily according to dams' body weight, recorded with an electronic precision balance (*Mod. Kern-440-47N, resolution 0.1g*).
We tested the same dose for both BPA and BPS to allow a precise comparison of the effects of the two bisphenols. Moreover, at present, although BPS is one of the most used BPA substitutes and it has already been detected in environmental and human samples (Catenza et al., 2021), at

present no user guidelines are available. Dams were treated starting at GD0, throughout pregnancy and lactation, until weaning of the offspring at postnatal day 28 (PND28). To resemble human exposure conditions, the daily treatment or the vehicle was given orally to the dams, by means of a pipette, to minimize dams' stress (Bo et al., 2016; Palanza et al., 2002). This type of administration allowed us to perform a perinatal treatment (covering both pre-natal and post-natal critical window of development) (Neier et al., 2019) on the offspring. In fact, it is known that both BPA and BPS can pass first through the placenta and then into the milk during the lactation (Cimmino et al., 2020; Mao et al., 2020).

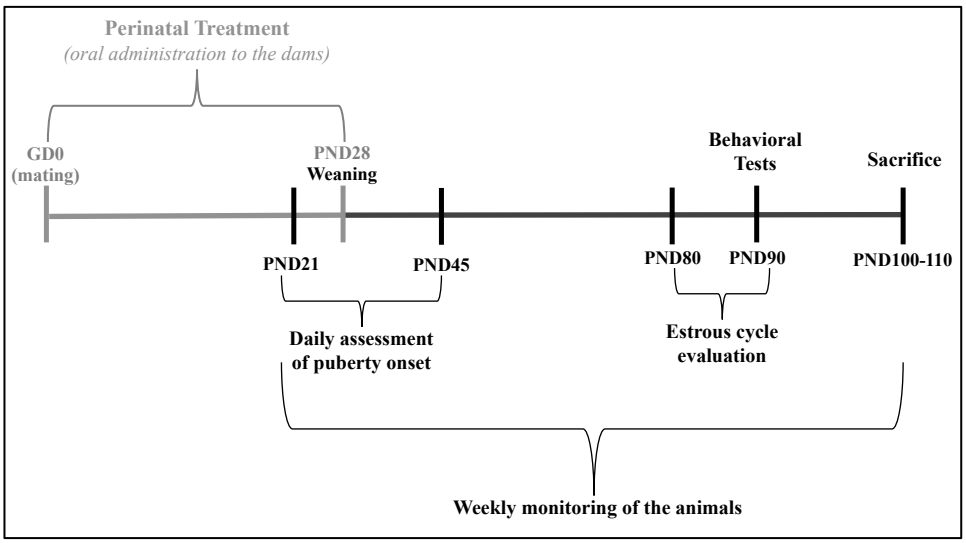

Figure 1. *Schematic temporal representation of experimental procedures.*

Litters were reduced to 8 pups at birth, to obtain an equal number of pups of both sexes, sexed *via* the measurement of the anogenital distance (AGD) (Manno 3rd, 2008). The pups were weaned at PND28 and housed in monosexual groups of 4 mice. They were monitored weekly until adulthood when the behavioral tests were performed.
We monitored the mice weekly, from the weaning until the sacrifice, evaluating in particular:
- Body weight (BW), recorded once a week.
- Food intake (FI, measured as g of food/animal/day) once a week.
- AGD: measured before the puberty onset (PND21), after the puberty onset (PND45), at PND60 (young adult) and PND90 (adult).
- Puberty onset, assessed by the Vaginal Opening (VO) in females (Gaytan et al., 2017) and by the balano-preputial Separation (BS) in males (Spears et al., 2013).
- The estrous cycle, evaluating the vaginal cytology smears (McLean et al., 2012), for at least 2 cycles (PND80-90 approximately).

Behavioral tests

Around PND90 the sexual-odor preference and the sexual behavior itself were evaluated performing the two-bedding T-maze test (Nunes, 2009; Yano, Sakamoto, and Habara 2012; Habedank, A.; KahnauP; Lewejohann 2021) and the observation of the spontaneous sexual behavior (Carvalho et al., 2018; Liu et al., 2020; McGill, 1962) (n=10±1 /group). The females, both testers and no-testers, were tested in estrus phase, assessed by vaginal smear (McLean et al., 2012). On the days of tests, mice were placed in the room in which the tests were performed at least 2 hours before starting, to allow the habituation to the room. Before starting and between

each trial, the testing apparatus were cleaned with 70% ethanol, being sure to thoroughly dry the apparatus to avoid exposure of mice to alcohol. The tests were performed in the dark, using only a 25-W red light, which mice cannot see, to help the operator to manipulate the animals (Palanza et al., 2002). Each test was recorded with an infrared camera placed above the apparatus in order to subsequently perform the behavioral analysis through the Ethovision XT Software (Noldus Information Technology, Wageningen, The Netherlands).

Two-bedding T-Maze

The test was performed using T-shaped maze (schematized in *Fig. 3A*), with 3 arms of equal size (50 × 10 cm x 15 cm walls). Before proceeding with the testing session, to exclude the preference for one of the two arms, the animals underwent to a first phase of habituation to the empty apparatus, in which they were placed at the base of the T and allowed to free explore it for 5 min. Then the mice were momentarily placed into a clean cage, while the operator added bedding obtained from unknown age-matched in estrus females' cage into the right arms of the apparatus and unknown age-matched males-derived bedding into the left one (Yano et al., 2012). For the testing session, the animals were positioned at the base of the T and were allowed to freely explore the apparatus for 10 minutes (Nunes, A C; da Luz Mathias, M; Ganem, 2009). After the recording, the parameters described in *Table 1* were analyzed thanks to Ethovision XT Software (Noldus Information Technology, Wageningen, The Netherlands). Some parameters (reported in *Table 1*) were evaluated for both the entire length of right or left arms, and specifically for the bedding spots placed within each arm. This allowed us to discriminate a more general, still odor-guided, exploration of the arms with a specific interaction with the female or male bedding.

Parameter	Description
Cumulative Duration (CD)	The cumulative time (s) spent by the tester in the central, in the right or in the left arm, and in the female or male bedding.
Latency to first entry	The time passed (s) until the mouse first entered the right and the left arm, or the female and male bedding.
Frequency of entrance	The number of times the tester mouse entered in the central, in the right or in the left arm, and in the female or male bedding. The mouse was considered to have entered right or left arm if all four paws had left the central arm.
Distance	The total distance traveled (cm) by the tester mice in the whole apparatus during the trial.
Mean velocity (v)	The mean velocity (cm/s) displayed by the tester mouse during the trial.

Table 1. *Parameters analyzed for each mouse in the two-bedding T-maze recorded trials.*

Sexual Behavior

The observation of spontaneous sexual behavior was performed into a square arena (30 × 30 × 38 cm walls), in which the male was introduced during the habituation phase to the room, 2 hours before the beginning of the test to ensure that the territoriality of the male inside the cage is established, which is necessary for a good expression of male sexual behavior (Carvalho et al., 2018). The test lasted 30 minutes: after the first 5 minutes, the receptive female was introduced into the arena and, after the recording, the parameters (Carvalho et al., 2018; Osakada et al., 2018) described in *Table 2* were analyzed thanks to Ethovision XT Software (Noldus Information Technology, Wageningen, The Netherlands). Unknown age-matched female (in estrus) or male mice were used as no-tester during the testing of experimental males and females respectively. The male- (*i.e.*, mount, intromission) and female- (*i.e.*, lordosis,

rejection) specific behaviors and were analyzed for either tester or no-tester mice depending on the recorded session.

Parameter	Description
Self-grooming	The number of times (frequency) the tester mouse licked or scratched its fur, washed its face, or licked its genitalia.
Allo-grooming	The number of times (frequency) the tester mouse licked or scratched the fur, washed the face, or licked the genitalia of no-tester mouse.
Sniffing	The number of times (frequency) the tester mouse olfactorily explored the no-tester one, either motionless or moving.
Anogenital sniffing	The number of times (frequency) the tester mouse olfactorily explored the genitalia of no-tester one.
Mount	Attempts of the male to mount the female, in the absence of intromission.
Intromission	Successful attempts of the male to mount the female, which led to ejaculation (avoided by the intervention of the operator).
Lordosis	Sexually receptive posturing in which the female presents its hindquarters by curving the lumbar region of the back towards the floor.
Protected rearing	The number of times the tester mouse reared on its hind paws in the border of the arena.
Unprotected rearing	The number of times the tester mouse reared on its hind paws in the center of the arena.
Rearing	Total number of times the tester mouse reared on its hind paws in the center or in the border of the arena.
Rejection	Sets of behavioral response (hiding the lumbar region of the back or attack the male) through which the females avoid or reject the male's exploring.

Table 2. *Parameters analyzed for each mouse in the sexual behavior recorded trials.*

Fixation and tissue sampling

At least 10 days after the performing of the behavioral tests, mice were sacrificed, by deep irreversible anesthesia (intraperitoneal injection of Zoletil 80 mg/kg/ Rompum 10 mg/kg) and transcardially perfused with 4% paraformaldehyde (PFA) solution. Females were sacrificed in estrus phase, assessed by vaginal smear (McLean et al., 2012). Brains were removed and stored in a 4% PFA solution for 24 hours, 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 (Marraudino et al., 2017).

Brains (n=4/group) were serially cut in the coronal plane at 30µm 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 (Paxinos et al., 2001). Sections were collected in a cryoprotectant solution (Watson Jr. et al., 1986) and stored at -20 °C.

Kisspeptin immunohistochemistry

The presence of kiss was detected by immunohistochemistry performed on free-floating sections from one series. Briefly, the sections were washed overnight in phosphate buffer (PBS) at pH 7.3. The following day, sections were first incubated with citrate buffer (citric acid 10 mM, 0.05% Tween, pH 6.0) previously heated at 95°C for antigen retrieval and then washed three times in PBS. Next, the sections were washed in PBS containing 0.5% 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 incubated for 30 min with blocking solution containing normal goat serum (Vector Laboratories, Burlingame, CA, USA) diluted in PBS, and then incubated one overnight at room temperature with polyclonal anti-kiss antibody (AC#566, a generous gift of Drs A. Caraty, I. Franceschini and M. Keller, Tours,

France; Rabbit, 1:5.000) diluted in PBS containing 0.5% Triton X-100. A biotinylated goat anti-rabbit secondary antibody (Vector Laboratories, Burlingame, CA, USA) diluted in PBS, pH 7.3–7.4, containing 0.2% Triton X-100 was then employed at a dilution of 1:200 for 60 min at room temperature. The antigen-antibody reaction was revealed by 60 min incubation with avidin–peroxidase complex (Vectastain ABC Kit Elite, Vector Laboratories, Burlingame, CA, USA). 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, Milano, Italy). The production, characterization, and specificity of used antibody has been described (Franceschini et al., 2006) and successfully used in previous studies (Marraudino et al., 2017, 2018). 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 covering the rostral periventricular area of the third ventricle (RP3V, Bregma 0.26 to –0.22 mm), the paraventricular nucleus (PVN, Bregma -0.58 to -0.94 mm) and the arcuate nucleus (Arc, Bregma -1.58 to -1.82 mm) were chosen according to the mouse brain atlas (Paxinos et al., 2001). Two sections of comparable levels 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.S., Firenze, Italy). Images were digitized by using a 20x objective for the nuclei acquisition. Digital images were processed and analyzed by ImageJ (version 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 (0.074 mm^2 for the RP3V and 0.118 mm^2 for the Arc). The PVN (total area 0.120 mm^2) was instead subdivided into four same-size squares (0.03 mm^2), following the different distribution within the nucleus of the analyzed system (Marraudino et al., 2017; Paxinos et al., 2001). In particular, we analyzed the distribution of kiss-ir within the dorsomedial (DM, 0.03 mm^2), the dorsolateral (DL, 0.03 mm^2) and the ventromedial (VM, 0.06 mm^2) regions of the PVN (Marraudino et al., 2021).

We evaluated the extension of the immunoreactivity (cell bodies, dendrites, fibers) in all the selected nuclei as fractional area covered by immunopositive material. In addition, we also counted the number of kiss-positive cells in the RP3V, but not in the Arc, as the high density of fibers does not allow to clearly see the cell bodies (the detailed procedures were described in (Marraudino et al., 2021; Viglietti-Panzica et al., 1994).

Statistical analysis

BW, FI and AGD were analyzed by three-way (sex, treatment, and time as independent variables) analysis of variance (ANOVA). All other quantitative data were analyzed by two-way way (sex and treatment as independent variables) ANOVA with SPSS 27 statistic software (SPSS Inc., Chicago, USA). If the ANOVA was significant, the *post-hoc* analysis was performed using the Tuckey's HSD test. Comparison between the estrous cycle evaluations was performed using the Student's *t*-test. Differences were considered statistically significant for values of $p \leq 0.05$. Data are shown as mean ± SEM (mean standard error).

Results

Effects of perinatal exposure to BPA or BPS on body weight, food intake and reproductive-related parameters

The analysis using three-way ANOVA for BW ($F_{(2,22)}=0.610$, $p=0.919$; *Fig.1A*), FI ($F_{(2,20)}=0.296$, $p=0.999$; *Fig.1B*), and AGD ($F_{(2,23)}=0.258$, $p=0.956$; *Fig.2C*,), with age, sex, and treatment considered as independent variables, showed no overall significant effects. In particular, no significant effects of BPA or BPS treatment on these parameters were observed ($F_{(2,22)}$=0.460, $p=0.985$, *Fig.2A*; $F_{(2,20)}=0.802$, $p=0.711$, *Fig.2B*; $F_{(2,23)}=0.426$, $p=0.861$, *Fig.1C*), while the sexual dimorphism was maintained ($F_{(2,22)}=22.929$, $p<0.001$, *Fig.2A*; $F_{(2,20)}=14.452$; $p=0.001$, *Fig.2B*; $F_{(2,23)}=396.435$, $p<0.001$; *Fig.2C*).

The perinatal treatment with both BPA or BPS resulted in a huge alteration of the puberty onset ($F_{(5,54)}=34.522$, $p<0.001$; *Fig.2D*). In particular, compared to the controls, the BPS caused in males a significant anticipation of the BS *(p=0.007; Fig.2D*), while the BPA caused in females a significant delay in VO (*p<0.001; Fig.2D*). Together these alterations caused the appearance of a sexual dimorphism in puberty onset between the BPs-treated males and females (*p<0.001*), which was not present between the controls (*p=0.112*).
Moreover, the subsequent analysis of estrous cycle in adult females revealed that both BPA and BPS treatments caused an alteration of the time spent in the different phases of the estrous cycle (*Fig.2E*). In particular, both BPA- (*p=0.02*) and BPS-treated (*p=0.004*) females spent more time in estrus and less time in metestrus (BPA, *p=0.046*; BPS, *p=0.037*), with a tendency to decrease also the time spent in diestrus (BPA, *p=0.058*; BPS, *p=0.083*), compared to control females (*Fig.2E*).

Effects of perinatal exposure to BPA or BPS on sexual-related behaviors

Results obtained from the analysis of the two-bedding T-maze test and of the sexual behavior are summarized in *Table 3* and *Table 4* respectively. Here we highlight the most interesting results (*Figure 3 and 4*).

Two-bedding T-maze

The analysis of the Two-bedding T-maze test (*Figure 3*) highlighted some significant differences among the groups (summarized in *Table 3*).
First, we observed that control males spent less time in the left arm (*p=0.010, Table 3*) and tended to spend more time in the right arm *(p=0.090, Table 3*) and in the presence of the female bedding (*p=0.078, Fig.3E*) compared to control females.
The BPA treatment caused significant alterations in males. In fact, compared to control males, the BPA-treated ones spent more time and entered more frequently in the left arms (*p=0.001, Table 3; p=0.013, Table 3*) and within the male bedding (*p=0.004; Fig.3B; p=0.002, Fig.3D*). Conversely, they spent less time in the right arm (*p=0.003, Table 3*) and within the female bedding (*p=0.001,* Fig.3E) in which they entered less frequently *(p=0.046; Fig.3G*), compared to control males. Moreover, BPA-treated males displayed also higher latency to first entry in the right arm (*p=0.026, Table 3*) also in presence of the females bedding (*p=0.027, Fig.3F*) compared to control ones. These results suggest that BPA treatment in males caused an alteration in sexual preference driven by sexual odor, increasing that towards males and decreasing that towards females.

On the other hand, BPS-treated males showed only higher latency to first entry in the left arm (*p=0.001, Table 3*) and in the presence of the males bedding (*p=0.005, Fig.3C*) compared to control males, suggesting that they were less likely to explore the male odor marked area of the apparatus.
In females, both BPs did not cause any significant alterations *(Table 3* and *Fig.3*).

Parameter	Oil		BPA		BPS		ANOVA	
	Males	Females	Males	Females	Males	Females	$F_{(5,57)}$	p
Cumulative duration (s) in:								
Central arm	90.068±5.033	89.701±12.689	73.538±2.986	82.03±8.561	88.745±8.285	79.513±3.558	*0.364*	*0.871*
Right arm	255.716±20.58	179.755±25.642	146.381±10.087	212.597±26.983	226.998±17.664	179.71±15.624	*3.929*	***0.004***
Female bedding	216.331±20.645	146.63±19.918	104.452±9.337	179.409±24.083	175.742±17.5	158.933±14.37	*4.413*	***0.002***
Lefty arm	219.134±19.478	323.927±24.428	348.676±10.897	269.026±24.457	247.147±20.823	265.503±23.471	*5.411*	***0.000***
Male bedding	172.466±18.072	240.965±30.349	289.145±12.661	234.483±484	196.462±17.091	235.702±22.077	*3.566*	***0.007***
Latency (s) to first entry in:								
Right arm	19.396±5.296	25.771±1.099	85.227±20.182	58.82±25.912	18.45±6.464	36.821±10.622	*3.374*	***0.010***
Female bedding	27.294±8.313	59.924±23.757	130.11±40.143	75.403±26.182	20.474±6.376	37.685±10.639	*3.269*	***0.012***
Left arm	4.855±1.251	9.427±3.058	13.562±2.729	14.779±6.252	40.144±10.449	19.946±5.578	*4.624*	***0.001***
Male bedding	6.132±1.227	14.706±6.255	10.4±1.806	18.308±5.88	34.86±7.625	19.992±5.961	*3.448*	***0.009***
Frequency of entrance in:								
Central arm	25±1.626	23.182±2.071	24.273±0.764	22.778±1.847	24.182±1.705	25.364±1.512	*0.788*	*0.562*
Right arm	9.4±0.702	8±0.751	6.364±0.338	8.667±1.404	9.546±0.957	8.727±0.589	*2.098*	*0.079*
Female bedding	11±0.894	8.364±1.081	7.273±0.488	8.778±1.011	9.818±0.893	10.546±0.846	*2.595*	***0.035***
Left arm	9.2±0.757	10±1	13.182±0.749	9.556±0.868	9.182±0.615	11.818±0.861	*4.123*	***0.003***
Male bedding	10.1±0.823	10.091±0.879	15.364±1.012	10.889±1.419	10.273±0.675	11.272±0.74	*4.994*	***0.001***
Distance traveled (cm)	3202.172±156.819	3160.798±168.202	3316.475±109.292	2543.76±554.771	3167.388±124.673	3438.581±158.07	*1.211*	*0.316*
Mean velocity (cm/s)	5.337±0.261	5.268±0.28	5.529±0.182	9.44±.534	5.282±0.209	5.958±0.356	*2.851*	***0.023***

Table 3. *Results obtained from the analysis of the parameters during the two-bedding T-maze test.* Data are reported as Mean ± SEM. Two-way ANOVA (sex and treatment as independent variables) revealed a significant effect for $p \leq 0.05$, highlighted in bold.

Sexual Behavior

The analysis of the spontaneous sexual behavior (*Figure 4*) highlighted some significant differences among the groups (summarized in *Table 4*).
First, while we had no difference in grooming ($F_{(5.57)}=2.250$, *p=0.062; Table 4*), we observed higher allo-grooming (*p<0.001, Fig.4A*), higher anogenital sniffing (*p<0.001; Fig.4D*) and higher unprotected rearing (*p=0.044, Fig.4H*) in control males compared to control females. These sexual differences were generally maintained; however, some specific parameters were

affected by the treatments. In fact, BPA treatment in males seemed to cause a general reduction in the sexual behavior, even if not significantly, as we observed lower allo-grooming (*Fig.3A*), lower anogenital sniffing (*Fig.4D*), lower number of mounts (*Fig.4E*) and intromissions (*Fig.4F*). Moreover, we observed lower rejections displayed by the no-tester females used to test the BPA-treated males, due to their general lower attempts of approaching (*Fig.4B*). Conversely, in the BPS-treated males we observed a significant increase in the anogenital sniffing (*p<0.001, Fig.3D*) compared to control males, along with an increase, even if not significantly, in the number mounts (*Fig.3E*) and intromissions (*Fig.3F*). Furthermore, we observed higher rejections displayed by the no-tester females used to test the BPS-treated males, due to their general higher attempts of approaching them (*Fig.3B*).
In females, both BPs did not cause any significant alterations (*Fig.4*). However, the analysis of the no-tester males suggested, even if not significantly, a reduced interest towards the BPS-treated females, as they displayed both lower number of mounts (*Fig.4E*) and intromissions (*Fig.4F*).

