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Full Length Article

Perinatal bisphenol A (BPA) exposure alters brain oxytocin receptor (OTR) expression in a sex- and region- specific manner: A CLARITY-BPA consortium follow-up study

Shannah K. Witchey[a], Joelle Fuchs[a], Heather B. Patisaul[a,b,*]

[a] Department of Biological Sciences, NC State University, Raleigh, NC, 27695, United States
[b] Center for Human Health and the Environment, NC State University, Raleigh, NC, 27695, United States

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ABSTRACT

Bisphenol A (BPA) is a well-characterized endocrine disrupting chemical (EDC) used in plastics, epoxy resins and other products. Neurodevelopmental effects of BPA exposure are a major concern with multiple rodent and human studies showing that early life BPA exposure may impact the developing brain and sexually dimorphic behaviors. The CLARITY-BPA (Consortium Linking Academic and Regulatory Insights on BPA Toxicity) program was established to assess multiple endpoints, including neural, across a wide dose range. Studies from our lab as part of (and prior to) CLARITY-BPA have shown that BPA disrupts estrogen receptor expression in the developing brain, and some evidence of oxytocin (OT) and oxytocin receptor (OTR) disruption in the hypothalamus and amygdala. While BPA disruption of steroid hormone function is well documented, less is known about its capacity to alter nonapeptide signals. In this CLARITY-BPA follow up study, we used remaining juvenile rat tissues to test the hypothesis that developmental BPA exposure affects OTR expression across the brain. Perinatal BPA exposure (2.5, 25, or 2500 μg/kg body weight (bw)/day) spanned gestation and lactation with dams gavaged from gestational day 6 until birth and then the offspring gavaged directly through weaning. Ethinyl estradiol (0.5 μg/kg bw/day) was used as a reference estrogen. Animals of both sexes were sacrificed as juveniles and OTR expression assessed by receptor binding. Our results demonstrate prenatal exposure to BPA can eliminate sex differences in OTR expression in three hypothalamic regions, and that male OTR expression may be more susceptible. Our data also identify a sub-region of the BNST with sexually dimorphic OTR expression not previously reported in juvenile rats that is also susceptible to BPA.

1. Introduction

Neuronal development is heavily dependent on steroid and other hormones, particularly for sexual differentiation, which makes it an especially vulnerable target for endocrine disruption (McCarthy et al., 2009; Nesan et al., 2018; Patisaul, 2017; Schug et al., 2011; Wolstenholme et al., 2011a). Bisphenol A (BPA) is a well-characterized EDC, known to interfere with endogenous hormone signaling and metabolism, particularly during development, and to induce long-term impairments to brain structure and function in multiple species (reviewed in: (Nesan et al., 2018; Patisaul, 2019; Schug et al., 2015; Wolstenholme et al., 2011a)). Robust experimental and epidemiological evidence has repeatedly linked developmental BPA exposure to sex-dependent socioemotional behavioral outcomes such as anxiety, hyperactivity, externalizing behaviors, and cognitive deficits, even at doses below the current US Food and Drug Administration No Observed Adverse Effect Level (NOAEL) of 5 mg/kg body weight (bw)/day (Braun et al., 2011; Cao et al., 2014; Jasarevic et al., 2013; Kinch et al., 2015; Nesan et al., 2018; Patisaul et al., 2012; Rebuli et al., 2015; Sullivan et al., 2014; Wolstenholme et al., 2011a, b). In its 2012 report, the Food and Agriculture Organization of the United Nations and the World Health Organization identified “changes in anxiety and convergence of anatomical brain sex differences” as a potential human-relevant health risk of developmental BPA exposure (FAO/WHO, 2011). Along with the evolutionarily related nonapeptide, vasopressin (AVP), oxytocin (OT) is a critical modulator of socioemotional behaviors including anxiety (Johnson and Young, 2017; Patisaul, 2017). Here we tested the hypothesis that developmental BPA exposure sex-specifically alters oxytocin receptor (OTR) density across multiple brain regions.

This study was conducted as part of the inter-agency research program known as the consortium linking academic and regulatory insights on BPA toxicity (CLARITY-BPA). A collaborative effort between

* Corresponding author at: NC State University, 127 David Clark Labs, Raleigh, NC, 27695, United States.
E-mail address: hbpatisa@ncsu.edu (H.B. Patisaul).