Parameter	Oil		BPA		BPS		ANOVA	
	Males	Females	Males	Females	Males	Females	$F_{(5, 57)}$	p
Self-grooming	15.1±1.59	33.8±10.499	12±1.635	33.222±11.232	14.91±3.004	23.636±3.86	*2.250*	*0.062*
Allo-grooming	54.4±4.622	8.7±1.919	42.636±2.107	8.667±1.405	50.364±5.066	8.455±1.186	*47.625*	***<0.001***
Sniffing	21.1±1.506	26.7±2.433	21.909±1.412	27.889±3.225	19.273±1.356	28.364±2.688	*3.202*	***0.013***
Anogenital sniffing	22.3±3.377	2±0.6	15.091±3.38	3±0.85	33.634±3.904	2.363±0.528	*23.888*	***<0.001***
Mount	6.1±2.488	4.5±2.07	1.455±0.824	5.111±2.27	5.727±2.701	1.636±0.704	*1.064*	*0.390*
Intromission	1.2±0.759	3.8±1.561	0.273±0.273	2.556±2.31	1.819±0.952	0.636±0.453	*1.210*	*0.317*
Lordosis	1.1±0.095	1±0	1±0	1±0	1±0	1±0	*1.044*	*0.401*
Protected rearing	70.2±3.773	62.5±7.468	41.09±3.515	65.778±9.902	53.818±5.582	70±7.81	*6.391*	***<0.001***
Unprotected rearing	28.8±4.228	9.2±1.81	31.545±3.904	16.556±5.352	35.636±6.647	10.727±3.32	*3.004*	***0.018***
Rearing	99±7.34	71.7±9.278	72.636±4.243	82.333±14.172	89.455±10.325	80.727±9.892	*1.184*	*0.329*
Rejection	5.5±1.394	26.7±7.412	3.272±1.301	19.556±6.912	15.727±2.562	15.909±3.822	*2.837*	***0.023***

Table 4. *Results obtained from the analysis of the parameters during the sexual behavior.* The male- (*i.e.*, mount, intromission) and female- (*i.e.*, lordosis, rejection) specific behaviors are reported for either tester or no-tester (*gray box*) mice depending on the recorded session. Data are reported as Mean ± SEM. Two-way ANOVA (sex and treatment as independent variables) revealed a significant effect for $p \leq 0.05$, highlighted in bold.

Kiss-ir analysis

The analysis of the immunoreactivity for kiss (summarized in *Table 5*) revealed that both BPs treatment affected the analyzed hypothalamic nuclei, differently in the two sexes.

First, we corroborated the presence of sexual dimorphism within all the analyzed nuclei in control mice (*Table 5*). In fact, oil-treated female mice displayed higher kiss-ir in RP3V, both in terms of number of Kiss-positive cells (*p<0.001, Fig.5B*) and FA (*p<0.001, Fig.5C*), in the

PVN (*p<0.001, Fig.4E*), and particularly within all its regions (DM, *p<0.001, Fig.5F*; DL, *p=0.015, Fig.5G*; VM, *p<0.001, Fig.5H*), and also in Arc (*p=0.001, Fig.5L*) compared to control males. The treatments did not alter the presence of the sexual dimorphism (*Table 5* and *Fig.5*), except for the BPA-treated males and females in the Arc, which resulted in the abolition of sexual differences (*p=0.126, Fig.4L*), due to a significant increase of kiss-ir among BPA-treated males compared to control males within the nucleus (*p=0.013, Fig.5L*).

Number of kisspeptin positive cells								
Zone	**Oil**		**BPA**		**BPS**		**ANOVA**	
	Males	**Females**	**Males**	**Females**	**Males**	**Females**	**F (5, 18)**	**p**
RP3V	3.75±0.322	17.75±1.8	4.75±0.25	26.75±2.287	3.625±0.315	28.75±1.613	*73.319*	***<0.001***
Fractional Area (%)								
Zone	**Oil**		**BPA**		**BPS**		**ANOVA**	
	Males	**Females**	**Males**	**Females**	**Males**	**Females**	**F (5, 18)**	**p**
RP3V	2.413±0.443	15.889±1.01	3.605±0.236	22.624±1.992	2.813±0.3	27.394±2.092	*75.457*	***<0.001***
PVN	5.156±0.205	20.575±0.945	3.6±0.112	26.289±0.751	3.883±0.23	15.865±0.245	*350.278*	***<0.001***
- DM	1.241±0.063	3.87±0.322	0.856±0.075	4.983±0.329	0.946±0.075	3.477±0.199	*70.552*	***<0.001***
- DL	0.818±0.22	1.493±0.142	0.367±0.054	1.499±0.105	0.234±0.05	0.922±0.108	*17.893*	***<0.001***
- VM	3.097±0.44	15.213±0.891	2.377±0.162	19.929±0.858	2.704±0.217	11.292±0.107	*186.919*	***<0.001***
Arc	15.013±1.136	24.652±1.12	22.539±2.365	27.833±1.01	10.963±0.893	29.616±1.29	*27.849*	***<0.001***

Table 5. *Results obtained from the analysis of kiss-ir in all selected nuclei.* Data are reported as Mean ± SEM, both as number of positive cells and fractional area for the RP3V and as fractional area alone for PVN and Arc. Two-way ANOVA (sex and treatment as independent variables) revealed a significant effect for $p \leq 0.05$, highlighted in bold. RP3V = rostral periventricular area of the third ventricle, PVN = paraventricular nucleus, DM = dorsomedial region of the paraventricular nucleus, DL = dorsolateral region of the paraventricular nucleus, VM = ventromedial region of the paraventricular nucleus, Arc = arcuate nucleus.

The major alterations were found in BPs-treated female mice. BPA-treated females showed a significant increase in RP3V, in both number of kiss-positive cells (*p=0.002, Fig.5B*) and FA (*p=0.016, Fig.5C*) and in the total PVN (*p<0.001, Fig.5E*), which was mainly due to the DM (*p=0.016, Fig.5F*) and VM (*p<0.001, Fig. 5H*) regions of the nucleus. The BPS-treated females showed likewise a significant increase in RP3V, in both number of kiss-positive cells (*p<0.001, Fig.5B*) and FA (*p<0.001, Fig.5C*), compared to control ones. Conversely, in the PVN, they showed a significant reduction of kiss-ir (*p<0.001, Fig.5E*), which was mainly due to the VM (*p=0.001, Fig.5F*) region of the nucleus, but was significant also in the DL (*p=0.05, Fig.5G*) part. Both treatments did not affect kiss-ir within the Arc in the females (*Fig.5H*).

Particularly interesting is that in the BPA-treated males, as we said, we observed a significant increase in kiss-ir only in the Arc (*p=0.013, Fig.5L*) compared to control ones, while no significant effects were found in RP3V (*p=0.995*, *Fig.5B; p=0.994, Fig.5C*) neither in PVN (*p=0.323, Fig.5E*) or its DM (*p=0.785, Fig.5F*), DL (*p=0.173, Fig.5G*) and VM (*p=0.934, Fig.5H*) regions. On the other hand, BPS-treated males displayed a significant decrease of kiss-ir in the DL (*p=0.043, Fig.5G*) compared to control males, while no significant effects were

found in RP3V (*p=1.000, Fig.5B; p=1.000, Fig.5C*), in total PVN (*p=0.531*, *Fig.5E*) or its DM (*p=0.915, Fig.5F*) and VM (*p=0.995, Fig.5H*) regions, neither in Arc (*p=0.350, Fig.5L*).

Discussion

The results of this study highlighted that perinatal exposure to low-dose of both BPA or BPS is altering, in a sexually differentiated way, not only some reproductive-relevant parameters but also sexual behaviors and kiss-ir within the RP3V, PVN, and Arc hypothalamic nuclei.

First, we corroborated previous results describing alterations in the puberty onset and in the estrous cycle due to BPs exposure (den Braver-Sewradj et al., 2020; Rubin, 2011; You & Song, 2021). Our work, however, highlights different effects of the two tested BPs in the two sexes. In fact, we showed that BPA-treated females displayed a delay in the VO, while BPS-treated males displayed an anticipation of the BS. BPA exposure during critical periods is known to cause alterations either in terms of anticipation (Nikaido et al., 2004; Ruiz-Pino et al., 2019) or delay (Franssen et al., 2016; Naule et al., 2014; Vandenberg et al., 2007) of the puberty onset in female rodents. Perinatal low-dose BPA exposure did not seem to affect puberty onset in males, as emerged also by little evidence present in the literature (Hass et al., 2016). Interestingly, our data showed that also BPS exposure affected pubertal timing, but only in males, anticipating the BS. Even if there is some evidence of BPS effects on reproductive functions (den Braver-Sewradj et al., 2020), accelerated puberty has been reported only in females (Shi et al., 2019). We observed this effect in males. Effects of BPs exposure described in the literature appeared to be quite variable, and this variance in the results had been linked to differences in experimental models, time windows, doses, and routes of exposure, and potentially to different effects of BPA at central (*e.g.*, the kiss-mediated regulation of GnRH pulsatile secretion) and peripheral (*e.g.,* canalization which led to VO) levels (Franssen et al., 2021; Ruiz-Pino et al., 2019).
Another important point related to the analyzed reproductive parameters regards the alterations of the estrous cycle. The analysis of the estrous cycle in adult females revealed that both BPA and BPS treatments caused an alteration of the time spent in the different phases of the estrous cycle, increasing the time spent in estrus in spite of non-estral phases. These results are in line with available literature, which described prolonged estrous cycle in female mice treated in developmental-relevant time windows with both BPs (den Braver-Sewradj et al., 2020; Rubin, 2011; You & Song, 2021).

As described in the introduction, the effects of BPA exposure on sexual behavior described in the literature appear not to be consistent (Bakoyiannis et al., 2021; Palanza et al., 2021), and almost nothing is known about the potential effects of BPS. In the present study, we reported that both BPs partially altered some aspects of sexual and sexual-related behaviors, mainly in males.
The Two-bedding T-maze test allows investigating the sexual-odor preference of the tester mice (Habedank, A.; KahnauP; Lewejohann, 2021; Nunes, A C; da Luz Mathias, M; Ganem, 2009). BPs treatments induced, in our experiment, some alterations of these behaviors chiefly in males. In particular, BPA-treated males seemed to be the most affected, spending more time within the left arms and particularly in the presence of male bedding compared to the controls, suggesting an alteration in sexual preference driven by sexual odor, increasing that towards

males and decreasing that towards females. On the other hand, BPS-treated males showed higher latency to first enter in the left arm with or without the male bedding compared to control ones, suggesting that they were less likely to explore the male odor-marked area of the apparatus. We found no significant alterations in treated females.
The analysis of spontaneous sexual behavior suggested that sex differences in peculiar behaviors were generally maintained among the treated group. However, some specific behaviors were affected by the treatments. Once again, BPA treatment in males seemed to cause a general reduction (lower allo-grooming, anogenital sniffing, lower number of mounts and intromissions) in the approach towards no-tester females, which displayed lower rejections towards them due to their lower insistence. Conversely, in the BPS-treated males, we observed a general increase (higher anogenital sniffing, tendency to higher number of mounts and intromissions) in the approach towards no-tester females, which, for their part, displayed higher rejections towards them due to their higher insistence. Also in this case, we did not observe any significant alterations in the treated females. However, the analysis of the no-tester males suggested a reduced interest (lower number of mounts and intromissions) towards the BPS-treated females.

Thus, our study suggested that both BPs partially altered some aspects of sexual and sexual-related behaviors, mainly in males. Overall, BPA-treated males displayed the major alterations in both indirect (odor-driven) and direct sexual behaviors. This could be in line with the general reduction in the sex differences, which are usually observed in the socio-sexual responses, due to EDCs exposure (Palanza et al., 2021). Furthermore, it has been recently described that the exposure of male mice to BPA led to increased socio-sexual exploration with the same sex (Gao et al., 2020) and to the elimination of the preference for opposite-sex urine odor (J. Wang et al., 2021). Furthermore, it has been reported that low-dose BPA caused also decreased number of intromissions, lower copulatory efficiency and higher mount and intromission latency in male rats, while female sexual behavior is not affected (Jones et al., 2011) or is even potentiated (Farabollini et al., 2002). On the other hand, even if some behavioral outcomes of BPS exposure started to become clearer (Naderi & Kwong, 2020), such as an impairment in social behavior (Mornagui et al., 2019), there are no precise evidence concerning its effects on sexual behavior either in males or females. Despite this, we observed both a potentiated male sexual behavior in BPS-treated mice and an interesting reduction in no-tester males' approach towards BPS-treated females, which could be linked to alteration of female pheromone production which is highly important in the control of a wide set of behavioral responses in mice (Stowers & Liberles, 2016).

Previous studies revealed that the kisspeptin system is particularly sensitive to exposure to several EDCs including BPA (Bateman & Patisaul, 2008; Bellingham et al., 2009; Cao et al., 2012). In the present study, we demonstrate that kiss-ir is differentially affected in both sexes by BPs treatment in all analyzed hypothalamic nuclei. We showed that BPA-treated females had a significant increase in RP3V, in the total PVN (mainly due to the DM and VM regions of the nucleus), while the BPS-treated ones showed a significant increase in RP3V and a significant reduction in the PVN (mainly due to the VM region but present also in the DL). In the BPA-treated males, we observed a significant increase in the Arc, while the BPS-treated ones displayed a significant decrease in the DL. However, all these changes had no effects on the sexual differences of the system. Our results corroborated the idea that kiss system is a

target of BPA action (Ruiz-Pino et al., 2019; X. Wang et al., 2014) and highlighted, for the first time, that it could be affected also by BPS exposure during the perinatal period.

In females, BPA and BPS had the same effect in RP3V, causing an increase in kiss-ir, which has been already described for BPA (Ruiz-Pino et al., 2019; X. Wang et al., 2014). Both BPA and BPS could act as positive modulators of ERα (Park et al., 2020), contributing to the observed alterations in puberty onset and in the estrous cycle. Considering the delicate positive/negative feedback control of the reproductive functions (Kaprara & Huhtaniemi, 2018), those alterations could lead to an impairment of the HPG axis (Pivonello et al., 2020; Santoro et al., 2019), which could give rise to a self-maintained distorted mechanism.
Conversely, we observed the opposite effect of the treatments within the female PVN. In the BPA-treated females, we observed an increased kiss-ir in the medial region of the nucleus, where corticotropin-releasing hormone (CRH) and thyrotropin-releasing hormone (TRH) parvocellular neurons, highly involved in the control of organism homeostasis, are located (Kondo et al., 2021; Wamsteeker Cusulin et al., 2013), and in which the nucleus displayed the highest expression of newly identified kisspeptin receptor (*Npffr1*) (Higo et al., 2021). This alteration could be in line with the previous one, considering the role of kiss in integrating metabolic inputs in order to regulate reproductive functions (Harter et al., 2018). On the other hand, we observed a kiss-ir reduction in VM of BPS-treated females: these opposite effects could be due to the fact that estrogen receptors are differentially impacted by the two BPs (Catanese & Vandenberg, 2017; Nourian et al., 2020), even if both are known to act on all estrogen receptors (Park et al., 2020). BPS seems to impact less on ERβ, which is fundamental for estrogen pathway regulation in magnocellular neurons of the hypothalamus (Mitra et al., 2003), and more on ERα compared to BPA (Catanese & Vandenberg, 2017; Nourian et al., 2020). Considering the different expression of the two ERs within PVN (Mitra et al., 2003), it is possible that BPA could act directly on PVN population through ERβ locally expressed, while BPS could act indirectly on PVN, through ERα present in neurons projecting to this nucleus (Catanese & Vandenberg, 2017; Grassi et al., 2010; Nourian et al., 2020; Park et al., 2020). Interestingly, we observed that BPS decreased kiss-ir in the DL of both treated males and females. In this region, are located not only part of the neurosecretory magnocellular neurons (Otero-Garcia et al., 2016), but also long-projecting pre-autonomic neurons, which send their axons to the brainstem and the spinal cord (lateral gray horn of the spinal column), and GABA-ergic interneurons (Ferguson et al., 2008; Geerling et al., 2010). The long-projecting pre-autonomic neurons in DL are known to project to the Nucleus of the Solitary Tract (NTS) (Ferguson et al., 2008; Geerling et al., 2010), which is mainly involved in life-sustaining functions (*e.g.*, appetite, digestion, breathing, and blood pressure) (Gasparini et al., 2020) but it has potential roles in the regulation of both reproduction and stress axis (for a review see (Brunton & Russell, 2008). Thus, if in females the alterations could be linked to altered reproductive parameters (NTS is involved in circuits controlling estrous cyclicity) (Feng et al., 2007), in males an alteration could be hypothesized of NTS integration of reproductive and stress axis (Grover et al., 2020), resulting in a partially altered sexual behavior.

Last, the observed increase in kiss-ir within the Arc of BPA-treated males could be linked to the different alterations observed in the sexual behaviors. First, kiss in Arc is hugely involved in the control of metabolism and energy balance (Dudek et al., 2018; Padilla et al., 2019; Patel & Smith, 2020): reproduction is strictly related to the metabolism of the organism and

impairment in the perception of the energy state could lead to alterations in the sexual behavior (Patel & Smith, 2020). Furthermore, even if it seems that less-described kisspeptin neurons located in the amygdala play a central role in the olfactory control of the gonadotropic axis (R Pineda et al., 2017; Rafael Pineda et al., 2021), also kiss population in the Arc appear to be a target of pheromone actions (Boehm et al., 2005), and in particular of male ones (Sakamoto et al., 2013), potentially underlying the altered behavior observed both during the Two-bedding T-maze and the sexual one.

In conclusion, our results suggest that perinatal exposure to both BPA and BPS, even at low dose, leads to alterations in reproductive-related parameters in the two sexes and also in the displaying of sexual behavior, especially in males. Both BPs, differentially in the two sexes, affected kiss system in the RP3V, PVN, and Arc hypothalamic nuclei, and these impairments could be partially linked to the observed physiological and behavioral alterations. The possible health implications of exposure to BPs must be avoided, or at least limited, by new and more stringent regulations on the use of these compounds.

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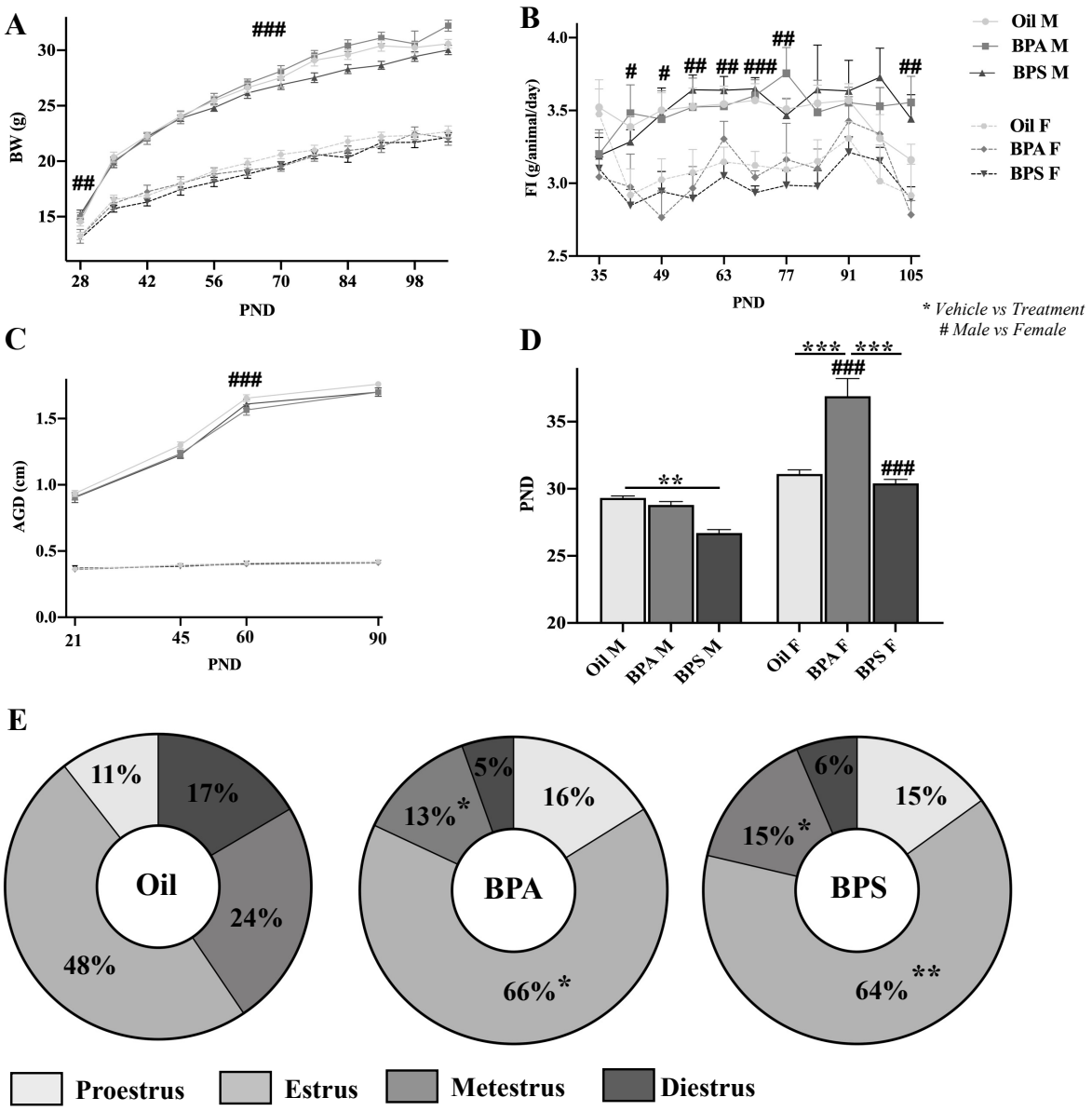

Figure 2*. Effects of BPA and BPS exposure on body weight, food intake and reproductive-related parameters.** Body weight ***(A) and food intake ***(B)*** weekly evaluation from the weaning (PND28) until the sacrificed of the animals. ***(C)*** Measurement of anogenital distance (AGD) before puberty (PND21), after puberty (PND45), in young adult (PND60) and adult (PND90) mice. ***(D)*** Evaluation of puberty onset through the assessment of the PND of vaginal opening (VO) for females and balano-preputial separation (BS) for males. ***(E)*** Mean percentage of time spent in the different phases of the estrous cycle, assessed by vaginal cytology smears, in the Oil- (*left*), BPA- (*center*) or BPS- (*right*) treated females. Data are expressed as mean ± SEM. Statistical analysis revealed a significant effect for $p \leq 0.05$ (* = vehicle vs treatment; # = male vs female). BW = body weight; FI= food intake; AGD = anogenital distance; PND = postnatal day.

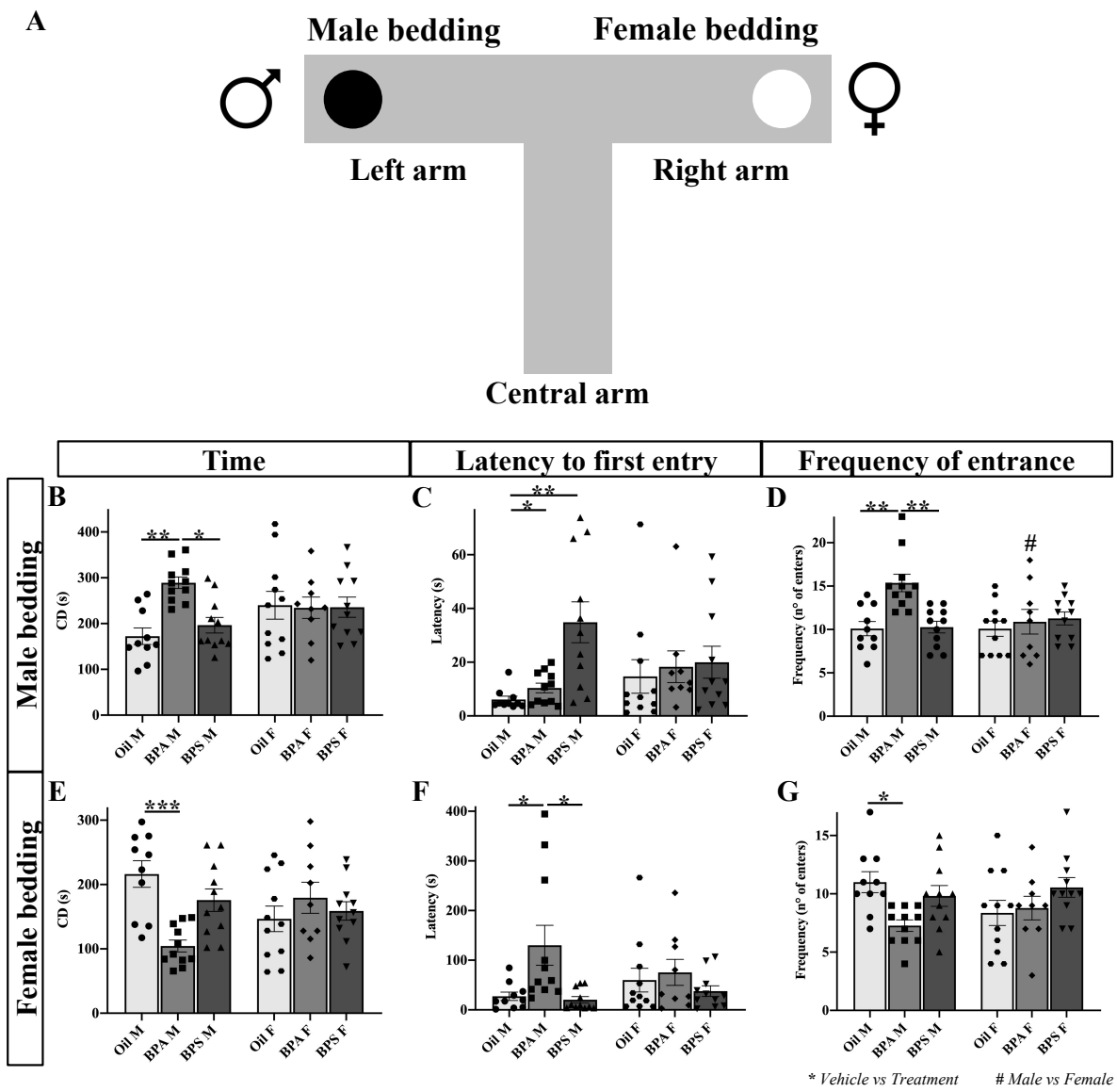

Figure 3*. Analysis of Two-bedding T-maze test in control and BPs-treated mice.** ***(A) Schematic representation of the two-bedding T-maze apparatus. Time spent ***(B, E)***, latency to first entry ***(C, F)***, and frequency of entrance ***(D, G)*** in the male (*up*) or female (*bottom*) bedding by the Oil-(*light gray*), BPA-(*gray*) or BPS-(*dark gray*) treated male (*left side of the graphs*) or female (*right side of the graphs*) mice. Data are expressed as mean ± SEM. Two-way ANOVA revealed a significant effect for $p \leq 0.05$ (* = vehicle vs treatment; # = male vs female). CD = cumulative duration.

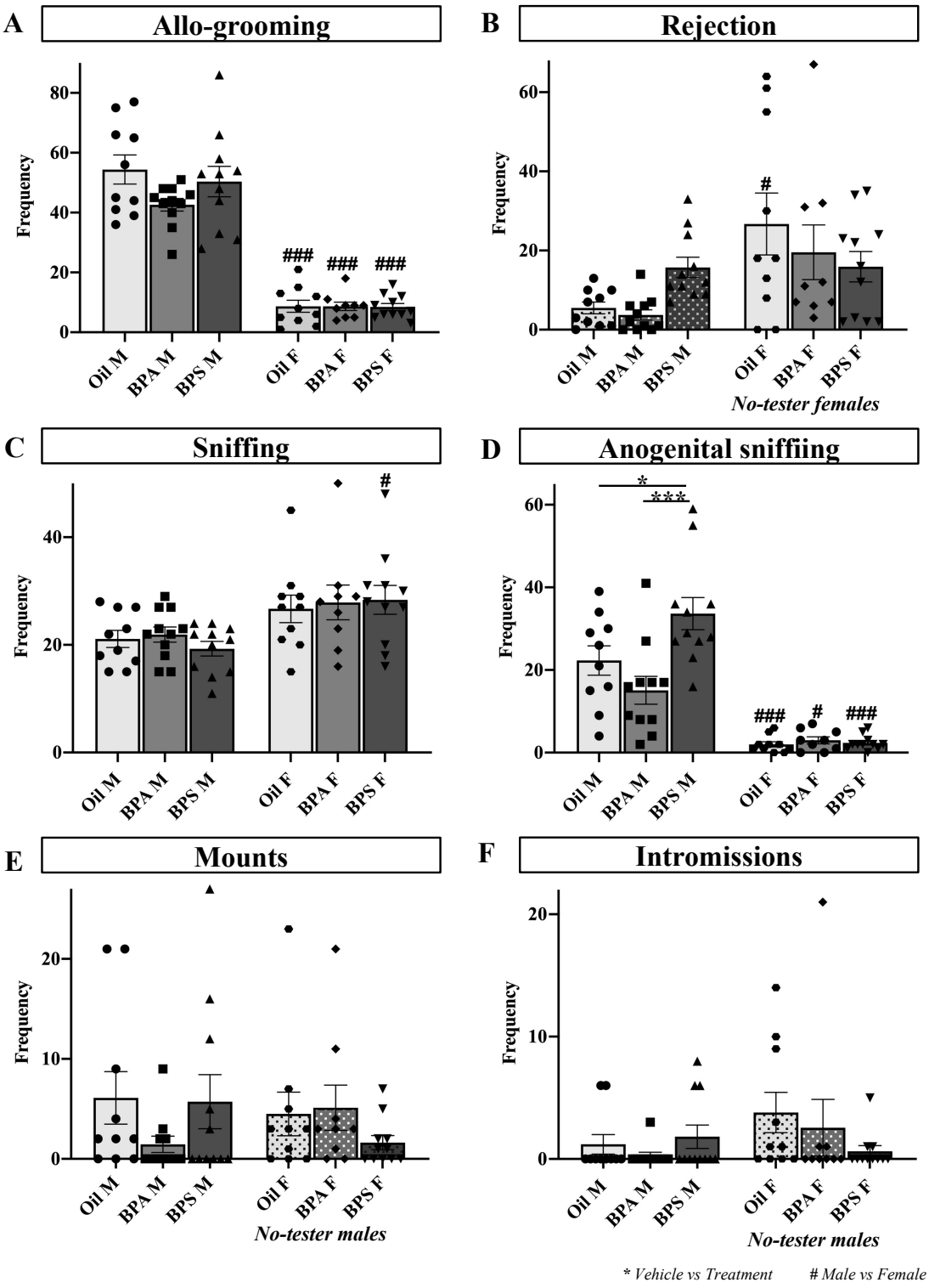

Figure 4*. Analysis of spontaneous sexual behavior in control and BPs-treated mice.** The histograms show the frequency (number of times) the Oil-(*light gray*), BPA-(*gray*) or BPS-(*dark gray*) treated male (*left side of the graphs*) or female (*right side of the graphs*) mice displayed the following specific behaviors during the recorded session: ***(A) allo-grooming, ***(B)*** rejection, ***(C)*** sniffing, ***(D)*** anogenital sniffing, ***(E)*** mounts and ***(F)*** intromissions. Male- (*i.e.*, mount, intromission) and female- (*i.e.*, rejection) specific behaviors are reported for either tester (*fill bar*) or no-tester (*dotted bar*) mice depending on the recorded session. Data are expressed as mean ± SEM. Two-way ANOVA revealed a significant effect for $p \leq 0.05$ (* = vehicle vs treatment; # = male vs female).

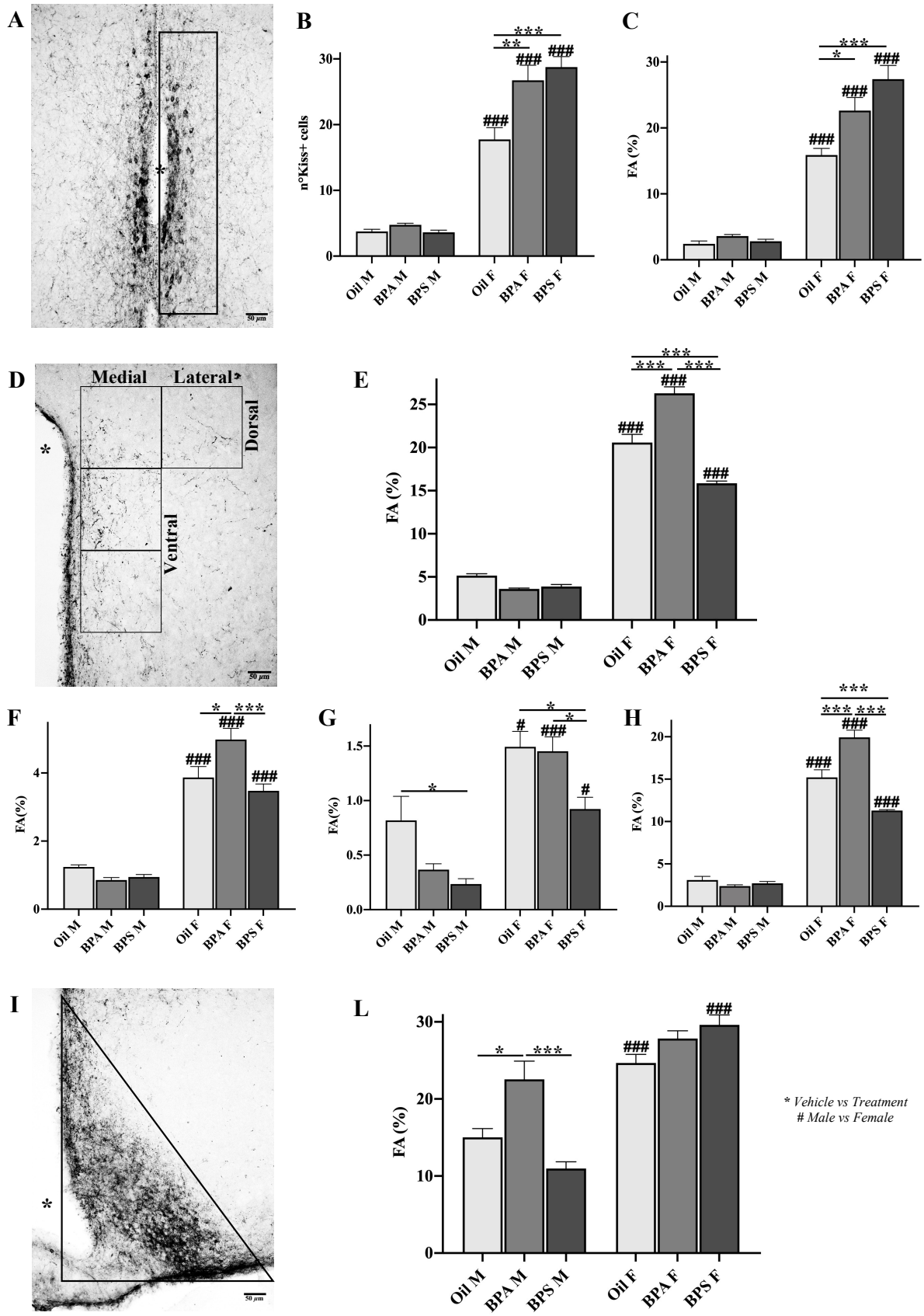

Figure 5. **Kisspeptin immunoreactivity in the analyzed hypothalamic nuclei of control and BPs-treated mice**. Representative images of kisspeptin immunoreactivity in a coronal section of ***(A)*** RP3V, ***(D)*** PVN and ***(I)*** Arc of control female. Analysis of kiss-ir in RP3V, expressed both as ***(B)*** number of kiss+ cells and ***(C)*** fractional area (FA). Analysis of kiss-ir, expressed as

fractional area (FA), in ***(E)*** total PVN, in ***(F)*** DM, in ***(G)*** DL, in ***(H)*** VM and in ***(L)*** Arc. In the histograms ***(B, C, E, F, G, H, L)*** the oil-treated mice are shown in light gray, BPA-treated mice are shown in gray, and BPS-ones are shown in dark gray. Male mice are shown on the left side of the graph, while females are on the right side. Data are expressed as mean ± SEM. Two-way ANOVA revealed a significant effect for $p \leq 0.05$ 05 (* = vehicle vs treatment; # = male vs female). Scale bar = 50μm. + = positive; kiss = kisspeptin; RP3V = rostral periventricular area of the third ventricle, PVN = paraventricular nucleus, DM = dorsomedial region of the paraventricular nucleus, DL = dorsolateral region of the paraventricular nucleus, VM = ventromedial region of the paraventricular nucleus, Arc = arcuate nucleus; FA = fractional area; * = third ventricle.

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Perinatal exposure to bisphenol A or S: effects on anxiety-related behaviors and serotonergic system

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ABSTRACT

Bisphenols (BPs), organic synthetic compounds used in the production of plastics, are an extremely abundant class of Endocrine Disrupting Chemicals, *i.e.,* exogenous chemicals, or mixtures of chemicals, that can interfere with any aspect of hormone action. Exposure to BPs can lead to a wide range of effects and it is especially dangerous if it occurs during specific critical periods of life. Focusing on the effects of perinatal exposure to BPA or to its largely used substitute BPS, we treated C57BL/6 dams orally with a dose of 4µg/kg body weight/day (*i.e.*, EFSA Tolerable Daily Intake dose) of BPA or BPS dissolved in corn oil or with vehicle alone, starting with mating and continuing until the weaning of the offspring. In adulthood (PND90), the offspring of both sexes performed the elevated plus maze and the open field tests. Both testes highlighted alterations in some parameters in both BPA- and BPS-treated mice, suggesting different effects of the BPs exposure on anxiety-related behavior in males (anxiolytic) and females (anxiogenic). Therefore, thanks to immunohistochemical techniques, we analyzed the serotonergic system in dorsal (DR) and median (MnR) raphe nuclei, which are highly involved in the control of anxiety-related behavior. In control mice, we detected sex dimorphism of the system in the DR only, with control females showing higher values of 5-HT-ir when compared to control males. BPA-treated males displayed a significant increase of 5-HT-ir in all analyzed nuclei, whereas BPS-treated males showed an increase in ventral DR only. In females, both BPA- and BPS-treated groups showed a significant increase of 5-HT-ir in dorsal DR compared to the controls, and BPA-treated females also showed a significant increase in MnR. In conclusion, exposure during early phases of life to both BPA or BPS is altering, in a sexually differentiated way, both anxiety-related behavior and the Raphe population of serotonin neurons which is involved in the control of this behavior.

Keywords: endocrine disrupting chemicals, BPA, BPS, serotonin, 5-HT, Raphe Nucleus

Author's contributions
BB designed and performed experiments, analyzed data, and wrote the draft. AC, MTO, MB and SN performed experiments and analyzed data. SG revised the draft. MM and GCP revised the draft and wrote the paper.

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All authors have no conflicts of interest to declare with respect to the research, authorship, and/or publication of this article.

Introduction

Growing literature considered the Endocrine Disrupting Chemicals (EDCs), *i.e*., "exogenous chemicals, or mixtures of chemicals, that interfere with any aspect of hormone action", as substantial and costly public health problems due to their pervasiveness and their associations with chronic disease (Gore et al., 2015; Kahn et al., 2020).
More recent concerns regard the possibility of EDCs to alter brain development, neurochemistry, and behaviors (Bakoyiannis et al., 2021). Among EDCs, exposure to bisphenols (BPs), and in particular to bisphenol A (BPA), seems to impact the behavioral outcome, especially when the exposure occurs during critical periods of development, such as the pre- or perinatal one (Bakoyiannis et al., 2021; Rebolledo-Solleiro et al., 2021).
However, because of several health issues, the European Food Safety Authority (EFSA), after completing a full risk assessment in 2006 (EFSA, 2006), established a tolerable daily intake (TDI) for BPA of 50 μg/kg of body weight (BW)/day, which has been reduced in 2015 from 50 to 4 μg (EFSA, 2015). Contextually, the European Commission has imposed BPA removal from some consummatory goods, such as infant feeding bottles (European Commision, 2011) or other foodstuffs (Andersson et al., 2018). Considering BPA limitations, some substitutes have been proposed. Thanks to its increased stability (Kuruto-Niwa et al., 2005), one of the most used is bisphenol S (BPS), which unfortunately shares with BPA not only some structural similarities but also the endocrine-disrupting properties (Eladak et al., 2015; Gramec Skledar & Peterlin Masic, 2016; Naderi & Kwong, 2020; Rochester & Bolden, 2015). In fact, emerging evidence highlighted that BPS seems not to be a safe alternative to BPA: despite this, it is still poorly investigated, and no specific limitations are currently available (den Braver-Sewradj et al., 2020; Mustieles et al., 2020; Naderi & Kwong, 2020; Thoene et al., 2020).

Anxiety consists of several, and mostly conserved among mammals, reactions (somatic, cognitive, emotional, and behavioral) combined to achieve a successful evolutionary mechanism to survive or to cope with really or potentially threatening stimuli (Hohoff, 2009). Correct activation of the anxiety state is needed to react properly to those stimuli. Thus, anxiety enables an individual to adapt to environmental challenges (Gold, 2015; Hohoff, 2009). If anxiety responses are inappropriate, the ability to adapt to environmental conditions is compromised (Gold, 2015).

There is some evidence that exposure to BPA at any time of life alters the anxiety responses in rodents (Bakoyiannis et al., 2021; Rebolledo-Solleiro et al., 2021), producing anxiogenic or anxiolytic effects, depending on the considered dose, period of exposure, sex, and experimental model (Rebolledo-Solleiro et al., 2021). Interestingly, perinatal exposure to BPA, which induces alteration in anxiety, has been associated with changes in hormones receptors' levels within the brain.
In particular, BPA anxiogenic effects, seen mainly in developmentally exposed females in rats (Gioiosa et al., 2013; Poimenova et al., 2010; Zhou et al., 2015), seem to be linked to increased levels of plasma corticosterone (Chang et al., 2016; Poimenova et al., 2010) and to decreased

estrogen receptor α (ERα) (Chang et al., 2016), estrogen receptor β (ERβ), and melanocortin receptors (Patisaul et al., 2012) in different regions of the brain.
More recent works also investigated the effects of BPS in anxiety-related behaviors. It has been recently demonstrated that 10-weeks post-lactational exposure of male mice to 100 µg/kg/day BPS mediated anxiogenic effects (Mornagui et al., 2019); juvenile exposure of male mice to either BPA or BPS (dose of 1 mg/kg BW/day or 100 µg/kg BW/day) also led to an increase in the anxiety state, along with hyperactivity of basolateral amygdala (BLA), strongly involved in fear and anxiety responses (Hu et al., 2022). Interestingly, few works investigated the effects of perinatal exposure to BPS in mice (dose of 0.2 mg/kg BW/day) or rats (dose of 10 µm/kg BW/day or 50 mg/kg BW/day), associating it to an increase in anxiety-like behaviors, mainly in males (Kim et al., 2015).

The serotonergic system, within the dorsal (DR) and median (MnR) raphe nuclei, is particularly relevant for the control of anxiety behaviors (Ren et al., 2018; Zangrossi & Graeff, 2014). Serotonin (5-hydroxytryptamine, 5-HT) is a monoamine neurotransmitter, synthesized from the amino acid tryptophan (Zhang et al., 2004). In the central nervous system (CNS), 5-HT is produced into small clusters of cells, defined as raphe nuclei, extending from the midbrain to the medulla oblongata and reaching several CNS areas (Hornung, 2010). Of the nine raphe nuclei (B1-B9), five (rostral) are in the midbrain and in the rostral pons, and four (caudal) are in caudal pons and medulla (Hornung, 2010). From the rostral nuclei, and especially from DR and MnR, the projections reached different forebrain structures, while from the caudal ones projections are primarily sent to the spinal cord (Ren et al., 2019).

Recent evidence supports the view of a dual role of 5-HT (anxiolytic and anxiogenic) in the control of anxiety-like behaviors (Gordon & Hen, 2004). Griebel has proposed that the dual effects, anxiolytic and anxiogenic, of 5-HT, are due to the different involvement and distribution within brain areas of 5-HT receptors (Griebel, 1995). Daekin and Graeff proposed that 5-HT increase or decrease anxiety-related responses depending on the targeted brain areas (*i.e.*, enhancing it in the forebrain areas and reducing it in subcortical structures) (Deakin & Graeff, 1991). Last, Gray and McNaughton supposed that 5-HT, on one hand, promotes fear response activating the amygdala and the periaqueductal gray, on the other one it decreases anxiety, inhibiting the hippocampus (Davidson & Jarrard, 2004).
Notably, DR and MnR display high specialization in terms of functional connectivity (Ren et al., 2018). This is in line with the theory proposed by Deakin and Graeff theory, according to which two main pathways are involved in the control of anxiety behaviors (Deakin & Graeff, 1991). The first originates in the DR, goes through the medial forebrain bundle, and reaches the amygdala and the frontal cortex, facilitating avoidance behaviors (Deakin & Graeff, 1991). The second starts in the MnR and reaches the hippocampus, promoting resistance to chronic stress (Deakin & Graeff, 1991).