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academic and government scientists, CLARITY-BPA is coordinated by the National Toxicology Program (NTP) and involves the National Institute of Environmental Health Sciences (NIEHS), the U.S. Food and Drug Administration (FDA), the National Center for Toxicological Research (NCTR) and multiple academic investigators (Birnbaum et al., 2012; Heindel et al., 2015; Schug et al., 2013). The studies incorporated research recommendations identified by WHO and others to strengthen the robustness and reproducibility of EDC studies (Beronius et al., 2010; Chapin et al., 2008; FAO/WHO, 2011; FDA, 2012; NTP, 2008) including the strict use of blinding, use of a single animal strain (agreed to be a strain of Sprague Dawley rat bred at NCTR (NCTR-SD)), controlling for potential litter effects, testing and comparing effects in both sexes, minimizing exogenous EDC exposure, oral dosing, use of a reference estrogen (ethinylestradiol (EE)), and evaluation of multiple BPA doses, particularly levels at or below the FDA NOAEL. CLARITY-BPA consists of two main components, a "core study" conducted at NCTR using endpoints typical of a classic guideline toxicology study, and "grantee studies" conducted by academic labs, all using shared tissues obtained from NCTR.

In our prior BPA-CLARITY studies, we demonstrated that perinatal exposure to BPA, below the FDA NOAEL, alters the volume of sexually dimorphic brain regions in juvenile NCTR-SD rats (Arambula et al., 2017). We have further shown that prenatal BPA exposure changes the expression of multiple hormones and hormone receptors, including estrogen receptors (ERs) and androgen receptors, in the hippocampus, hypothalamus, and amygdala as early as PND 1 (Arambula et al., 2016, 2018)(reviewed in (Patisaul, 2019)). Evidence from a similarly designed study, using the same rat strain and experimental procedures, revealed that some of these effects are persistent (Cao et al., 2014; Rebuli et al., 2014). Of particular interest for the present studies was the observed dose-dependent disruption of OT expression in both male and female PND1 hippocampus and hypothalamus (Arambula et al., 2016), and OTR expression in the amygdala (Arambula et al., 2018). The present study used remaining available tissue from our juvenile CLARITY-BPA rats (Arambula et al., 2017) to quantify OTR levels.

Classically considered to be estrogen disrupting, BPA has also been observed to bind androgen receptors, thyroid receptors, glucocorticoid receptor and PRARγ (reviewed in (MacKay and Abizaid, 2018)). BPA crosses the placental barrier and is routinely detected in cord blood, fetal tissue, placenta and amniotic fluid (Gerona et al., 2013; Padmanabhan et al., 2008; Vandenberg et al., 2010), thus effects on the developing human brain are plausible. BPA is used in plastics and epoxy resins for a variety of consumer products ranging from food and beverage containers to medical devices and thermal paper (Geens et al., 2011). Human exposure primarily occurs from the ingestion of contaminated food and beverages (Konieczna et al., 2015; Rudel et al., 2011; Vandenberg et al., 2007), with nearly everyone having measurable bodily levels of BPA (Corrales et al., 2015; Geens et al., 2012). Exposure estimates vary but are generally under 1 μg/kg/day for North American adults (Lakind et al., 2012), but higher in children and infants (Corrales et al., 2015; Geens et al., 2009; Lakind et al., 2012). The United States Environmental Protection Agency (EPA) reference dose for oral BPA exposure is 50 μg/kg/day (Lakind et al., 2012), with equivalent benchmarks lower in Canada (25 μg/kg/day) (Eladak et al., 2015) and the European Union (4 μg/kg/day) (Hessel et al., 2016). Because exposure is nearly ubiquitous and increasing, possible consequences of human exposure at levels at or below these presumed "safe" levels are of concern (Schug et al., 2015; Wolstenholme et al., 2011a).

OT is synthesized primarily by the paraventricular (PVN) and supraoptic nuclei (SON) and released in multiple areas of the mesolimbic dopamine system and other brain nuclei that play a role in food intake, social recognition, mate choice, anxiety, empathy, parental care and other socioemotional behaviors (Caldwell, 2017; Carter et al., 2009; Johnson and Young, 2015; King et al., 2016; Patisaul, 2017). OT directly released into the periphery via the posterior pituitary (neurohypophysis) coordinates the milk "let down" reflex, uterine contraction at birth, and aspects of cardiovascular development and physiology. Many aspects of the OT signaling pathways are sexually dimorphic and can sex-specifically influence sociosexual behaviors including anxiety (Caldwell, 2017; Johnson and Young, 2015; King et al., 2016; Scott et al., 2015; Smith et al., 2017). Exquisitely sensitive to steroid hormones, responsivity of OT and OTR to estrogens and androgens can also be sex specific. For example, in the adult rodent brain, OTR is upregulated by estradiol in the female ventrolateral portion of the ventromedial nucleus (VMNvl), while testosterone can suppress OT release (Coirini et al., 1992; Johnson, 1992).

In rodents, manipulation of perinatal OT levels can have long-lasting, sexually dimorphic consequences on behaviors including altered anxiety, alloparental behavior and pair-bond formation (Carter et al., 2009). Neonatal OT administration can also heighten vasopressin receptor (V1a) binding in multiple brain regions including the bed nucleus of the stria terminalis (BNST), cingulate cortex (CgCtx), medial preoptic area of the hypothalamus (MPOA), and lateral septum (LS) (Bales et al., 2007). While there are well-documented species differences, function and consequences of OT and AVP manipulation are thought to have similar effects on human behavior and social development (Dumais and Veenema, 2016; Gao et al., 2016). Although disruption of steroid hormone signaling is well documented for many EDCs, including BPA, far less is known about the capacity for environmental chemicals to alter nonapeptide signaling (Patisaul, 2017). This knowledge gap, in addition to evidence of OT and OTR disruption in prior studies (Adewale et al., 2011; Sullivan et al., 2014; Wolstenholme et al., 2012), including our CLARITY-BPA studies (Arambula et al., 2016, 2017), prompted us to further examine possible effects of developmental BPA exposure on OTR binding across the brain.

The present study leveraged and built upon past research by examining OTR binding in regions known to be susceptible to estrogen modulation during development, plus additional regions fundamental for coordinating socioemotional behaviors including the dorso lateral BNST (BNSTdl), lateral septum (LS), central amygdala (CeA), hippocampus (Hipp), paraventricular thalamic nucleus (PVT) and medial preoptic area (mPOA). By adulthood, rat brain OTR expression is known to be sexually dimorphic in some regions including the posterior bed nucleus of stria terminalis (BNSTp), ventromedial hypothalamus (VMH), and the paraventricular hypothalamic nucleus (PVN). Some of these differences are also present in juvenile Wistar rats, thus disruption of sex differences was also of interest (Dumais and Veenema, 2016; Smith et al., 2017; Tribollet et al., 1991). We hypothesized that any disruption of OTR density by BPA would be region and sex-specific and sex differences may be eliminated.

2. Materials and methods

This study used brain tissue collected as part of the CLARITY-BPA program (Birnbaum et al., 2012; Schug et al., 2013). Brain sections came from the same juvenile NCRT-SD rats from which our lab has previously published behavioral (Rebuli et al., 2015) and volumetric data, and reported exposure-related changes to the anteroventral periventricular nucleus (AVPV) and the posterodorsal aspect of the medial amygdala (MePD) (Arambula et al., 2017).

Comprehensive CLARITY-BPA experimental design details are described elsewhere (Heindel et al., 2015; Prins et al., 2018; Schug et al., 2013; Vandenberg et al., 2019), thus only the most relevant methods are summarized herein. Since this study leveraged remaining tissues to explore an unplanned endpoint, it is considered a "post-hoc" CLARITY-BPA study. All aspects of this study were approved by the NCTR Institutional Animal Care and Use Committee (IACUC). The ARRIVE Guidelines Checklist for Reporting Animal Research was used in the construction of this manuscript with all elements met (Kilkenny et al., 2010). The ARRIVE guidelines were developed in consultation with the

scientific community as part of an NC3Rs (National Centre for the Replacement Refinement and Reduction of Animals in Research) initiative to improve the standard of reporting of research using animals.