As BPA has been reported to alter anxiety-related behaviors (Bakoyiannis et al., 2021; Rebolledo-Solleiro et al., 2021), some studies have investigated its impact on the serotonergic system. In particular, it has been shown, in male rats, that a single intracranial injection of BPA (0.1-10 µg/kg BW) on postnatal day 2 led to an increase 5-HT levels in the hippocampus, appreciable not only 5 days but also 28 days after the injection (Matsuda et al., 2010). Another study, performed in mice, highlighted that perinatal exposure to BPA (20 µg/kg BW/day) led,

both in males and females, to an increase in 5-HT in different brain areas (caudate and putamen nuclei, dorsal raphe nucleus, thalamus, and substantia nigra) appreciable at 3, 10 and 15 postnatal weeks (Nakamura et al., 2010). Furthermore, it has been demonstrated, in female mice perinatally exposed to a low-dose BPA (250 ng/kg/day), an increase in the 5-HT turnover in the hippocampus (Matsuda et al., 2013). All these results suggest that perinatal exposure to BPA may perturb 5-HT metabolism and signaling also in a long-term fashion.

Considering the increasing level of exposure to both BPs, due to their persistence in the environment (Chen et al., 2016; Vasiljevic & Harner, 2021; Wu et al., 2018), and the lack of data regarding the BPS, in this study we aimed to evaluate the potential sexually dimorphic effects on anxiety-related behaviors (tested through the Elevated Plus Maze and the Open Field test) (Carola et al., 2002; Lezak et al., 2017) of adult male and female mice perinatally exposed to low-dose (4 µg/kg BW/day) of BPA or BPS. In parallel, we investigated the possible alterations of serotonergic systems in the DR and MnR, which is known to be involved in the control of different aspects of anxiety-related behaviors (Ren et al., 2018; Zangrossi & Graeff, 2014) and to be targeted at least by BPA exposure (Castro et al., 2015; Matsuda et al., 2010, 2013; Nakamura et al., 2010).

Materials and methods

Animals

Adult C57BL/6J mice from our 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 10:00 AM). Food (standard mouse chow 4RF21, Mucedola srl, Settimo Milanese, Italy) and water were provided *ad libitum*. One male and two female mice (3-month-old) were housed together to achieve a successful mating, assessed by the evaluation of the presence of the vaginal plug (assumed as gestational day 0, GD0) (Hasegawa et al., 2017).

Animal care and handling were according to the European Union Council Directive of 22nd September 2010 (2010/63/UE); all the procedures reported in the present study were approved by the Italian Ministry of Health (407/2018-PR) and by the Ethical Committee of the University of Torino (Project n° 360384).

Treatments

BPA (Sigma Aldrich, 239658, CAS 80-05-7) or BPS (Sigma Aldrich, 103039, CAS 80-09-1) were prepared for oral administration by dissolving them in corn oil (Sigma-Aldrich, C8267). 12 pregnant dams were assigned randomly to three experimental groups: control dams (receiving only vehicle, corn oil; n=4), BPA-treated dams (receiving 4 µg/kg BW/day of BPA, corresponding to the European TDI; n=4) and BPS-treated dams (receiving 4 µg/kg BW/day of BPS; n=4). The dose was calculated daily according to dams' body weight, recorded with an electronic precision balance (*Mod. Kern-440-47N, resolution 0.1g*).
We decided to test the same dose for both BPA and BPS to allow a precise comparison of the effects of the two bisphenols. Moreover, at present, although BPS is one of the most used BPA substitutes and it has already been detected in environmental and human samples (Catenza et al., 2021), at present no user guidelines are available. Dams were treated starting at GD0,

throughout pregnancy and lactation, until weaning of the offspring at postnatal day 28 (PND28). To resemble human exposure conditions, the daily treatment or the vehicle was given orally to the dams, by means of a pipette, to minimize dams' stress (Bo et al., 2016; Palanza et al., 2002). This type of administration allowed us to perform a perinatal treatment (covering both prenatal and postnatal critical window of development) (Neier et al., 2019) on the offspring. In fact, it is known that both BPA and BPS can pass first through the placenta and then into the milk during the lactation (Cimmino et al., 2020; Mao et al., 2020).

Litters were reduced to 8 pups at birth, to obtain an equal number of pups of both sexes, sexed *via* anogenital distance (AGD) (Manno 3rd, 2008). The pups were weaned at PND28 and housed in monosexual groups of 4 mice. They were monitored weekly until adulthood when the behavioral tests were performed.

Behavioral tests

Around PND90 the anxiety-related behaviors were evaluated performing the Elevated Plus Maze (EPM) test and the Open Field (OF) test (n=10±1/group) (Carola et al., 2002; Kraeuter et al., 2019; Kulesskaya & Voikar, 2014; Lezak et al., 2017; Seibenhener & Wooten, 2015; Walf & Frye, 2007). The females were tested in estrus phase, assessed by vaginal smear (McLean et al., 2012). On the day of the test, mice were placed in the room in which the test was performed at least 2 hours before starting, to allow the habituation to the room. Before starting and between each trial, the testing apparatus were cleaned with 70% ethanol, being sure to thoroughly dry the apparatus to avoid exposure of mice to alcohol. The EPM is particularly sensitive to testing conditions (Albani et al., 2015; Shoji & Miyakawa, 2021) and so it was the first performed test, followed by the OF, after at least 1 hour (Carola et al., 2002; Schmitt & Hiemke, 1998). The tests were performed in the dark, using only a 25-W red light, which mice cannot see, to help the operator to manipulate the animals (Palanza et al., 2002). Each test was recorded with an infrared camera placed above the apparatus in order to subsequently perform the behavioral analysis through the Ethovision XT Software (Noldus Information Technology, Wageningen, The Netherlands).

Elevated Plus Maze (EPM)

The EPM test apparatus was a plus-cross shaped platform comprising two open arms (30 cm x 5 cm) and two closed arms (30 cm x 5 cm x 15 cm walls) originating from a central platform (5 cm x 5 cm) and raised 60 cm above the floor (Longo et al., 2014). To start, the tester mouse was gently placed in the center of the platform. Then it was allowed to freely explore the apparatus for 10 minutes. After the recording, the parameters (Carola et al., 2002) described in *Table 1* were analyzed for the first 5 minutes of the test thanks to Ethovision XT Software (Noldus Information Technology, Wageningen, The Netherlands).

Open Field (OF)

OF apparatus consisted in an unfamiliar arena (45 cm × 45 cm x 38 cm walls), which is divided into a central (20 x 20 cm) and a peripheral zone (Longo et al., 2014). At the beginning, the mouse was placed in the corner of the apparatus and was allowed to explore the arena for 10 minutes. After the recording, the parameters (Carola et al., 2002) described in *Table 2* were analyzed for the first 5 minutes of the test thanks to Ethovision XT Software (Noldus Information Technology, Wageningen, The Netherlands).

Parameter	Description
Cumulative Duration (CD)	The cumulative time (s) spent by the tester mouse in the center, in the open arms or in the closed ones.
Frequency of entrance	The number of times the tester mouse entered in the center, in the closed or open arms. The mouse was considered to have entered an arm if all four paws had left the center square.
Distance	The total distance traveled (cm) by the tester mice in the center, in each arm and in the total arena.
Latency to first entry in open arms	The time passed (s) until the mouse first entered the open arms.
Mean velocity (v)	The mean velocity (cm/s) displayed by the tester mouse during the trial.
Protected head-dipping	The number of times the tester mouse scanned over the side of the center of the platform towards the floor.
Unprotected head-dipping	The number of times the tester mouse scanned over the side of the open arms of the platform towards the floor.
Head-dipping	Total number of times the tester mouse scanned over the side of the platform (center and open arms) towards the floor.

Table 1. *Parameters analyzed for each mouse in the EPM recorded trials.*

Parameter	Description
Cumulative Duration (CD)	The cumulative time (s) spent by the tester mouse in the center or in the border of the arena
Frequency of entrance	The number of times the tester mouse entered in center or in the border The mouse was considered to have entered the zone if all four paws had overtaken the borderline between the two.
Distance	The total distance traveled (cm) by the tester mice in the center, in the border and in the total arena.
Latency to first entry in the center of arena	The time passed (s) until the mouse first entered the center of the arena.
Mean velocity (v)	The mean velocity (cm/s) displayed by the tester mouse during the trial.
Grooming	The number of times the tester mouse licked or scratched its fur, washed its face, or licked its genitalia.
Protected rearing	The number of times the tester mouse reared on its hind paws in the border of the arena.
Unprotected rearing	The number of times the tester mouse reared on its hind paws in the center of the arena.
Rearing	Total number of times the tester mouse reared on its hind paws in the center or in the border of the arena.

Table 2. *Parameters analyzed for each mouse in the OF recorded trials.*

Fixation and tissue sampling

At least 10 days after the performing of the behavioral tests, mice were sacrificed, by deep irreversible anesthesia (intraperitoneal injection of Zoletil 80 mg/kg/ Rompum 10 mg/kg) and transcardially perfused with 4% paraformaldehyde (PFA) solution. Females were sacrificed in the estrus phase, assessed by vaginal smear (McLean et al., 2012). Brains were removed and stored in a 4% PFA solution for 24 hours, 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 (Marraudino et al., 2017).

Brains (n=4/group) were serially cut in the coronal plane at 30 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 (Paxinos & Franklin, 2001). Sections were collected in a cryoprotectant solution (Watson Jr. et al., 1986) and stored at -20 °C.

Serotonin immunohistochemistry

The presence of serotonin (5-HT) was detected by immunohistochemistry performed on free-floating sections from one series. Briefly, the sections were washed overnight in 0.01 M phosphate buffer (PBS), pH 7.3. The following day, sections were first incubated with citrate buffer (citric acid 10 mM, 0.05% Tween, pH 6.0) previously heated at 95°C for antigen retrieval and then washed three times in PBS. Next, the sections were washed in PBS containing 0.5% 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 first incubated for 30 min with blocking solution containing normal horse serum (Vector Laboratories, Burlingame, CA, USA) and bovine serum albumin (Sigma-Aldrich, Milan, Italy) diluted in PBS 0.5% Triton X-100, and then incubated two overnight at +4°C with anti-5-HT antibody (Immunostar,#20079, Goat, 1:2.500) diluted in the blocking solution. A biotinylated horse anti-goat secondary antibody (Vector Laboratories, Burlingame, CA, USA) diluted in PBS, pH 7.3–7.4, containing 0.2% Triton X-100 was then employed at a dilution of 1:200 for 60 min at room temperature. The antigen-antibody reaction was revealed by 60 min incubation with avidin–peroxidase complex (Vectastain ABC Kit Elite, Vector Laboratories, Burlingame, CA, USA). 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, Milano, Italy). This antibody was successfully used in previous studies (García-González et al., 2017; Li et al., 2020) and its specificity was tested by the factory (*https://www.biocompare.com/9776-Antibodies/2874940-5-HT-Serotonin-Goat-Antibody/#citations*). 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, a selected standardized section covering the Dorsal Raphe Nucleus (DR, Bregma -4.60 to -4.84 mm) and Median Raphe Nucleus (MnR, Bregma -4.36 to -4.48 mm), were chosen according to the mouse brain atlas (Paxinos & Franklin, 2001). Single section for each nucleus was 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.S., Firenze, Italy). Images were digitized by using a 20x objective for the nuclei acquisition. Digital images were processed and analyzed by ImageJ (version 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 (456.706 mm^2 for the DR; 238.818 mm^2 for the MnR). The DR was also divided into two subregions, the dorsal region (DRD, 375.348 mm^2) and the ventral regiont (DRV, 81.268 mm^2), following the different distribution within the nucleus of the analyzed system (Paxinos & Franklin, 2001; Ren et al., 2018).

We evaluated the extension of the immunoreactivity (cell bodies, dendrites, fibers) in all the selected nuclei as fractional area (FA) covered by immunopositive material (Marraudino et al., 2021; Viglietti-Panzica et al., 1994). In addition, we also counted the number of 5-HT-positive cells in the two analyzed nuclei.

Statistical analysis

Data were analyzed by two-way analysis of variance (ANOVA) (sex and treatment used as independent variables) with SPSS 27 statistic software (SPSS Inc., Chicago, USA). If the ANOVA was significant, the *post-hoc* analysis was performed using the Tuckey's HSD test. Differences were considered statistically significant for values of $p \leq 0.05$. Data are shown as mean ± SEM (mean standard error).

Results

Effects of perinatal exposure to BPA or BPS on anxiety-related behaviors

Results obtained from the analysis of the EPM and OF tests are summarized in *Table 3* and *Table 4* respectively. Here we highlight the most interesting results (*Figure 1 and 2*).

Elevated Plus Maze (EPM)

The statistical analysis of the EPM highlighted some significant differences among the groups (summarized in *Table 3*). First, as expected, we observed that control males are more anxious compared to control females, as they spent less time in open (*p=0.003I*, *Fig.1B*) and more time in closed arms (*p=0.012*, *Fig.1C*), displayed higher latency to first entry in open arms (*p=0.008, Fig.1H*) and lower number of head-dipping (*p=0.021*, *Fig.1I*). Interestingly, the treatment with both BPs seemed to disrupt these sex-driven differences (*Table 3* and *Fig.1*).

In fact, perinatal treatment with BPA in males caused a significant decrease in time spent in closed arms (*p=0.012, Fig.1C*) together with an increased number of entries (*p=0.023, Fig.1G*) and a decrease in the latency to first entry (*p=0.002, Fig.1H*) in open arms, in which they tended to travel more distance (*p=0.053, Fig.1E*), compared to control males. On the other hand, BPA treatment in females caused a significant decrease in time spent in open arms (*p=0.048*, *Fig.1B*) together with an increase in the distance traveled (*p=0.025*, *Fig.1E*), compared to control females.

Perinatal treatment with BPS in males is linked, even if not significantly, to the increase in time spent in open arms (*p=0.471*, *Fig.1B*) and to the decrease in time spent in the closed ones (*p=0.667*, *Fig.1C*) compared to control males. It also caused a significant decrease in the latency to first entry in open arms (*p=0.010, Fig.1C*), in which they tended to travel more distance (*p=0.058, Fig.1E*) compared to control ones. Conversely, BPS-treated females, compared to control ones, seemed to spend less time in open arms (*p=0.086, Fig.1B*) and displayed a significant increase in time spent in the closed ones (*p=0.011, Fig.1C*), in which they traveled more distance (*p=0.006, Fig.1F*).

Finally, both BPs set aside the differences in number of head-dipping (*Fig. 1I*).

Parameter	Oil		BPA		BPS		ANOVA	
	Males	Females	Males	Females	Males	Females	$F_{(5, 57)}$	p
Cumulative duration (s) in:								
Center	44.641 ± 4.819	82.66 ± 7.826	69.489 ± 3.63	84.891 ± 8.627	52.651 ± 3.252	57.28 ± 6.562	*6.628*	***<0.001***
Open arms	27.883 ± 4.144	68.966 ± 8.602	53.626 ± 8.771	39.276 ± 9.299	43.54 ± 5.782	41.145 ± 5.620	*3.760*	***0.005***
Closed arms	226.986 ± 8.499	148.516 ± 10.878	174.604 ± 10.286	175.994 ± 13.437	199.824 ± 8.059	199.842 ± 11.084	*6.390*	***<0.001***
Frequency of entrance in:								
Center	24.6 ± 1.31	32.727 ± 2.232	31.455 ± 2.006	29.75 ± 2.372	28 ± 2.284	27.455 ± 2.738	*1.656*	*0.160*
Open arms	8.9 ± 0.888	15.909 ± 1.786	16.909 ± 2.395	12.25 ± 1.287	12.636 ± 1.439	15.364 ± 1.835	*2.973*	***0.019***
Closed arms	14.5 ± 0.764	16.455 ± 1.021	16.455 ± 0.666	17.875 ± 1.623	16.636 ± 1.771	14.091 ± 1.131	*1.123*	*0.359*
Distance (cm) traveled in:								
Arena	46603.921±3156.563	45885.65 ±7783.543	77262.609±2833.946	80483.599±841.290	73579±8679.094	75878.268± 5133.699	*5.715*	***<0.001***
Center	22599.16±1673.799	21562±291±3815.782	36798.473±1267.868	37800.025±4469.577	34287.418±	37853±4582±414	*4.058*	***0.003***
Open arms	22014.96±1688.617	21488.58 ±4214.155	36475±1956±229	38163.05 ±4214.903	36293.245±4337.169	31545.582± 2915.674	*4.485*	***0.002***
Closed arms	1989.801± 339.334	2834.779 ±580.471	3988.336 ± 611.876	4520.524 ±603.451	2999.208 ±5 84.489	6479.432 ± 1086.564	*5.168*	***0.001***
Latency (s) to first entry in open arms	22.938 ± 5.370	5.790 ± 1.650	3.470 ± 1.034	7.775 ± 1.947	6.152 ± 1.885	15.646 ± 5.217	*4.923*	***0.001***
Mean velocity (cm/s)	4.066 ± 0.257	3.690 ± 0.149	4.893 ± 0.233	4.893 ± 0.356	4.369 ± 0.257	5.242 ± 0.284	*5.114*	***0.001***
Protected head-dipping	7.8 ± 0.49	11.091±0.986	11.182 ± 0.903	9.375 ± 0.979	10.182 ± 1.750	8.636 ± 1.28	*1.279*	*0.286*
Unprotected head-dipping	3.5 ± 1.186	10.182 ± 2.296	6.546 ± 1.841	6.875 ±s 2.282	3.903 ± 1.221	6.364 ± 1.011	*1.929*	*0.104*
Head-dipping	11.3±1.325	21.272±1.893	17.728±1.799	16.25±2.927	14.091±2.343	15±2.074	*2.524*	***0.039***

Table 3. *Results obtained from the analyzed parameters within the first 5 minutes of the EPM test.* Data are reported as Mean ± SEM. Two-way ANOVA (sex and treatment used as independent variables) revealed a significant effect for $p \leq 0.05$, highlighted in bold.

Open Field (OF)

The statistical analysis of the OF highlighted some significant differences among the groups (summarized in *Table 4*). The analysis confirmed the sex differences, emerged also in the analysis of the EPM, between control males and females. In fact, males spent less time in the center (*p=0.019, Fig.2A*) and more time in the border (*p=0.019, Fig.2B*), in which they traveled lower distance (*p=0.003*, *Fig.2D*), and displayed higher latency to first entry in the center (*p=0.003*, *Fig.2F*) along with a tendency of enter the center less frequently (p=*0.065, Fig.1E*) compared to females. Moreover, males traveled, in the entire arena, significantly (*p=0.003*) more distance compared to females (*Table 3*). Once again, the treatment with both BPs seemed to disrupt these sex-driven differences.

In fact, BPA treatment in males is linked, even if not significantly, to more time spent in the center (*p=0.615, Fig.2A*) and less time spent in the border (*p=0.614*, *Fig.2B*) of the arena

compared to controls. Furthermore, they displayed a significant decrease in latency of first entry in the center (*p=0.001, Fig.2F*) compared to control males. On the other hand, BPA-treated females spent, even if not significantly, less time in the center (*p=0.834, Fig.2A*) and more time in the border (p=*0.832, Fig.2B*) of the arena compared to the controls.

The BPS treatment in males caused, even if not significantly, similar alterations compared to the BPA one, decreasing the time spent in the center (*p=0.319, Fig.2A*) and increasing that spent in the border (p=*0.318, Fig.2B*), compared to controls. Moreover, BPS-treated males showed a significant decrease in latency of first entry in the center (*p=0.041, Fig.2F*) compared to control ones. Conversely, BPS-treated females spent, even if not significantly, less time in the center (*p=0.834*, *Fig.2A*) and more time in the border (*p=0.536*, *Fig.2B*) of the arena compared to the control ones.

Finally, both BPs set aside the differences in frequency of entries in the center of the arena (*Fig. 2E*).

Parameter	Oil		BPA		BPS		ANOVA	
	Males	Females	Males	Females	Males	Females	$F_{(5, 57)}$	p
Cumulative duration (s) in:								
Center	12.295 ± 1.909	24.470 ± 3.226	18.109 ± 2.679	19.928 ± 1.83	19.896 ± 3.032	18.376 ± 2.175	*2.283*	*0.058**
Border	287.84 ± 1.91	275.649 ± 3.226	282.928 ± 2.681	280,212 ± 1.831	280.235 ± 3.033	281.758 ± 2.177	*2.287*	*0.058**
Frequency of entrance in:								
Center	11.5 ± 1.551	19.636 ± 2.325	17 ± 2.183	16.778 ± 2.080	18 ± 2.195	18.376 ± 2.175	*2.365*	*0.051**
Border	12.5 ± 1.551	20.545 ± 2.302	17.909 ± 2.125	17.667 ± 2.021	19 ± 2.195	21.273 ± 1.556	*2.387*	***0.049***
Distance traveled (cm) in:								
Arena	55078.65 ± 1456.051	51255.73 ± 939.741	51996.06 ± 1332.43	49384.66 ± 1625.357	51413.93 ± 1332.43	53713.63 ± 1313.416	*2.072*	*0.082*
Center	54216.45 ± 1540.589	49436.682 ± 954.328	51144.028 ± 1428.175	47927.611 ± 1707.159	50050.982 ± 1488.990	52460.318 ± 1414.765	*2.366*	*0.053**
Border	862.203 ± 178.520	1819.045 ± 207.095	852.031 ± 143.619	1457.047 ± 145.799	1362.946 ± 182.690	1253.307 ± 155.909	*4.692*	***0.001***
Latency to first entry in the center (s)	40.496 ± 10.606	12.268 ± 4.131	8.589 ± 2.464	10.044 ± 3.961	18.689 ± 3.402	5.716 ± 1.967	*6.118*	***<0.001***
Mean velocity (cm/s)	11.542 ± 0.514	13.064 ± 0.739	14.021 ± 1.074	11.381 ± 0.785	13.401 ± 0.816	14.328 ± 0.796	*2.254*	*0.061*
Grooming	3.3 ± 0.423	2.455 ± 0.718	3.818 ± 0.761	4.222 ± 1.267	4.091 ± 0,623	2.182 ± 0.519	*1.373*	*0.248*
Protected rearing	31.2 ± 2.284	33.364 ± 3.619	33.363 ± 2.487	27.667 ± 2.609	34.273 ± 2.512	33.455 ± 2.146	*0.765*	*0.579*
Unprotected rearing	3.8 ± 1.254	6.455 ± 1.391	4.273 ± 1.685	6.111 ± 1.670	6.818 ± 2.231	4.455 ± 0.888	*0.667*	*0.650*
Rearing	35.9 ± 2.834	39.819 ± 3.968	37.455 ± 2.774	33.778 ± 3.179	41.091 ± 4.564	38.182 ± 2.354	0.573	*0.720*

Table 4. *Results obtained from the analyzed parameters within the first 5 minutes of the OF test*. Data are reported as Mean ± SEM. Two-way ANOVA (sex and treatment as independent variables) revealed a significant effect for $p \leq 0.05$, highlighted in bold. * Tendency towards significance ($0.05 < p < 0.06$).