2.1. Animal husbandry

All CLARITY-BPA Sprague-Dawley rats were obtained from the National Center for Toxicological Research colony (NCTR-SD rats) and all experiments were conducted in an Association for Assessment and Accreditation of Laboratory Animal Care (AALAC) accredited facility at NCTR. Animals rooms were kept on a 12:12 h light dark cycle with lights on at 0600 h, 23 ± 3 °C, 50 ± 20% relative humidity with food and water provided ad libitum. Housing conditions and diet were specifically designed to minimize unintended exposure to BPA and other endocrine disruptors. This included the use of glass water bottles with filtered water, thoroughly washed polysulfone caging, woodchip bedding and a soy- and alfalfa free diet (5K96 verified casein diet 10 IF, round pellets, g-irradiated; Cat. 1810069, Purina Mills, Richmond IN). To ensure diet and other study materials were not contaminated, all materials were monitored for BPA and myco/phytoestrogens by liquid chromatography/mass spectrometry (Delclos et al., 2014) and all had levels below the average analytical method blanks (Heindel et al., 2015). For this specific study, all juvenile rats were generated from the same colony as the CLARITY-BPA studies, but not obtained from the mainline study. They were also housed separately after weaning because these animals were bred for behavioral testing and the behavior testing facility was in different building (for further details see: (Rebuli et al., 2015)).

2.2. Reagents and dosing

The BPA (CAS # 80-05-7, catalog # B0494, TCI America, Portland, OR) and ethinylestradiol (EE2; CAS # 57-63-6, catalog #E4876, Sigma-Aldrich, St. Louis, MO) were more than 99% pure. Treatments were administered in 0.3% aqueous carboxymethyl cellulose (CMC; catalog # C5013, Sigma- Aldrich, St. Louis, MO) by gavage daily at a volume of 5 ml/kg bw using a modified Hamilton Microlab ML511C programmable 115 V pump (Hamilton Co., Reno, NV) (Lewis et al., 2010).

To ensure body weights were equivalent across all groups, two weeks prior to mating female NCTR-SD rats were randomly assigned to exposure groups stratified by body weight. No sibling or first cousin mating occurred as previously described (Delclos et al., 2014). Dams were orally gavaged daily with vehicle (0.3% CMC), 2.5, 25, or 2500 μg BPA/kg bw/day, or 0.5 μg EE2/kg bw/day from GD 6 until the onset of labor (note: the full CLARITY-BPA study has additional exposure groups, see (Heindel et al., 2015)). No dosing occurred on the day of birth (PND 0). All litters were randomly culled on PND 1 to a maximum litter size of 10 (minimum size of 6). Following culling, pups were directly gavaged daily through weaning (PND 21).

2.3. Weaning and tissue collection

Following the last daily gavage on PND 21, all offspring were weaned and tail tattooed with a unique identifier. As previously published, the offspring used for this study were from litters with at least 9 pups that had a balanced sex ratio at birth (no litter had more than a 4 pup sex difference except for 2 litters, which had a 5 pup sex difference: 9 males and 4 females) (Rebuli et al., 2015). At the time of weaning, animals were transferred to new rooms and housed in groups of 2–3 (same-exposure group, same-sex, same-age, non-siblings) under conditions identical to the preweaning rooms described above, except the light cycle, which was adjusted to accommodate behavioral testing (23:00–11:00). No animals were housed alone. Twelve animals per sex per group (1/sex/litter) were used. Prior to puberty on PNDs 25–27, animals were tested for anxiety-like behaviors using the open field and elevated plus maze, the outcomes of which are published (Rebuli et al., 2015). The animals (n = 120) were then sacrificed on PND 28 by CO_2 asphyxiation followed by rapid decapitation. Brains were collected, flash frozen on crushed dry ice and shipped from NCTR to North Carolina State University (NCSU) where they were stored at −80 °C.

2.4. Tissue processing

Each brain was cryosectioned (Leica CM1900, Nußloch, Germany) into three serial sets of 20 μm coronal sections, mounted onto Superfrost plus slides (Fisher Scientific, Pittsburgh, PA) and stored at −80 °C. Each set encompassed the hypothalamus (0.72 through -3.60 mm from Bregma). Sections from the first and, to some degree, second set were used in a prior study to analyze volumetric differences in sexually dimorphic nuclei (Arambula et al., 2017). For the current study, we were limited to the remaining sections, and did not have, for example, tissues anterior to the AVPV.

2.5. Control for risk of bias

In the prior studies, all work was done fully blinded. Only after all behavioral or volumetric measurements were complete, and the coded raw data submitted to the NTP Chemical Effects in Biological Systems (CEBS), were the NCSU investigators unblinded (for more details see (Arambula et al., 2017; Rebuli et al., 2015)). For the present study, although the experimental groups had previously been unblinded, slide selection through autoradiographic analysis was done by investigators blinded to sex and exposure groups using unique identifiers. Thus, all information obtained from these animals was collected under blinded conditions.

2.6. Receptor autoradiography

Slides were thawed at room temperature and allowed to dry for approximately one hour. OTR receptor binding and autoradiography were performed as previously published (Young et al., 1998) using well validated materials and methods (Elands et al., 1988; Smith et al., 2017; Tribollet et al., 1989). Briefly, sections were fixed in 0.1% paraformaldehyde in phosphate-buffered saline (pH 7.2) for 2 min at room temperature and washed twice in 50 mM Tris−HCl (pH 7.4) for ten minutes. Sections were then incubated in a tracer buffer containing 50 mM Tris with 10 mM MgCl2 (pH 7.4), 0.1% bovine serum albumin, 0.05% bacitracin, and 50 pM of selective 125I OXTR ligand: ornithine vasotocin analog (vasotocin, d(CH2)5[Tyr(Me)2,Thr4,Orn8,[125I]Tyr9 NH2]; ([125I]-OVTA, NEX254, Perkin-Elmer, Inc., Boston, MA)) for one hour at room temperature. Following incubation, all sections were washed in 50 mM Tris with 10 mM MgCl2 (pH 7.4) four times for 5 min each time, and finally, for 30 min while gently shaking. Sections were then washed in cold dH20 and allowed to fully air-dry. Sections were laid, in random order, in a cassette (no closer than 1 cm to the edge) with 14 × 17″ Carestream BioMax MR film (Sigma Aldrich, Rochester, NY) for either 9 (CeA, Hipp, PVP, PVN, VMH and mPOA) or 7 (pBNST, dlBNST LSv) days. The films were then developed using a Konica SRX-101A film processor (Konica, Tokyo, Japan) and analyzed for optical density. To prevent batch effects, all slides were run simultaneously. The ligand ([125I]-OVTA) used in this study is highly selective for OTR in mice, rats, and voles as determined by displacement with a competitive unlabeled ligand (Elands et al., 1988; Insel and Shapiro, 1992) and the absence of specific binding in the OTR KO mouse (Takayanagi et al., 2005)

2.7. Quantification

All measurements were made by investigators blind to exposure group. A monochrome QICAM 1394 12-bit camera (QImaging, Surry, BC, Canada) mounted above a light-box (NorthernLights; Bert-hold, Australia) was used for imaging the X-ray films. OTR expression was