5-HT-ir analysis

The statistical analysis of the immunoreactivity for 5-HT (summarized in *Table 5*) revealed that both BPs treatment affected the analyzed nuclei, differently in the two sexes.

First, we corroborated the presence of sexual dimorphism within the DR in oil-treated mice, in terms of FA (*p=0.001*, *Fig.3E*), both in the dorsal (*p=0.050*, *Fig.3F*) and in the ventral regions (*p=0.004*, *Fig.3G*) of the nucleus, with males showing lower FA compared to females (*Fig.3A*, *upper level*).

The treatment with BPA caused the highest alterations in males within all the analyzed nuclei (*Fig.3 left side-central level; Fig.4 central column-upper level*). In fact, we observed an increase of 5-HT-ir, both in terms of number of cells and FA, in DR (*p=0.*001, Fig.*3B*; *p<0.*001, Fig.*3E*), in the DRD (*p=0.007*, *Fig.3C*; *p<0.001*, *Fig.3F*), in DRV (*p=0.001*, *Fig.3D*; *p<0.001*, *Fig.3G*) and also in the MnR (*p=0.006, Fig.4B*; *p=0.003*, *Fig.4C*) of BPA-treated males compared to the control ones. In BPA-treated females (*Fig.3 right side-central level*; *Fig.4 central column-lower leve*l), we noticed a significant increase in FA in DRD (*p=0.009*, *Fig.3F*) and in MnR (*p<0.001*, *Fig.4C*), compared to control females.

The perinatal treatment with BPS caused in males a significant increase in the FA of DR (*p=0.001*, *Fig.3E*), due to a significant increase in the ventral component of the nucleus, appreciable both in terms of number of cells (*p=0.031*, *Fig.3D*) and FA (*p=0.004*, *Fig.3G*), compared to controls.

In BPS-treated females (*Fig.3 right side-lower level*; *Fig.4 left column-lower leve*l), we noticed a significant increase in FA in DRD (*p=0.020*, *Fig.3F*), compared to the control ones.

Number of serotonin positive cells								
Zone	**Oil**		**BPA**		**BPS**		**ANOVA**	
	Males	**Females**	**Males**	**Females**	**Males**	**Females**	**F (5, 18)**	**P**
DR	133.75 ± 7.793	150.75 ± 3.613	209.25 ± 8.702	138.5 ± 13.295	158.5 ± 16.795	174.75 ± 4.423	*7.106*	***0.001***
DRD	101.25 ± 4.644	107.5 ± 4.806	154 ± 7.223	87.5 ± 11.449	111.25 ± 14.585	127.75 ± 7.204	*6.576*	***0.001***
DRV	32.5 ± 3.663	43.25 ± 2.496	55.25 ± 3.544	51 ± 3.189	47.25 ± 2.358	47 ± 2.858	*6.479*	***0.001***
MnR	27.5 ± 1.936	39 ± 3.629	46.25 ± 2.780	46.25 ± 5.391	32.25 ± 1.436	37.5 ± 2.062	*5.055*	***0.005***
Fractional Area (%)								
Zone	**Oil**		**BPA**		**BPS**		**ANOVA**	
	Males	**Females**	**Males**	**Females**	**Males**	**Females**	**F (5, 18)**	**p**
DR	19.964 ± 0.701	34.835 ± 1.472	47.689 ± 0.907	43.58 ± 4.123	34.657 ± 1.424	43.047 ± 1.813	*22.874*	***<0.001***
DRD	7.871 ± 0.569	12.881 ± 0.627	20.149 ± 0.761	19.172 ± 1.344	12.694 ± 0.988	18.601 ± 1.832	*18.917*	***<0.001***
DRV	12.094 ± 0.866	21.954 ± 1.214	27.540 ± 0.567	24.408 ± 3.087	21.963 ± 1.576	24.446 ± 0.51	*11.382*	***<0.001***
MnR	4.518 ± 0.406	7.795 ± 0.924	10.605 ± 1.456	17.68 ± 1.163	6.992 ± 0.462	5.145 ± 0.861	*26.013*	***<0.001***

Table 5. *Results obtained from the analysis of 5-HT-ir in all selected nuclei.* Data are reported as Mean ± SEM, both as number of positive cells and fractional area. Two-way ANOVA revealed a significant effect for $p \leq 0.05$, highlighted in bold. DR = dorsal raphe; DRD = dorsal region of the dorsal raphe; DRV = ventral region of the dorsal raphe; MnR = median raphe.

Discussion

To date, the effects of BPs on various aspects of endocrine control, such as reproduction (den Braver-Sewradj et al., 2020; Tomza-Marciniak et al., 2018) or metabolism (den Braver-Sewradj et al., 2020; Rubin et al., 2019), are widely debated, but very little is known about the possible effects of potential effects of BPs on anxiety-like behaviors. The results of this study highlighted that exposure to both BPA and BPS during early phases of life is altering, in a sexually differentiated way, both anxiety-related behaviors and serotonin population within the Raphe nucleus, which is involved in the control of these behaviors, in the adult animals.

During the EPM test, BPA-treated males showed a significant increase in the time spent in the open arms compared to the control ones and a decrease of the latency of the first entry in the open arms, that was also displayed by BPS-treated males, while among females a significant decrease in time spent in open arms was observed in the BPA-treated group, together with an increase in time spent in the closed ones among the BPS-treated ones. The OF test showed that both treatments disrupted the sexual-driven differences in the analyzed behaviors, mainly decreasing anxiety-related behaviors in males and increasing them in females. These behavioral alterations suggested different effects of the BPs exposure in the two sexes: anxiolytic in males and anxiogenic in females.
Therefore, we analyzed the serotonergic system in the Raphe complex, which is highly involved in the control of anxiety-related behaviors. We performed an immunohistochemical analysis of the 5-HT-ir, both in terms of number of cells and fractional area, in the DR, distinguishing its dorsal (DRD) and ventral (DRV) component, and in the MnR. In control mice, we detected sexual dimorphism of the system in the DR only, with control females showing higher values of 5-HT-ir when compared to control males. BPA-treated males displayed a significant increase of 5-HT-ir in all analyzed nuclei, whereas BPS-treated males showed an increase in DRV only. In females, both BPA- and BPS-treated groups showed a significant increase of 5-HT-ir in DRD compared to the controls, and BPA-treated females also showed a significant increase in MnR. Interestingly, both treatments caused alterations within the analyzed nuclei, increasing 5-HT-ir. However, while in males the increase seemed to be due to an increased number of 5-HT-positive cells, in females the alterations were appreciable only in terms of fractional area.

The EPM test allows to evaluate different aspects of anxiety-related behaviors, leaning on the balance between the natural tendency of rodents to avoid open or elevated spaces and their innate curiosity to explore unknown new areas (Carola et al., 2002; Lezak et al., 2017). A less anxious mouse will spend more time in the open arms compared to a more anxious one (Carola et al., 2002; Lezak et al., 2017). In the OF test, instead, mice with lower anxiety tend to spend more time in the center of the arena compared to the border, exploring more the open space (Carola et al., 2002; Lezak et al., 2017).
Our results firstly confirmed previous evidence of sexual dimorphism in anxiety-related behaviors (Cover et al., 2014; Donner & Lowry, 2013). In fact, both the EPM and the OF test showed that control, in estrus, females displayed less anxious behaviors, basically spending more time in open and unprotected zones compared to control males. The perinatal treatment with both BPs disrupted these sex-driven behavioral differences. On one hand, treated males seemed to be less anxious and more explorative; on the other one, treated females became more anxious compared to control ones. These results suggest an anxiolytic effect of tested BPs in

males and an anxiogenic one in females. It has already been shown that exposure to BPA, during critical periods, alters anxiety-related behaviors. In particular, males exposed *in utero* to low-dose BPA displayed a decrease in anxiety-related behavior (Kundakovic et al., 2013), while some anxiogenic effects have been shown in females exposed *in utero* (Kundakovic et al., 2013) or during the pre- or postnatal periods (Gioiosa et al., 2013) to low-dose BPA. Our data corroborated previous results, in which perinatal exposure to low-dose BPA has anxiolytic effects in males and anxiogenic effects in females. Interestingly, we noticed that the same effects are also mediated by BPS. Until now, BPS effects on anxiety in rodents have been poorly investigated. There is some evidence of anxiogenic effects of BPS exposure in females (Hu et al., 2022), while available studies suggest an increase in anxiety-related behaviors also in males (da Silva et al., 2019; Hu et al., 2022; Kim et al., 2015; McDonough et al., 2021; Mornagui et al., 2019). However, this discrepancy could be due to the different timing, dose, and way of BPS administration, which are known to be particularly accountable for the observable effects mediated by EDCs (Gioiosa et al., 2013).

The analysis of the serotonin system within the DR and MnR showed some significant effects of both BPs. First, we corroborated previous results, that described a sexual dimorphism in rodents' DR (Domínguez et al., 2003; Rubinow et al., 1998), with control females showing higher 5-HT-ir compared to control males.
Then, we demonstrated that perinatal exposure to either low-dose BPA or BPS caused an increase in 5-HT-ir in the analyzed nuclei, which impacts differentially the two sexes.
We observed an increase in both number of cells and FA in all analyzed nuclei in the BPA-treated males, while the increase was limited to DRV in the BPS-treated ones. These results are coherent with the anxiolytic effect of both BPs on males. In fact, 5-HT neurons located in DR are particularly involved in regulating the anxiety response, mainly promoting a decrease in the anxiety-like behaviors (Bocchio et al., 2016; Ren et al., 2018), thanks to the projections of the DRD to the subcortical regions and of the DRV to the cortical ones (Ren et al., 2018). Moreover, also the increase in the 5-HT-ir in the MnR, observed in the BPA-treated males, may be responsible for the anxiolytic effects, acting similarly to a 5-HT antagonist and so activating negative feedback in presynaptic serotonergic neurons located within the nucleus (Domínguez et al., 2003).
In both BPA- and BPS-treated females the effects were appreciable only in terms of FA. In fact, we observed an increase in 5-HT-ir in the DRD in both BPA- and BPS-treated females, and also in MnR of the BPA-treated ones, compared to controls. Since we did not notice any significant alterations in terms of number of cells, the increase in FA seems to be mainly due to an increase in serotonergic fibers and dendritic branching. In the literature, it is well described the impact of sex hormones on brain development and plasticity, and particularly the structural effects on neurite outgrowth, synaptogenesis, and dendritic branching mediated, especially in females, by estrogen and progesterone (Barth et al., 2015; Giannini et al., 2019). It could be possible that the opposite effect on anxiety-related behaviors of BPA and BPS in the two sexes is linked to a differential targeting of the serotonin neurons, which involved the cell bodies in males and the fibers and the dendritic branching in the females. It has been observed that perinatal BPA exposure can alter 5-HT metabolism and signaling: it increases 5-HT production in DR in both male and female rats (Nakamura et al., 2010), and it induces 5-HT increase in the hippocampus of female mice, accompanied by increased fear memory (Matsuda et al., 2013). However, the mechanisms by which BPA alters 5-HT expression have not been clearly

understood, even if some studies suggested dysregulation of tryptophan hydroxylase (TPH) (Castro et al., 2015; Yao et al., 2020). On the other hand, the effects of BPS on serotonin are not widely reported. Despite this, we showed that BPS mediates similar effects, suggesting that it could act similarly to BPA. Moreover, both BPA and BPS are known to act on a wide set of hormone receptors (Delfosse et al., 2012; den Braver-Sewradj et al., 2020; Murata & Kang, 2018; Park et al., 2020; Rochester & Bolden, 2015), detectable in brain areas involved in the control of anxiety-related behaviors (Landgraf, 2001; Nomura et al., 2005; Walf & Frye, 2006). Considering this, the observed effects could be linked to the high presence of ERβ (Mitra et al., 2003; Nomura et al., 2005), which is efficiently targeted by both BPA and BPS (Liu et al., 2019; Molina-Molina et al., 2013; Naderi & Kwong, 2020), within analyzed Raphe nuclei and especially in the DR one (Sheng et al., 2004). In fact, ERβ plays a crucial role in the regulation of anxiety-related behaviors in mice (Borrow & Handa, 2017; Imwalle et al., 2005). Furthermore, it is known that the expression of ERβ is sexually dimorphic (Mogi et al., 2015; Zhang et al., 2002) and that *in utero* exposure to BPA could lead to region-specific changes in the expression of genes encoding estrogen receptors within the brain (Kundakovic et al., 2013). Interestingly, ERβ gene expression decreases in the hypothalamus of female mice exposed to low-dose BPA, but it increases in the males (Kundakovic et al., 2013). Thus, we can speculate that, on one hand, both BPA and BPS could cause a decrease in the expression of ERβ in female Raphe nuclei, which is known to led to an increase in anxiety-like behaviors (Imwalle et al., 2005), while, on the other one, they can cause an increase in the males along with a consequent decrease in the anxiety. The potential alterations of ERβ expression within the Raphe nuclei could particularly impact the serotonergic neurons, given the high co-expression (Nomura et al., 2005). Those serotonergic neurons are richly connected to other brain regions, and in particular to several hypothalamic nuclei (Ogawa et al., 2014). Particularly, corticotropin-releasing hormone (CRH) neurons located in the paraventricular nucleus of the hypothalamus (PVN) are extremely integrated with serotonin signaling, influencing stress-related responses which are fundamental in the control of the anxiety (Jørgensen et al., 2002). The potential disruption of the stress axis, well-known target of BPs' action (Michael Caudle, 2016), is another mechanism possibly involved in the observed behavioral alterations.

In this study, we suggest that perinatal exposure to both BPA and BPS, even at low dose, leads to a disruption of sexual differences in the displaying of anxiety-related behaviors, which could be partially linked to alterations of the serotonergic system within the dorsal and median Raphe nuclei.
As the EFSA TDI for BPA appears not to be completely safe and the regulation in BPS use is still not available, our results support the need to further regulate the use of both BPs, in order to limit, or possibly avoid health implications.

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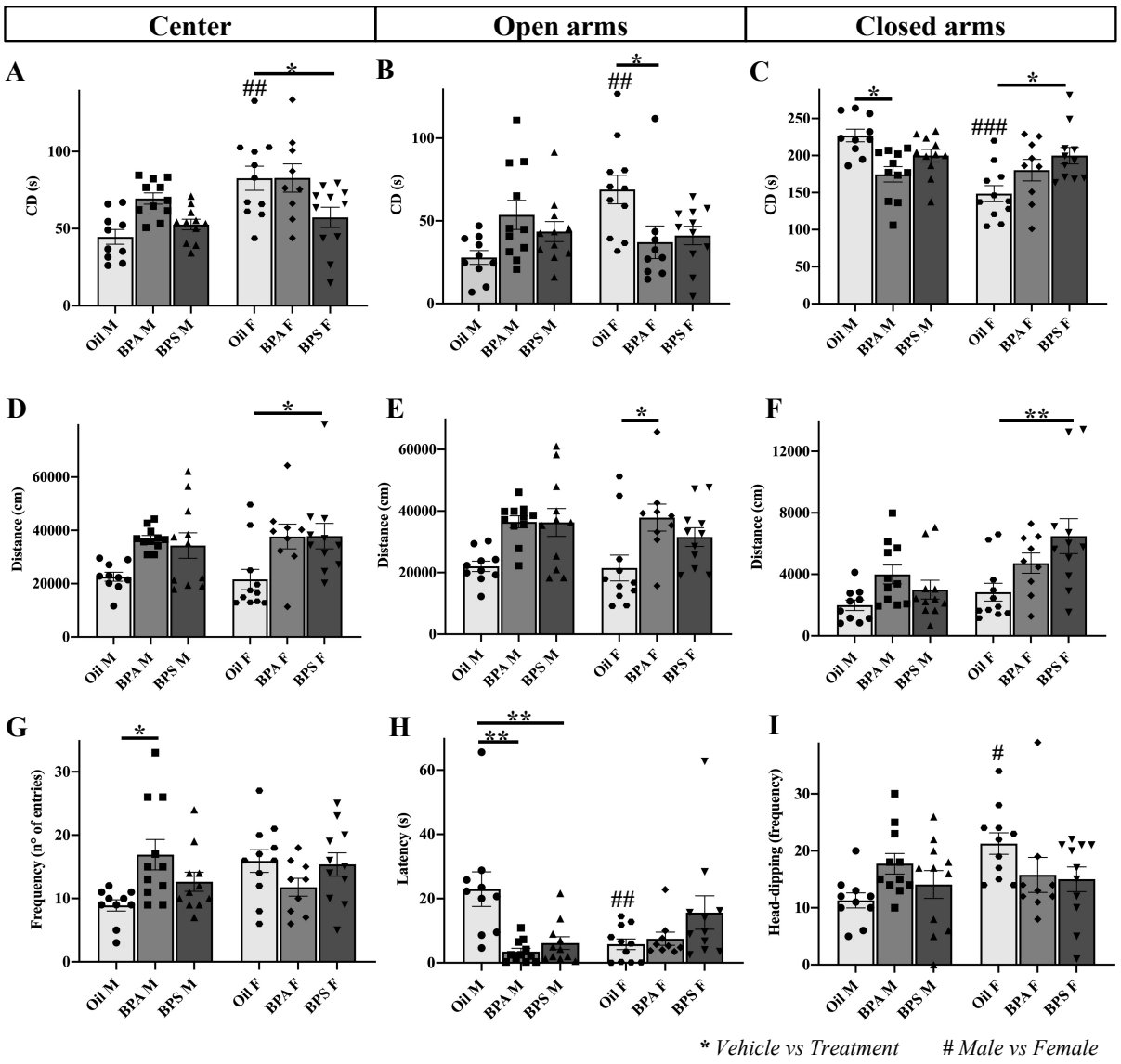

Figure 1. **Analysis of anxiety-related behaviors of control and treated mice through Elevated Plus Maze Test.** Time spent in the center ***(A)***, in open arms ***(B)*** or in closed arms ***(C)*** by oil- (*light gray*), BPA- (*gray*) or BPS- (*dark gray*) treated male mice (*left side of the graph*) or female mice (*right side of the graph*). Total distance traveled in the center ***(D)***, in open arms ***(E)*** or in closed arms ***(F)*** by oil- (*light gray*), BPA- (*gray*) or BPS- (*dark gray*) treated male mice (*left side of the graph*) or female mice (*right side of the graph*). ***(G)*** Number of entries in open arms displayed by oil- (*light gray*), BPA- (*gray*) or BPS- (*dark gray*) treated male mice (*left side of the graph*) or female mice (*right side of the graph*) within the first 5 minutes of the test. ***(H)*** Latency to first entry in open arms displayed by oil- (*light gray*), BPA- (*gray*) or BPS- (*dark gray*) treated male mice (*left side of the graph*) or female mice (*right side of the graph*) within the first 5 minutes of the test. (I) Total number of head-dipping performed by oil- (*light gray*), BPA- (*gray*) or BPS- (*dark gray*) treated male mice (*left side of the graph*) or female mice (*right side of the graph*) within the first 5 minutes of the test. Data are expressed as mean ± SEM. Two-way ANOVA revealed a significant effect for $p \leq 0.05$ (* = vehicle vs treatment; # = male vs female). CD = cumulative duration.

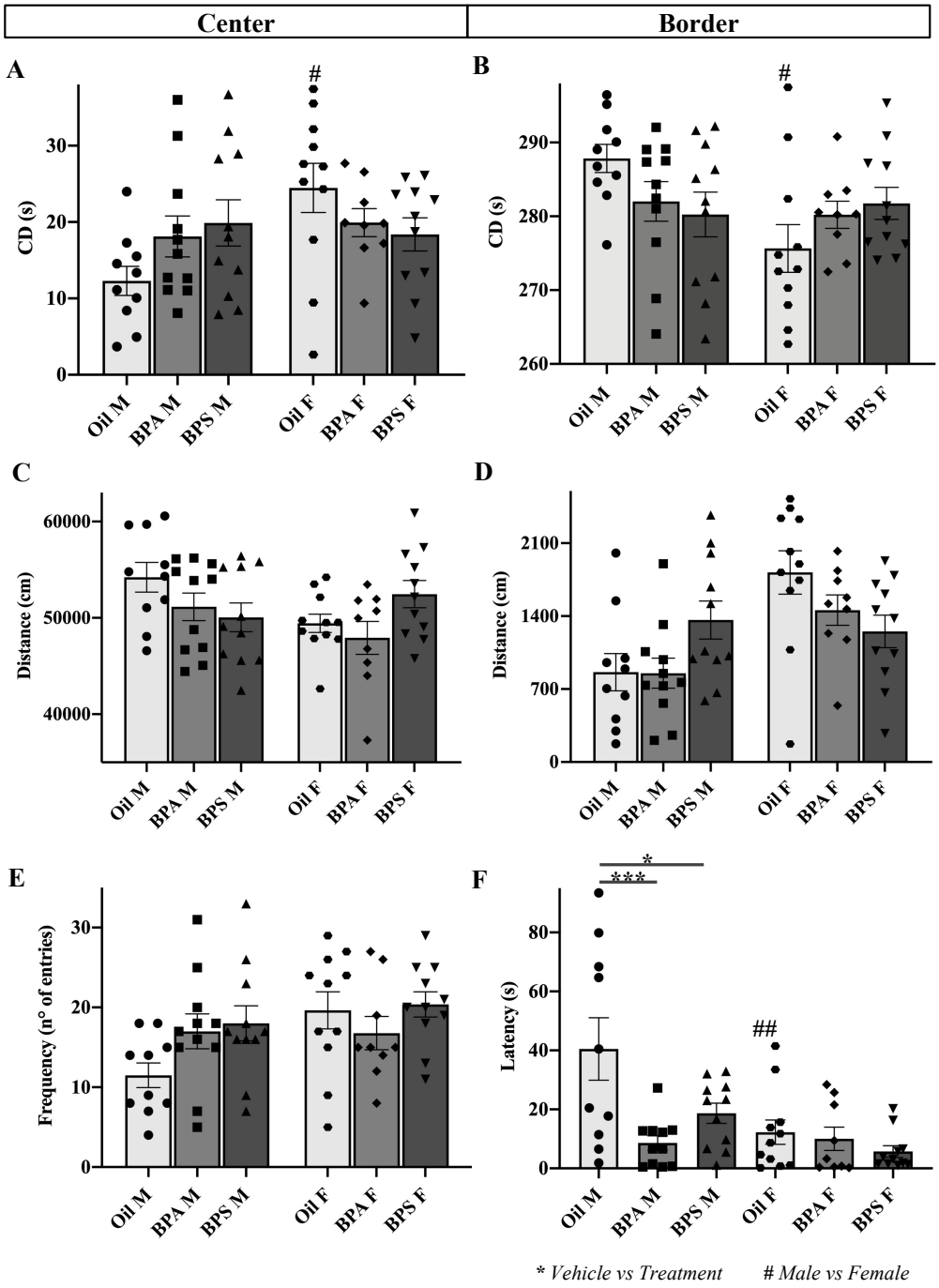

Figure 2. **Analysis of anxiety-related behaviors of control and treated mice through Open Field Test.** Time spent in the center ***(A)*** or in the border ***(B)*** of the arena by oil- (*light gray*), BPA- (*gray*) or BPS- (*dark gray*) treated male mice (*left side of the graph*) or female mice (*right side of the graph*). Total distance traveled in the center **(C)** or in the border ***(D)*** of the arena by oil- (*light gray*), BPA- (*gray*) or BPS- (*dark gray*) treated male mice (*left side of the graph*) or female mice (*right side of the graph*). ***(E)*** Number of entries in the center of the arena displayed by oil- (*light gray*), BPA- (*gray*) or BPS- (*dark gray*) treated male mice (*left side of the graph*) or female mice (*right side of the graph*) within the first 5 minutes of the test. ***(H)*** Latency to first entry in the center of the arena displayed by oil- (*light gray*), BPA- (*gray*) or BPS- (*dark gray*) treated male mice (*left side of the graph*) or female mice (*right side of the graph*) within the first 5 minutes of the test. Data are expressed as mean ± SEM. Two-way ANOVA revealed a significant effect for $p \leq 0.05$ (* = vehicle vs treatment; # = male vs female). CD = cumulative duration.

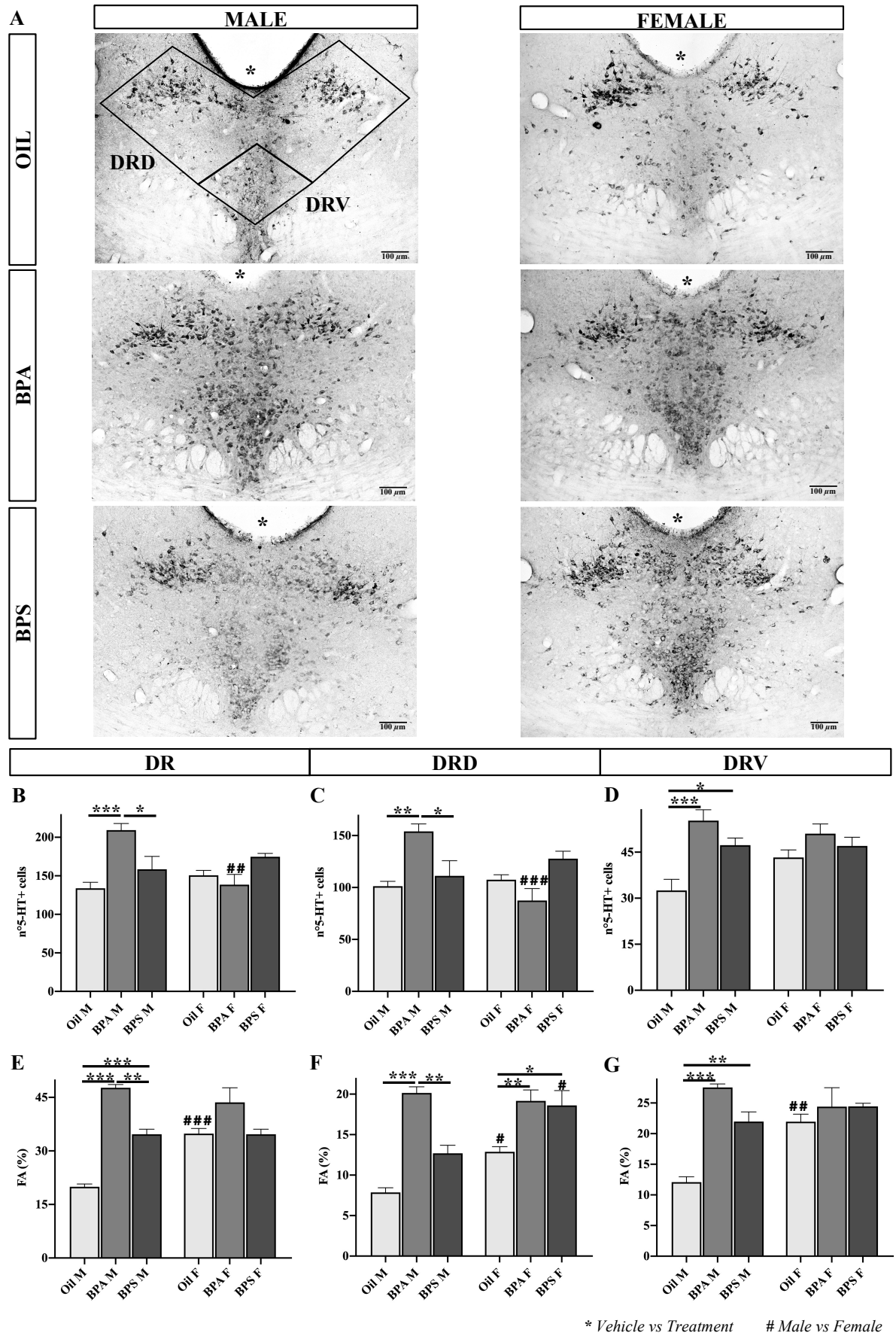

Figure 3. **Serotonin immunoreactivity in the DR of control and treated mice.** ***(A)*** Representative image of serotonin immunoreactivity in a coronal section of DR of oil-treated (*upper level*), BPA- (*central level*) or BPS- (*lower level*) treated male (*left*) or female (*right*)

mice. Analysis of 5-HT-ir in total DR (as ***(B)*** number of 5-HT+ cells and ***(E)*** fractional area), in DRD (as ***(C)*** number of 5-HT+ cells and ***(F)*** fractional area) and in DRV (as ***(D)*** number of 5-HT+ cells and ***(G)*** fractional area). In the histograms (B, C, D, E, F, G) the oil-treated mice are shown in light gray, BPA-treated mice are shown in gray, and BPS-ones are shown in dark gray. Male mice are shown on the left side of the graph, while females are on the right side. Data are expressed as mean ± SEM. Two-way ANOVA revealed a significant effect for $p \leq 0.05$ (* = vehicle vs treatment; # = male vs female). Scale bar = 100μm. 5-HT = serotonin; FA = fractional area; DR = dorsal raphe; DRD = dorsal region of dorsal raphe; DRV = ventral region of dorsal raphe; * = cerebral aqueduct.

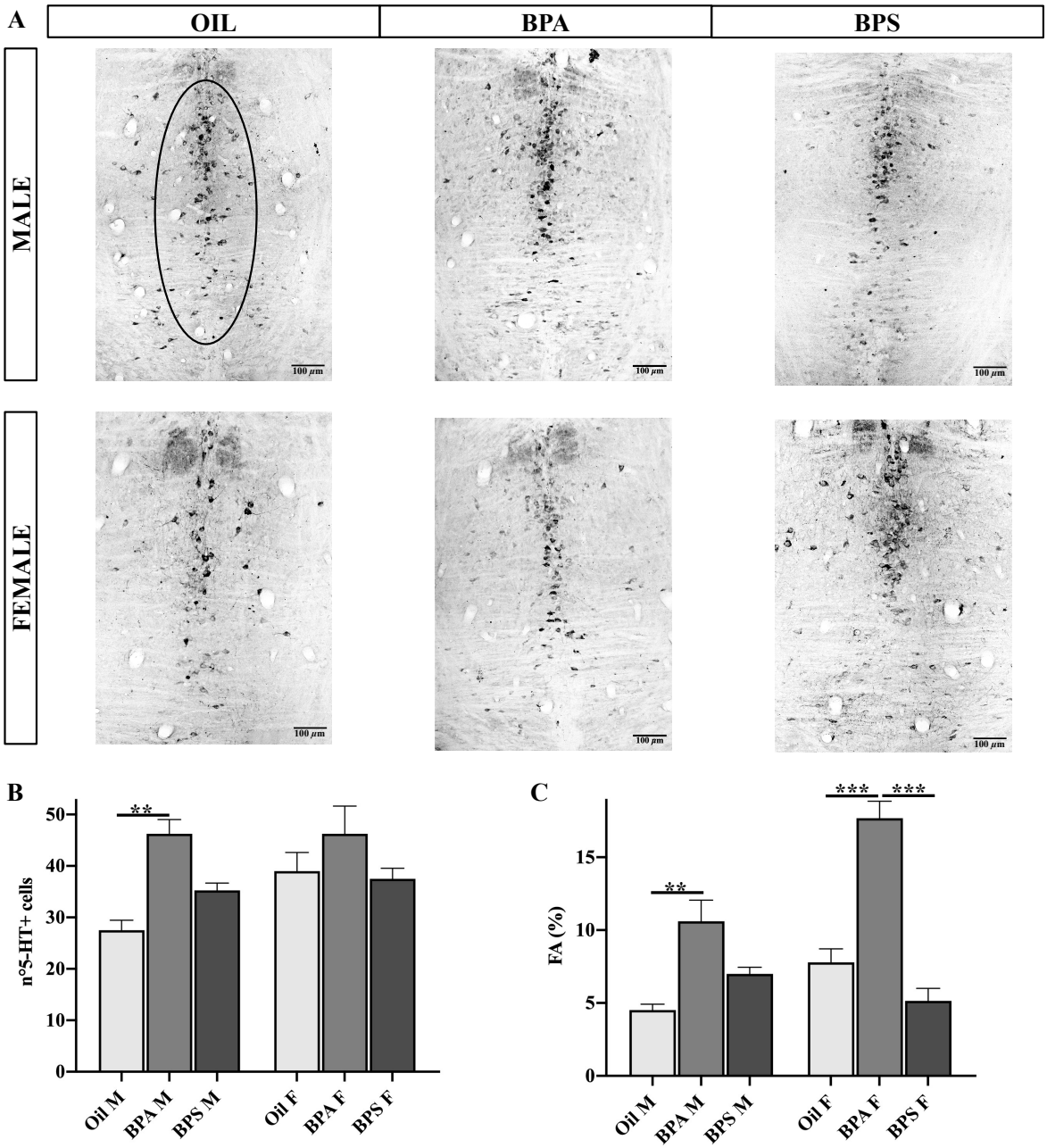

Figure 4. **Serotonin immunoreactivity in the MnR of control and treated mice.** ***(A)*** Representative image of serotonin immunoreactivity in a coronal section of MnR of oil-treated (*left*), BPA- (*center*) or BPS- (*right*) treated male (*up*) or female (*down*) mice. Analysis of 5-HT-ir in MnR, expressed both as ***(B)*** number of 5-HT+ cells and ***(C)*** fractional area. In the histograms ***(B, C)*** the oil-treated mice are shown in light gray, BPA-treated mice are shown in gray, and BPS-ones are shown in dark gray. Male mice are shown on the left side of the graph, while females are on the right side. Data are expressed as mean ± SEM. Two-way ANOVA revealed a significant effect for $p \leq 0.05$. Scale bar = 100μm. 5-HT = serotonin; FA = fractional area; MnR = median raphe; * = cerebral aqueduct.

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Effects of perinatal exposure to bisphenol A or S in EAE model of multiple sclerosis

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ABSTRACT

Epidemiological studies support the idea that multiple sclerosis (MS) is a multifactorial disease, overlapping genetic, epigenetic, and environmental factors. A better definition of environmental risks is critical to understand both etiology and the sex-related differences of MS. Exposure to Endocrine Disrupting Compounds (EDCs) fully represents one of these risks. EDCs are natural or synthetic exogenous substances (or mixtures) that alter the functions of the endocrine system. Among synthetic EDCs, exposure to bisphenol A (BPA) has been implicated in the etiology of MS, but to date, controversial data has emerged. Furthermore, nothing is known about bisphenol S (BPS), one of the most widely used substitutes for BPA. As exposure to bisphenols will not disappear soon, it is necessary to clarify their role also in this pathological condition defining their role in disease onset and course in both sexes. In this study, we examined, in both sexes, the effects of perinatal exposure to BPA and BPS in one of the most widely used mouse models of MS, experimental autoimmune encephalomyelitis (EAE). Exposure to bisphenols seemed to be particularly deleterious in males. In fact, both BPA- and BPS-treated males showed anticipation of the disease onset and an increased motoneuron loss in the spinal cord. Overall, BPA-treated males also displayed an exacerbation of EAE course and an increase in inflammation markers in the spinal cord. Analyzing the consequences of bisphenols exposure on EAE will help to better understand the role of both xenoestrogens and endogenous estrogens on the sexually dimorphic characteristics of MS.

Keywords: endocrine disrupting chemicals, environmental risk factor, BPA, BPS, Experimental Autoimmune Encephalomyelitis, MS

Author's contributions
BB designed and performed experiments, analyzed data, and wrote the draft. AC, FM, MB and FN performed experiments. MM and GCP revised the draft and wrote the paper.

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Introduction

Multiple sclerosis (MS) is a chronic autoimmune disease of the central nervous system (CNS), characterized by perivascular infiltration of inflammatory cells, demyelination, axonal loss, and gliosis (Thompson et al., 2018). MS has a different prevalence in the sexes and the female-to-male *ratio* varies between 1.5:1 and 2.5:1, with a trend toward higher values in more recent studies (2.3 - 3.5:1) (Harbo et al., 2013; Ortona et al., 2016). This data, indicating an increase in MS among women but not men, has led to in-depth studies of differences in the immune system or nervous system between women and men, which could be caused by gonadal hormones, genetic differences, different environmental exposures, and/or modern lifestyle (Alfredsson & Olsson, 2019; Ascherio & Munger, 2016; Olsson et al., 2017).
In general, women exhibit stronger humoral and cellular immune responses than men and this is thought to influence the different susceptibility to develop autoimmune disease (Rubtsova et al., 2015). This rapid increase could likely yet unidentified changes in the environment or nutrition (Alfredsson & Olsson, 2019; Ascherio & Munger, 2016). The effect of sex on the clinical features of MS is unclear, but there is evidence that women generally have an early onset of disease, have a slightly lower prevalence of primary progressive disease course, and generally show less progression to disability than men (Bergamaschi, 2007; Harbo et al., 2013; Olsson et al., 2017).

Among the main factors affecting these sex differences, gonadal hormones and different responses to environmental factors appear to be particularly significant (Rubtsova et al., 2015). The role of sex hormones in MS appears to be limited to women, however, the situation is much more complex (Ortona et al., 2016). Due to the presence of hormone receptors on immune cells, sex hormones can affect the activities of the immune system and potentially influence the risk, activity, and progression of autoimmune diseases (Moulton, 2018). In general, estrogen and prolactin act as humoral immunity enhancers, while testosterone and progesterone act as natural immunosuppressants (Pierdominici et al., 2010). In the case of estrogen, there are different effects depending on the dose: lower levels stimulate specific immune activities while higher levels (such as those in pregnancy) inhibit them (Whitacre et al., 1999). Hence, sex hormones have different effects depending not only on the concentration but also on the cell type and receptor subtype expressed on a given cell type (Ortona et al., 2016).

In view of the wide range of effects that sex hormones play within the CNS (Spence & Voskuhl, 2012), it is possible to hypothesize these hormones has a role in MS, acting not only on immune system cell populations but also on the CNS ones (Spence & Voskuhl, 2012): some endogenous and exogenous estrogens are useful in MS patients both during pregnancy (Gilli et al., 2010) and when using oral contraceptives (Sena et al., 2012). However, their action appears to be effective only in the early stages of MS (Spence & Voskuhl, 2012). Estrogen can also act on astrocytes which modulate neuronal death and inflammation through several pathways. The action of estrogens is mediated through the estrogen receptor α (ERα) that reduces inflammation, demyelination and axonal loss (Spence et al., 2011; Tiwari-Woodruff et al.,

2007), while the estrogen receptor β (ERβ) has a more controversial role. It is not involved in endogenous estrogen protection but can respond to exogenous ligands, protecting against demyelination and axonal loss and stimulating endogenous myelination (Crawford et al., 2010; Spence & Voskuhl, 2012). In women, studies are underway for estriol treatment with anti-inflammatory, neuroprotective and immunomodulatory effects (Voskuhl et al., 2016). The role of ERβ in MS is less known, but it is an attractive therapeutic candidate in association with some anti-inflammatory drugs. Indeed, estriol binds to ERα and ERβ weaker than estradiol, but it binds to ERβ stronger than to ERα, the main cause of estrogenic effects on breast cancer and cardiovascular disease (Voskuhl et al., 2016). Interestingly, a more recent study, performed in the Experimental Autoimmune Encephalomyelitis (EAE) model of MS, demonstrates that in this model, along with inflammation and demyelination in the spinal cord, is present inflammation of the hypothalamic tissue, in both females and males. This inflammation results in the downregulation of different genes in males and female, leading to sex-specific changes downstream in the hypothalamic-pituitary-axis (HPA) (Milosevic et al., 2020), supporting the idea that EAE partially also modeled sex-specific characteristics of the disease (Ryan & Mills, 2021).

In addition to endogenous estrogens, the organism can be targeted by natural (phytoestrogens and mycoestrogens) or synthetic (xenoestrogens) compounds with estrogenic activity, *i.e.*, Endocrine Disruptors Compounds (EDCs). There are thousands of chemicals, including pesticides and herbicides, dust, plastics, medical and/or dietary components, that have been classified as EDCs (Gore et al., 2015). 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, however, exposure to EDCs in adulthood also can alter physiology (Frye et al., 2012).
Environmental estrogens may display a synergic/additive effect with endogenous estrogens potentially also affecting the immune response. Moreover, there is a considerable burden of evidence *in vitro* and *in vivo* that these compounds may exert immunotoxic effects (Chighizola & Meroni, 2012; Ortona et al., 2016). There is also robust evidence that EDCs change the expression, abundance, and distribution of steroid hormone receptors in the developing CNS. Research on different EDCs consistently shows effects on mRNA levels, protein expression, and neuroanatomical distribution of nuclear hormone receptors studied to date, as well as functional consequences of altered receptor action (Gore et al., 2015).
The link between the CNS and the immune system is bidirectional, in fact receptors for neuropeptides, neurotransmitters, and hormones are located also in lymphoid organs and the activation of the immune system leads to changes in hypothalamic, autonomic, and endocrine functions (Bahadar et al., 2015). Furthermore, the autonomic and neuroendocrine outflow interacts with the immune system *via* pituitary-adrenal axis, modulating immune functions, thus representing a crucial link of CNS-immune interaction and autoimmune diseases (Bahadar et al., 2015). Therefore, the exposure to EDCs could increase risks or intensifying aggressiveness of autoimmune diseases affecting the CNS, above all MS (Ascherio et al., 2012).

One of the most known and studied EDCs is bisphenol A (BPA), a synthetic compound present into a variety of common consumer goods made by plastics and epoxy resins (Abraham & Chakraborty, 2020). BPA-based plastic is clear and tough and is made into a variety of common consumer goods (Abraham & Chakraborty, 2020). There is some evidence that BPA exposure

can alter the function of some systems, including the immune system (Kimber, 2017; Rochester, 2013). In fact, BPA exposure in mice is associated with enhanced cytokine and antibody production, and decreased numbers of regulatory T cells, although many of the reports focused on adult, as opposed to gestational, exposure to BPA (Kimber, 2017; Rochester, 2013). The risk to public health due to BPA exposure was recognized by EFSA in the 2015 (EFSA, 2015): the tolerable daily intake (TDI) for BPA was reduced from 50 to 4 µg/kg body weight/day, but the BPA substitutes, such as bisphenol S (BPS), have no specific limitations, even if they seem to have the same, or even worse, endocrine disrupting properties as the BPA (den Braver-Sewradj et al., 2020; Eladak et al., 2015; Gramec Skledar & Peterlin Masic, 2016; Rochester & Bolden, 2015).
Data on the effects of BPA exposure on different MS animal models appear to be particularly controversial, either excluding (Krementsov et al., 2013) or supporting (Brinkmeyer-Langford et al., 2014; Rogers et al., 2017) its potential effects on peculiar aspects of the disease.

Considering the increasing exposure to EDCs, and in particular to BPs, and that environmental components have been implicated in the etiology of MS, it is important to properly examine their role in the onset and course of the disease. Thus, taking the advantage of the EAE mouse model of MS, this study aimed to better understand the consequences of perinatal exposure to BPA and to evaluate and compare the one of BPS, in mice of both sexes. We assessed daily the severity of the disease both by carrying out a clinical evaluation and testing the motor symptoms, evaluating the performance with the rotarod. Finally, we evaluated the degree of inflammation and the motoneuron loss, thanks to histological investigations.

Materials and methods

Animals

Adult C57BL/6J mice from our 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 10:00 AM). Food (standard mouse chow 4RF21, Mucedola srl, Settimo Milanese, Italy) and water were provided *ad libitum*. One male and two female mice (3-month-old) were housed together to achieve a successful mating, assessed by the evaluation of the presence of the vaginal plug (assumed as gestational day 0, GD0) (Hasegawa et al., 2017).

Animal care and handling were according to the European Union Council Directive of 22nd September 2010 (2010/63/UE); all the procedures reported in the present study were approved by the Italian Ministry of Health (407/2018-PR) and by the Ethical Committee of the University of Torino (Project n° 360384).

Treatments

BPA (Sigma Aldrich, 239658, CAS 80-05-7) or BPS (Sigma Aldrich, 103039, CAS 80-09-1) were prepared for oral administration by dissolving them in corn oil (Sigma-Aldrich, C8267). 12 pregnant dams were assigned randomly to three experimental groups: oil-treated dams (receiving only vehicle, corn oil; n=4), BPA-treated dams (receiving 4 µg/kg BW/day of BPA, corresponding to the European TDI; n=4) and BPS-treated dams (receiving 4 µg/kg BW/day of

BPS; n=4). The dose was calculated daily according to dams' body weight, recorded with an electronic precision balance (*Mod. Kern-440-47N, resolution 0.1g*).
We decided to test the same dose for both BPA and BPS to allow a precise comparison of the effects of the two bisphenols. Moreover, at present, although BPS is one of the most used BPA substitutes and it has already been detected in environmental and human samples (Catenza et al., 2021), at present no user guidelines are available. Dams were treated starting at GD0, throughout pregnancy and lactation, until weaning of the offspring at postnatal day 28 (PND28). To resemble human exposure conditions, the daily treatment or the vehicle was given orally to the dams, by means of a pipette, to minimize dams' stress (Bo et al., 2016; Palanza et al., 2002). This type of administration allowed us to perform a perinatal treatment (covering both prenatal and postnatal critical window of development) (Neier et al., 2019) on the offspring. In fact, it is known that both BPA and BPS can pass first through the placenta and then into the milk during the lactation (Cimmino et al., 2020; Mao et al., 2020).

Litters were reduced to 8 pups at birth, to obtain an equal number of pups of both sexes, sexed *via* the measurement of the anogenital distance (AGD) (Manno 3rd, 2008). The pups were weaned at PND28 and housed in monosexual groups of 4 mice. They were monitored weekly until adulthood (PND56) when the experimental procedures were performed.

EAE induction and clinical evaluation
Chronic EAE has been induced in 8 weeks-old mice of both sexes (n=9/group) (Constantinescu et al., 2011). Briefly, mice have been immunized by subcutaneous immunization under the rostral part of the flanks and at the base of the tail with 300µl of 200 µg/mouse of myelin oligodendrocyte glycoprotein ($MOG_{35–55}$; Espikem, Florence, Italy) in incomplete Freund's adjuvant containing 8 mg/ml of Mycobacterium tuberculosis (strain H37Ra; Difco Laboratories Inc., St Henry, Detroit, Michigan, USA), and two intravenous injections of 500 ng of Pertussis toxin (Duotech, Milan, Italy) the day of immunization and 48 hour after (*i.e.*, 2 day post immunization, dpi) (Montarolo et al., 2014, 2015).

Body weight (BW) and clinical score (CS; 0=healthy; 1=limp tail; 2=ataxia and/or paresis of hind limbs; 3=paralysis of hind limbs and/or paresis of forelimbs; 4=tetra paralysis; 5=dying or death) have been recorded daily by a blind investigator. This analysis allows to evaluate the clinical differences in the onset and progression of the disease (Constantinescu et al., 2011; Montarolo et al., 2014, 2015).
Furthermore, since the rotarod test could be used as a more quantitative and precise clinical assessment of the disease course than the clinical score alone (van den Berg et al., 2016), mice underwent rotarod performance test daily (Mouse RotaRod, Ugo Basile 47600, Milan, Italy), starting from 6 dpi until the time of the sacrifice (28 dpi). The 1-5 dpi period has been used to train the animals in the use of the device and to obtain reference values (baseline). The test consisted in a single 300 second session during which the rod speed has been increased linearly from 4 rpm to 40 rpm (van den Berg et al., 2016). When the mouse was not capable of maintaining its balance and fell of the device, it fell and triggered a sensor, and the time (s) was recorded (latency).

Within the four weeks (0-28dpi) of EAE-follow up, we also monitored the food intake (FI, g/animal/day; once a week) and, in females, we checked the estrous cycle, for at least 2 cycles, evaluating the vaginal cytology smears (McLean et al., 2012).

Fixation and tissue sampling

At 28 dpi, mice were sacrificed, by deep irreversible anesthesia (intraperitoneal injection of Zoletil 80 mg/kg/ Rompum 10 mg/kg) and transcardially perfused with 4% paraformaldehyde (PFA) solution. Spinal cords were removed and stored in a 4% PFA solution for 24 hours, followed by several washings in 0.01 M saline phosphate buffer (PBS). Finally, they were embedded in paraffin. Paraffin-embedded spinal cords were cut in the transversal plane at 10 µm thickness with a microtome and collected on gelatin-coated slides. The plane of sectioning was oriented to match the drawings corresponding to the transversal sections of the mouse spinal cord atlas (Watson et al., 2009).

Histological evaluations

10µm-thick paraffin-embedded sections on gelatin-coated slides, representative of the entire spinal cord, were stained with Hematoxylin-Eosin (Montarolo et al., 2014, 2015) or Cresyl Violet (Nissl Staining) (Morales et al., 2006), to detect the presence of the perivascular inflammatory infiltrates (PvIIs; n=9 animals/group) and the motoneurons (MNs; n=5 animals/group) respectively. Presence of PvIIs and MNs loss are assessed as signs of the disease (Bolton & Smith, 2015; Constantinescu et al., 2011; Frezel et al., 2016; Gushchina et al., 2018).

Briefly, the staining was performed as follows: after deparaffinization, sections were stained with the Hematoxylin and Eosin procedure, by using Sigma-Aldrich (St. Louis, Missouri, USA) reagents, or they were Nissl-stained with 0.1% Cresyl Violet (Sigma-Aldrich, St. Louis, Missouri, USA). Dehydrated sections were covered with New-Entellan mounting medium (Merck, Milano, Italy).

Quantitative analysis

Neuropathological findings were quantified in 10 complete cross-sections of spinal cord per mouse representative of whole spinal cord levels. The sections were acquired and analyzed with the Neurolucida software connected to an E-800 Nikon microscope with a 20x objective (Glaser & Glaser, 1990). The number of PvIIs or MNs was calculated and expressed as the numbers of PvIIs or MNs per mm^2.

The representative images in *Figure 3* 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.S., Firenze, Italy). Images were digitized by using a 10x or 40x objective for the acquisition.

Statistical analysis

BW, FI, CS and latency at the rotarod performance test were analyzed by three-way (sex, treatment and time as independent variables) analysis of variance (ANOVA). All other quantitative data were analyzed by two-way (sex and treatment as independent variables) ANOVA with SPSS 27 statistic software (SPSS Inc., Chicago, USA). If the ANOVA was significant, the *post-hoc* analysis was performed using the Tuckey's HSD test. Comparison between the estrous cycle evaluations was performed using the Student's *t*-test. Differences

were considered statistically significant for values of p ≤0.05. Data are shown as mean ± SEM (mean standard error).

Results

Effects of BPs on body weight, food intake and estrous cycle of EAE-affected mice

The analysis of BW showed some differences ($F_{(2,48)}=1.498$, $p=0.011$; *Fig.1A*), due to the sex differences in the BW, which was always maintained ($p<0.001$; *Fig.1A*). The treatments had no effects on BW ($F_{(2,48)}=0.472$, $p=0.627$; *Fig.1A*). All the experimental groups displayed a similar BW trend, showing a decrease (*Fig.1A*) in the acute phase (within the second week post immunization) of the disease due to the increased EAE severity.
The analysis of FI did not show any differences between the groups ($F_{(2,8)}=0.950$, $p=0.485$; *Fig.1B*), but highlighted a significative decrease among all groups within the first and second post-immunization week ($p<0.001$), due to the increased EAE severity which caused difficult in reaching the food placed in an upper container in the cage. To avoid further stress to the animals, the food was then placed on the cage ground, and the FI returned to starter levels and the BW partially recovered (*Fig.1B*).

The analysis of estrous cycle in EAE-affected females revealed that both BPA and BPS treatments caused an alteration of the time spent in the different phases of the estrous cycle (*Fig.1C*). Both BPs-treated females spent more time in estral phases (proestrus and estrus; Oil vs BPA, $p<0.001$; Oil vs BPS, $p=0.004$), and less in non-estral ones (metestrus and diestrus; Oil vs BPA, $p=0.008$; Oil vs BPS, $p=0.004$) compared to oil-treated ones (*Fig.1C*). In particular, BPS-treated females spent more time in proestrus compared to oil-treated females ($p=0.031$; *Fig.1C*).

Effects of BPs on EAE onset and course

The clinical evaluation of EAE course was assessed daily, assigning both the CS and evaluating the rotarod performance (as latency of fall) in all experimental groups (*Figure 2*).

First, the analysis of the daily CS showed some significant differences in the disease course ($F_{(2,48)}=6.481$, $p=0.003$; *Fig.2A*). That is due to an increased CS among the BPA-treated males, which displayed also higher maximus reached CS (*Fig. 2C*) and a significantly higher cumulative CS ($p=0.004$, *Fig. 2D*) compared to oil-treated males. Interestingly, BPS-treated males displayed a significant increase in CS, compared to oil-treated ones, only at 7dpi ($p=0.44$) and 8 dpi ($p=0.004$). Furthermore, both BPA- ($p=0.027$) and BPS- ($p<0.001$) treated males showed an anticipation in disease onset (*Fig.2 B*) compared to the oil-treated ones, which disrupt the sexual dimorphism present among the oil-treated mice where the females displayed an anticipated onset compared to males ($p=0.011$, *Fig.2B*).

The analysis of the daily rotarod performance showed some significant differences among groups ($F_{(2,48)}=4.069$, $p=0.023$; *Fig.2E*), due to the fact that BPS-treated males displayed lower latency at 7dpi ($p=0.020$) compared to oil-treated ones. Among oil-treated groups, females showed lower latency at 27dpi ($p=0.020$) and 28 dpi ($p=0.043$) compared to males.

Effects of BPs on histological parameters in the spinal cord

The presence of PvIIs, observed in Hematoxylin-Eosin stained sections of spinal cord, and the MNs loss, measured in Cresyl Violet stained sections, are assessed as signs of disease severity (*Figure 3*).

The quantification of PvIIs highlighted some significant differences among the groups ($F_{(5,54)}=12.656$, $p<0.001$; *Fig.3C)*. First, oil-treated females displayed significantly higher values compared to oil-treated males ($p=0.013$, *Fig.3C*). This sexual dimorphism was disrupted in the treated groups. In fact, BPA-treated males showed a significant increase compared to oil-treated males ($p<0.001$, *Fig.3*), while BPA-treated females showed a significant decrease compared to oil-treated females ($p=0.006$, *Fig.3*), causing an opposite and extreme sexual difference ($p<0.001$, *Fig.3C*). On the other hand, BPS-treated males showed no difference compared to oil-treated ones ($p=0.999$, *Fig.3C*), while BPS-treated females showed a significant decrease compared to oil-treated females ($p=0.033$, *Fig.3C*), thus causing the disappearance of the sexual dimorphism in BPS-treated animals.

The analysis of MNs loss showed some significant differences among the groups ($F_{(5,24)}=3.189$, $p=0.024$; *Fig.3D)*. In particular, both BPA- ($p=0.044$) and BPS- ($p=0.027$) treated males displayed a decreased number of MNs compared to the oil-treated ones (*Fig.3D*), while we found no differences among the females (*Fig.3D*).

Discussion

MS is a multifactorial disease**,** which overlaps with genetic, epigenetic, and environmental factors (Ascherio et al., 2012; Ascherio & Munger, 2016; Waubant et al., 2019). Thus, defining the environmental risks is a crucial turning point to better understand the great variability of the diseases in terms of etiology, progression, and sexual prevalence (Ascherio et al., 2012; Ascherio & Munger, 2016).

In our study, we highlighted, in EAE model of MS, how exposure to either BPA or BPS, during a critical period of development, affected the disease onset and course, differentially in the two sexes. BPs treatment seemed to be particularly serious in males. In fact, BPA-treated males displayed the greatest alterations, showing a more aggressive disease in terms of anticipation of disease onset, clinical score, inflammation and motoneuron loss in the spinal cord. Furthermore, also BPS-treated males displayed an anticipation of the disease onset and a higher motoneuron loss in the spinal cord compared to oil-treated males. Among females, we did not notice any significant differences in the evaluated disease-related parameters, except for fewer PvIIs in the spinal cord, which did not come along with a recovered number of motoneurons.

The effect of sex on the clinical features of MS is unclear, but there is evidence that women generally have an early onset of disease, have a slightly lower prevalence of primary progressive disease course, and generally show less progression to disability than men (Bergamaschi, 2007; Harbo et al., 2004). So, even if MS is more prevalent in women compared to men, men generally developed a more aggressive and progressive form of the disease (Harbo et al., 2013; Ortona et al., 2016). As environmental exposures play a role in determining those differences (Alfredsson & Olsson, 2019; Ascherio & Munger, 2016), our results support the idea that exposure to BPs could lead to an exacerbation of the diseases in males.

Data on the effects of BPA exposure on different MS animal models appear to be controversial, while we have no information about the BPS. A 2013 study in EAE-affected female mice did not support the hypothesis that gestational BPA exposure represents a significant contributor to the increasing female MS risk (Krementsov et al., 2013). On the contrary, another 2013 study investigated, both in male and female mice, the effects of perinatal BPA exposure on Theiler's-virus-induced demyelination (TVID), another murine model of MS, showing that perinatal BPA exposure resulted in a decreased level of viral antibodies, accelerated the onset of TVID symptoms, increased inflammation in both the spinal cord and digestive tract, and amplified immune-related gene expression changes induced by viral infection (Brinkmeyer-Langford et al., 2014). The controversial results could be linked to the fact that MS is modeled using different animal models which reflect only partially the characteristic of the disease (Lassmann & Bradl, 2017; Procaccini et al., 2015), and also to the different periods and ways of administration, and dose selected for BPA treatment (Panzica & Melcangi, 2016).
However, our results are in line with the work of Rogers *et al.* (Rogers et al., 2017), which demonstrated that gestational exposure to BPA lowered the threshold for EAE onset, especially in male mice. It is interesting to notice that we observed the deleterious effects of the exposure at a lower dose (4 μg/kg BW/day vs 1 or 3 mg/kg BW/day). Moreover, we also highlighted the pathological signs of the disease at spinal cord levels. Finally, for the first time, we described the effect of BPS exposure in a murine model of MS. A more recent paper shows that subchronic exposure to BPA in mice led to deregulation of inflammatory cytokines and oxidative stress, possibly linked to neurotoxicity, axonal damage, and myelin degeneration (Khan et al., 2019). This mechanism could underlie the motoneuron loss in an immune system-independent way, which seemed to be the case, especially in BPS-treated males, displaying a significant decrease in the number of motoneurons without any increase in PvIIs compared to oil-treated ones. Increasing *in vitro* evidence describes the neurotoxic potential of BPS (Meng et al., 2021; Pang et al., 2019), while the inflammatory potential of BPS appears to be less compared to the one of BPA (Kobayashi et al., 2010; Profita et al., 2021).
In females, we did not observe any statistically relevant effects of both BPs on disease onset and course, except for a reduction in PvIIs in the spinal cord. This could be due to the effects of BPs on the estrous cycle. In fact, both BPA and BPS led to an increase of time spent in estral phases in treated females compared to oil-treated ones. This phase is characterized by increasing levels of estrogens which are known to exert an anti-inflammatory neuroprotective effect (Spence & Voskuhl, 2012). In particular, BPS-treated females spend more time in proestrus, which has been described as protective at least against neurological symptoms in the EAE models (Rahn et al., 2014).

It is important to underline the fact that the observed alterations are present in adult animals, following perinatal exposure. Perinatal BPA and BPS could cause an impairment either in immune system cell populations and in motoneurons which is maintained in adulthood or that could cause an altered response to stimuli. Moreover, both BPs could accumulate within some compartments of the organism and face a slow release (Charisiadis et al., 2018; Venisse et al., 2019). Finally, different effects observed in males and females could be due also to the fact that males appeared to be particularly vulnerable to developmental exposure to BPs (Kobayashi et al., 2010).

Studying the effects of exogenous compounds with estrogenic activity can help to better understand the role of endogenous hormones and to identify the mechanisms underlying sex differences in MS (Harbo et al., 2013; Ortona et al., 2016). Furthermore, a better definition of environmental risks is necessary. Investigating the effects of BPs exposure can help better determine their deleterious properties, which may be particularly relevant in pathological conditions. Additionally, defining BPs as a real risk of developing or worsening MS can help devise new strategies to reduce exposure for sensitive people or patients (*e.g*., avoiding specific environments, do not use plastic food/water containers, *etc.*).

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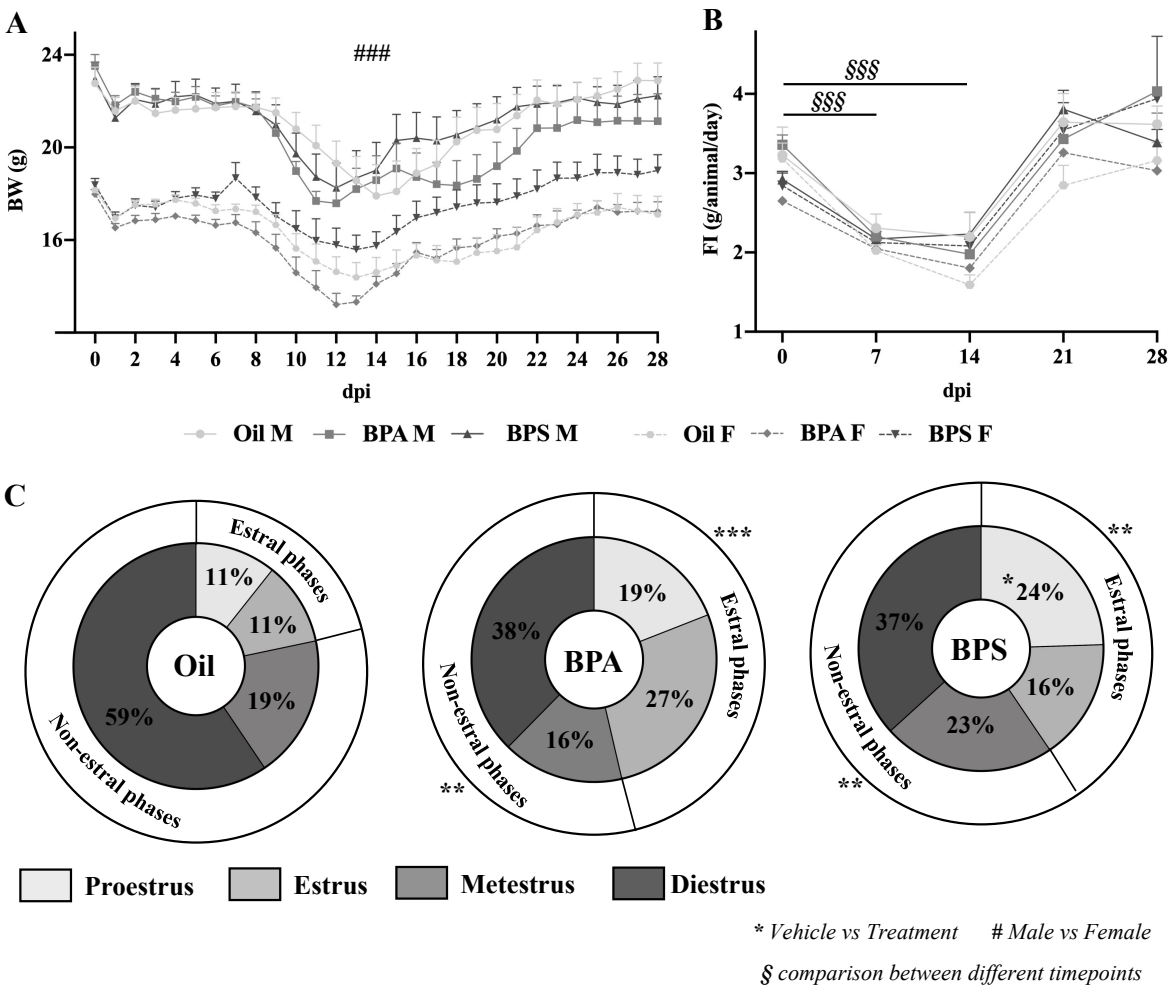

Figure 1*. Effects of BPA and BPS exposure on body weight, food intake and estrous cycle of EAE-affected mice.** Daily body weight ***(A) and weekly food intake ***(B)*** evaluation from the day of immunization (0 dpi) until the sacrifice (28 dpi) of the animals. ***(C)*** Mean percentage of time spent in the different phases of the estrous cycle, assessed by vaginal cytology smears, in the Oil- (*left*), BPA- (*center*) or BPS- (*right*) treated EAE-affected females. Data are expressed as mean ± SEM. Statistical analysis revealed a significant effect for $p \leq 0.05$ (* = vehicle vs treatment; # = male vs female; §=comparison between different timepoints). BW = body weight; FI= food intake; dpi = day post immunization.

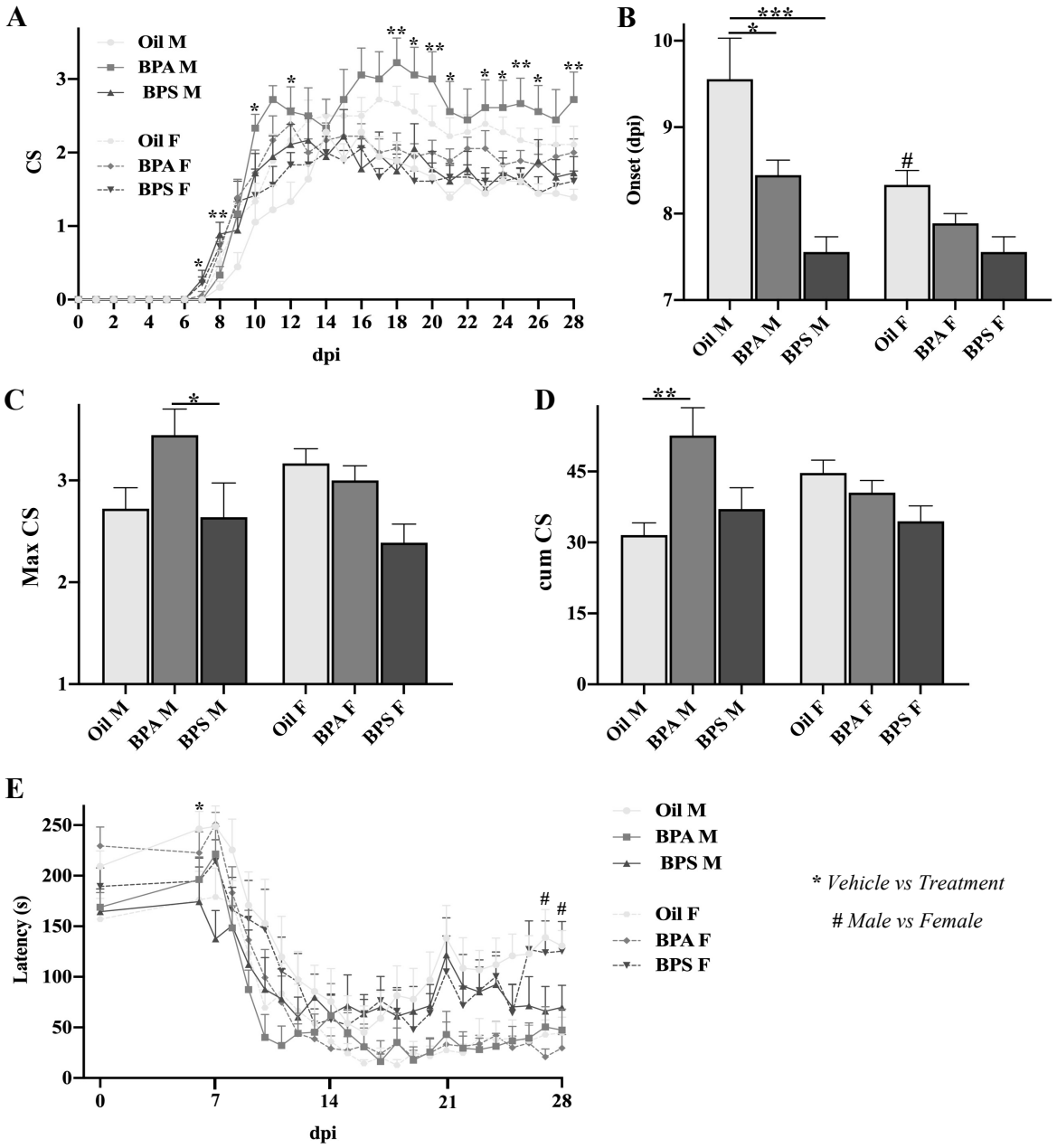

Figure 2*. Effects of BPA and BPS exposure on EAE clinical evaluations.** ***(A) Daily clinical score evaluation from the day of immunization (0 dpi) until the sacrifice (28 dpi) of the animals. ***(B)*** Mean dpi of disease onset in the Oil- (*left*), BPA- (*center*) or BPS- (*right*) treated EAE-affected male (*left side of the graph*) and female (*right side of the graph*) mice. Mean ***(C)*** maximus clinical score and ***(D)*** cumulative clinical score reached by the Oil- (*left*), BPA- (*center*) or BPS- (*right*) treated EAE-affected male (*left side of the graph*) and female (*right side of the graph*) mice. Data are expressed as mean ± SEM. Statistical analysis revealed a significant effect for $p \leq 0.05$ (* = vehicle vs treatment; # = male vs female). CS = clinical score; Max CS = maximus clinical score; cum CS = cumulative clinical score; dpi = day post immunization.

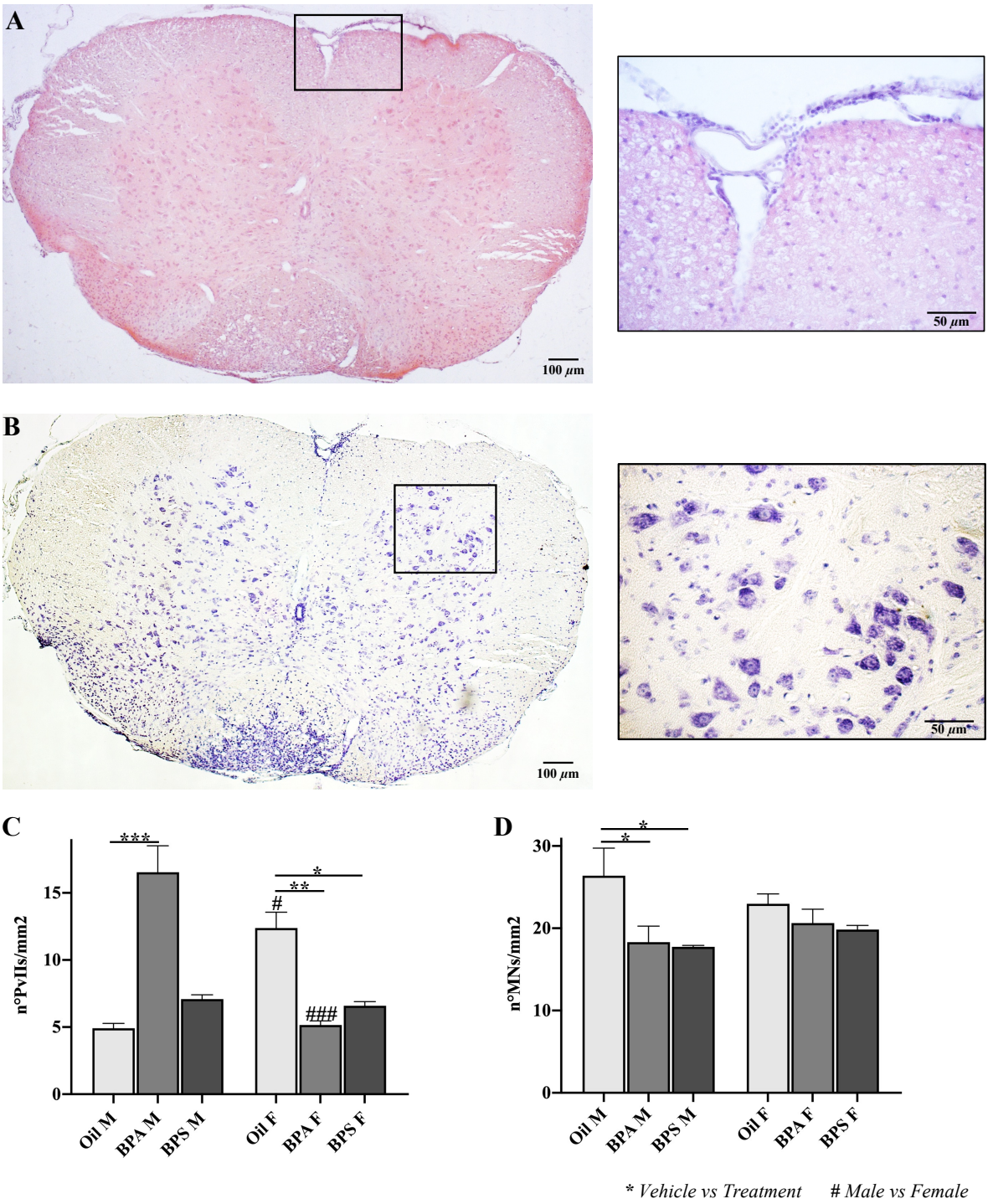

Figure 3*. Analysis of perivascular inflammatory infiltrates and motoneuron loss in spinal cord sections of Oil-, BPA- or BPS-treated EAE-affected mice.** Representative images of ***(A) Hematoxylin-Eosin and ***(B)*** Nissl staining in a transversal section of spinal cord from an Oil-treated EAE-affected male mice. Analysis of the ***(C)*** presence of PvIIs and ***(D)*** motoneuron loss in the spinal cords of Oil- (*left*), BPA- (*center*) or BPS- (*right*) treated EAE-affected male (*left side of the graph*) and female (*right side of the graph*) mice. Data are expressed as mean ± SEM. Statistical analysis revealed a significant effect for $p \leq 0.05$ (* = vehicle vs treatment; # = male vs female). Scale bar = 100 µm (10x) or 50 µm (40x). PvIIs = perivascular inflammatory infiltrates; MNs = motoneurons; *central canal.

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GENERAL CONCLUSIONS

Increasing exposure to Endocrine Disrupting Chemicals (EDCs, *i.e.*, exogenous chemicals, or mixture of chemicals, that interfere with any aspect of hormone action and cause adverse effects at the level of the organism, its progeny, populations, or subpopulations of organisms) (Zoeller et al., 2012) is linked to public health concerns (Yilmaz et al., 2020) and economic implications (Kassotis et al., 2020).
Among EDCs, bisphenols (BPs) represent an extremely abundant class of synthetic EDCs, present in plastic-based consumer goods (Catenza et al., 2021). The extensive exposure to them on a daily basis due to their wide use is the main cause of concern (Yilmaz et al., 2020).
Bisphenol A (BPA) is the first and still the most highly produced BP (Catenza et al., 2021), but, as the evidence of its deleterious effects increase, some substitutes have been proposed (Abraham & Chakraborty, 2020). One of the most used one is bisphenol S (BPS), which seems to display the same, or even worse, endocrine disrupting properties as the BPA (Catenza et al., 2021; den Braver-Sewradj et al., 2020).
Considering the variety of molecular targets of both BPA and BPS (den Braver-Sewradj et al., 2020; MacKay & Abizaid, 2018), their action can affect different tissues in the organism (Rochester, 2013; Rochester & Bolden, 2015). In particular, effects of BPA on brain and behavior are supported by a quite large number of experimental and epidemiological studies (Frye et al., 2012; Gore et al., 2019; Mustieles et al., 2015; Patisaul, 2020; Wolstenholme et al., 2011) and those investigating deleterious effects of BPS are alarmingly increasing (Naderi & Kwong, 2020).

The main aim of the thesis was to evaluate the effects, on both brain and behavior, of oral exposure, either during particularly sensitive period of adulthood (*i.e.,* pregnancy and lactation) or during development (*i.e.,* perinatal period), to low dose (*i.e*., 4µg/kg BW/day, EFSA TDI for BPA) (EFSA, 2015) BPA or BPS in C57BL/6J mice. To do so, five experiments were performed.

In the first experiment (*Chapter 3*) (Bonaldo et al., 2021), the long-term consequences of chronic exposure (covering pregnancy and lactation and reaching 20 weeks of treatment) to low-dose BPA on social behavior and related vasopressin (AVP) and oxytocin (OXT) systems of the direct exposed dams were investigated. Those dams displayed altered social behavior, interacting more with females and less with males compared to the controls. Interestingly, immunohistochemical analysis highlighted a decreased AVP-immunoreactivity (ir) in the hypothalamic paraventricular (PVN) and suprachiasmatic (SCh) nuclei, along with a reduction in G-protein-coupled estrogen membrane receptor 1 (GPER-1)-ir in the same nuclei, of the BPA-treated dams compared to controls. Conversely, no alterations were found in the OXT system.
In the second experiment (*Chapter 4*), the consequences of exposure throughout pregnancy and lactation to low dose of either BPA or BPS on the spontaneous maternal behavior and related hypothalamic OXT system, and how this can affect pups' survival within the first postnatal week, were investigated. In both BPA- and BPS-treated dams, a decrease in pup-related

behaviors, along with an increase in pup-unrelated behaviors, was observed. Interestingly, only the BPA-treated dams showed a significant increase in the OXT-ir in the PVN and in the supraoptic nucleus (SON). Moreover, within the first postnatal week, offspring mortality impacted differentially BPA and BPS litters, with more female dead pups among the BPA litters, while more male dead pups in the BPS litters, sharpening the difference in the sex *ratio* observed at birth (*i.e.*, lower number of females in BPA litters and lower number of males in the BPS ones).

In these two experiments, the effects of exposure to BPs in adulthood were evaluated. Even if in the first experiment we focused on BPA (still the most produced and widespread BP), performing a chronic treatment (20 weeks) which covered pregnancy and lactation, and in the second one we tested both BPA and BPS administered exactly during pregnancy and lactation, it can be assumed that, although exposure to EDCs is known to exert the most deleterious effects if it occurs during critical periods of development, exposure during adulthood is also matter of concerns (Frye et al., 2012; Rattan et al., 2017). In fact, pregnancy and lactation seem to represent particularly sensitive periods of adult life for endocrine disruption (Nesan & Kurrasch, 2020). Thus, adult exposure to BPs can impact not only on the mother-pup interaction and so, both directly and indirectly, on pups' survival, but also on the direct exposed dams, altering fundamental behaviors and related neuroendocrine circuits, essential for guaranteeing the quality of life of those animals (Beery & Kaufer, 2015; Cummings et al., 2010; Gore et al., 2019). Last, impairments in the maternal care could lead to long term epigenetic modifications (Curley & Champagne, 2016), which could be implied, along with the treatments themselves, in some alterations observed in the offspring (discussed below), such as altered response to stress and social stimuli and related neural circuits (Curley & Champagne, 2016), but also altered immune system's responses (Meagher et al., 2010; Parker & Douglas, 2010; Zajdel et al., 2019).

In the third (*Chapter 5*) and fourth (*Chapter 6*) experiments the effects on sexual and anxiety-related behaviors and on neural systems involved in the control of these behaviors were evaluated in adult male and female mice perinatally exposed to low-dose BPA or BPS.

The third experiment is focused on sexual behavior and related hypothalamic kisspeptin (kiss) system. The treatments resulted in sexually differentiated alterations of some reproductive-relevant parameters (*i.e.*, puberty onset and estrous cycle), sexual behaviors and kiss-ir within the rostral periventricular area of the third ventricle (RP3V), the PVN and the arcuate (Arc) hypothalamic nuclei. Exposure to BPs affected pubertal timing, with BPA causing a delay in females and BPS causing an anticipation in males. Both BPs altered estrous cycle in females, increasing the time spent in estrus in spite of non-estral phases. Furthermore, both BPs partially altered some aspects of sexual and sexual-related behaviors, mainly in males. In particular, BPA-treated males seemed to be the most affected, spending less time exploring the female bedding and showing fewer mounts and intromissions, while BPS-exposed males showed an increased number of mounts, intromissions and anogenital sniffing. Last, the immunohistochemical analysis highlighted that both BPs caused an increase of kiss-ir within the RP3V, while BPA led to an increase of the PVN innervation, and BPS induced a reduction in treated females compared to the control ones. Among males, BPA caused an increase of kiss-ir in the Arc while BPS caused a decrease of the PVN innervation, compared to controls.

The fourth experiment is focused on anxiety-related behavior and related serotonergic system within the raphe nucleus. Behavioral analysis suggested that both BPs altered anxiety-related

behaviors, mediating mainly anxiolytic effects in males and anxiogenic effects in females. The immunohistochemical analysis highlighted that BPA caused an increase of serotonin (5-HT) ir in the dorsal (DR) and median (MnR) raphe of males and in dorsal region of DR and in the MnR of females, while BPS led to an increase of 5-HT-ir in the ventral region of DR in males and in the dorsal region of DR in females. Interestingly those differences were evident in terms of number of 5-HT+ cells in males, and of fractional area in females.
Even if these two experiments are separately presented, in both the effects of exposure to BPs during the perinatal period were evaluated. In fact, it is interesting to point out that analyzed behaviors are highly interconnected. It is known the impaired response to stress stimuli has an impact also on sexual and socio-sexual behaviors (Magariños et al., 2018). Nevertheless, kiss and 5-HT play a role in the regulation of both behavioral aspects (Mills et al., 2021; Olivier et al., 2011). On one hand, the central administration of kiss to male mice mediates anti-depressive effects (Tanaka et al., 2013), while its role on anxiety is still controversial, even if its central administration in male rats resulted in promoting anxiety-like behaviors (Csabafi et al., 2013). On the other hand, 5-HT role in the control of sexual behaviors is more investigated. In fact, increased 5-HT, particularly in the amygdala and in the hypothalamus, leads to an inhibition of male sexual and emotional behaviors (Iovino et al., 2019), while it seems to play a more controversial role in female sexual behavior, linked to the receptor expression patterns in different brain regions (Snoeren et al., 2014). Interestingly, even if some studies supported the idea of minor or strictly time-depending role of 5-HT in the control of female sexual behaviors (Hegstad et al., 2020), the activation of 5-HT1A receptor (Kishitake & Yamanouchi, 2003; Snoeren, Chan, et al., 2011; Snoeren, Refsgaard, et al., 2011) or the increased 5-HT availability in the synapse (Adams et al., 2012; Uphouse et al., 2006) inhibits paracopulatory behavior and lordosis in female rats.
Finally, from these experiments, it can be generally assumed that:
- A confirmation that perinatal period is a critical time window during which exposure to both BPA and BPS leads to long-term consequences in brain and behaviors (Bakoyiannis et al., 2021; Frye et al., 2012) and that this is also true for BPS;
- Effects of exposure to BPA or BPS are different and sometimes opposite in the two sexes, underlying the necessity of including both sexes when potential effects of an EDC are evaluated (Gioiosa et al., 2013);
- The tested dose (*i.e.*, 4µg/kg BW/day, EFSA TDI for BPA) (EFSA, 2015) appeared not to be safe for BPA nor for BPS, which is still without regulation.

Last, in the fifth experiment (*Chapter 7*), taking the advantage of the Experimental Autoimmune Encephalomyelitis (EAE) mouse model of multiple sclerosis (MS), the effects on some aspects of the disease were evaluated in adult male and female mice perinatally exposed to low-dose BPA or BPS. As MS is a sexually dimorphic and multifactorial disease and various environmental components have been implicated in its etiology (Belbasis et al., 2015; Bergamaschi, 2007; Olsson et al., 2017), exposure to BPs is proposed as new environmental risk factor which can contribute to the different prevalence and clinical features of the disease observed in the two sexes (Harbo et al., 2013; Ortona et al., 2016). In fact, BPA led to the development of more aggressive disease in terms of anticipation of disease onset, clinical score, inflammation, and motoneuron loss in the spinal cord in males, and also BPS-treated males displayed an anticipation of the disease onset and a higher motoneuron loss in the spinal cord compared to the vehicle-treated ones. Among females, both BPA and BPS did not seem to alter

the evaluated disease-related parameters, except for fewer perivascular inflammatory infiltrates in the spinal cord, which did not come along with a recovered number of motoneurons. Considering the controversial data about BPA effect on MS (Krementsov et al., 2013; Rogers et al., 2017) and the lack of data about BPS, these results support the idea that exposure to both BPA and BPS represents a potential risk factor for MS, especially in males. Indeed, these results are in line with the fact that, even if MS is more prevalent in women compared to men, men generally develop a more aggressive and progressive form of the disease (Harbo et al., 2013; Ortona et al., 2016), and, as environmental exposures play a role in determining those differences (Alfredsson & Olsson, 2019; Ascherio & Munger, 2016), exposure to BPs could lead to an exacerbation of the diseases in males.

To conclude, our results strengthened previous data about deleterious effects of exposure, both in adulthood and during critical periods of development, to BPA and highlighted those of BPS, which, therefore, cannot represent a safe alternative. Furthermore, BPs exposure altered not only some physiological and behavioral parameters in healthy conditions but also exacerbated some aspects of MS pathology. Thus, a better definition of environmental risks represented by BPA and its analogue BPS is necessary to plan new strategies and draft updated guidelines to reduce, or possibly avoid, exposure to these compounds.

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List of papers

B. Bonaldo, L. Gioiosa, GC. Panzica, M. Marraudino "*Exposure to either bisphenol A or S represents a risk for crucial behaviors for pup survival, such as spontaneous maternal behavior and related oxytocinergic circuits in mice*" - *Submitted to Neuroendocrinology.*

M. Marraudino, **B. Bonaldo**, B. Vitiello, G.C. Bergui, GC. Panzica. "*Sexual differences in internet gaming disorder (IGD): from psychological features to neuroanatomical networks*" - Journal of Clinical Medicine – *Accepted (# jcm-1533364).*

B. Bonaldo, A. Casile, M. Bettarelli, S. Gotti, GC. Panzica, M. Marraudino (2021) "*Effects of chronic exposure to bisphenol A in adult female mice on social behavior, vasopressin system, and estrogen membrane receptor (GPER1)*" - European Journal of Histochemistry 2021 Nov 10;65(s1):3272. *[doi: 10.4081/ejh.2021.3272]*

S. Perga, F. Montarolo, S. Martire, **B. Bonaldo**, G. Bono, J. Bertolo, R. Magliozzi, A. Bertolotto (2021) "*Overexpression of the ubiquitin-editing enzyme A20 in the brain lesions of Multiple Sclerosis patients: moving form systemic to central nervous system inflammation*" Brain Pathology 2021 Mar;31(2):283-296. *[doi: 10.1111/bpa.12906]*

M. Marraudino, B. Carrillo, **B. Bonaldo**, R. Llorente, E. Campioli, I. Garate, H. Pinos, L.M. García-Segura, P. Collado, D. Grassi (2021) "*G protein-coupled estrogen receptor immunoreactivity in the rat hypothalamus is widely distributed in neurons, astrocytes and oligodendrocytes, fluctuates during the estrous cycle and is sexually dimorphic*" Neuroendocrinology 2021;111(7):660-677. *[doi: 10.1159/000509583]*

R. Llorente, M. Marraudino, B. Carrillo, **B. Bonaldo**, J. Simon-Areces, P. Abellanas-Pérez, M. Rivero-Aguilar, J.M. Fernandez-García, H. Pinos, L.M. García-Segura, P. Collado, D. Grassi (2020) "*G protein-coupled estrogen receptor immunoreactivity fluctuates during the estrous cycle and show sex differences in the amygdala and dorsal hippocampus*" Frontiers in Endocrinology 2020 Aug 7;11:537. *[doi: 10.3389/fendo.2020.00537]*

M. Marraudino, **B. Bonaldo**, A. Farinetti, GC. Panzica, G. Ponti, S. Gotti (2019) "*Metabolic disrupting chemicals and alteration of neuroendocrine circuits controlling food intake and energy metabolism*" Frontiers in Endocrinology 2019 Jan 9;9:766. *[doi: 10.3389/fendo.2018.00766]*

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"*Those who dwell, as scientists or laymen, among the beauties and mysteries of the earth are never alone or weary of life. Whatever the vexations or concerns of their personal lives, their thoughts can find paths that lead to inner contentment and to renewed excitement in living. Those who contemplate the beauty of the earth find reserves of strength that will endure as long as life lasts*."

(Rachel Carson, *The Sense of Wonder*, 1965)