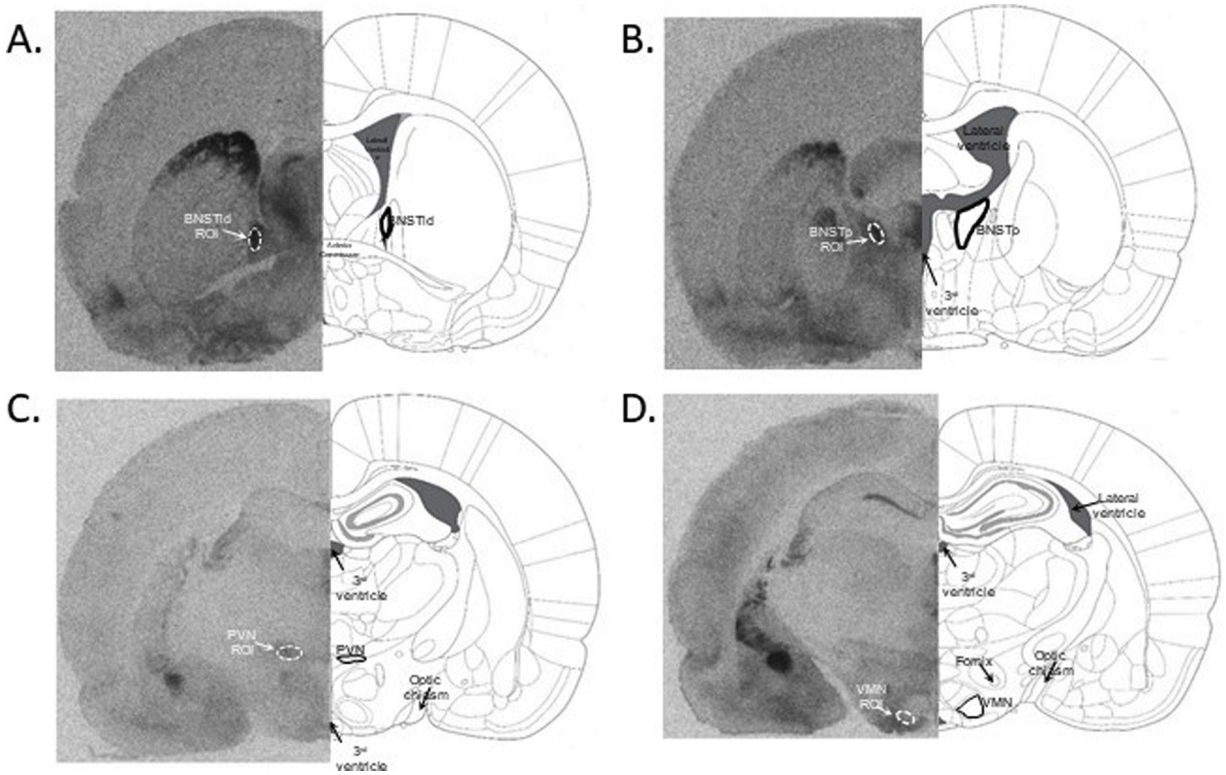

Fig. 1. Representative autoradiograms (left panels) of OTR binding in regions found to have sexually dimorphic expression and the corresponding sampling template created to define the area of interest (ROI, white dashed line) and quantify the autoradiographic signal within that brain area. Diagrams (right panels) depict the major anatomical landmarks used to identify each brain region of interest (circled in black). Diagrams were generated using (Paxinos and Watson, 2014). Regions of interest depicted and their distance from Bregma include: A) bed nucleus of stria terminalis, dorsolateral (BNSTld, -0.12 mm), B) bed nucleus of stria terminalis, principle (BNSTp, -0.72 mm C), paraventricular hypothalamic nucleus (PVN, -1.92 mm), and D) ventromedial hypothalamus (VMH, -2.52 mm).

quantified by optical density using the digital densitometry application of the MCID Core Image software program (InterFocus Imaging, Cambridge, UK). Sections were anatomically matched across subjects using well defined landmarks, the aid of a standard rat brain atlas (Paxinos and Charles, 2014), and a reference publication of juvenile rat OTR autoradiograms (Smith et al., 2017). To distinguish the LSv, BNSTld, BNSTp, mPOA, PVT, PVN, CeA, Hipp and VMH we used the shape of the lateral and 3rd ventricles, the anterior commissure, optic chiasm, median eminence and placement of the fornix as anatomical landmarks (Dong and Swanson, 2004, 2006; Hines et al., 1985; Ju and Swanson, 1989; Kirouac, 2015; Larsen et al., 1994; Risold and Swanson, 1997; Sa and Madeira, 2005; Swanson and Cowan, 1979). The way the BNST is anatomically subdivided differs across publications and atlases (Dong and Swanson, 2004, 2006). For the present studies, we used the same nomenclature for BNST subnuclei as Tribollet et al. and Smith et al., (BNSTp and BNSTld) (Smith et al., 2017; Tribollet et al., 1991). For each brain subnucleus to be analyzed, an oval region of interest (ROI) was defined such that it maximally contained the structure of interest, and three consecutive regions were averaged to obtain a representative measurement (for that region) for each animal (Fig. 1). Average background tissue levels, obtained by measuring levels in cortex or the lower portion of caudate putamen, neither of which have positive signal, were then subtracted to obtain a final value.

The CeA and BNST were independently defined and measured by two investigators blinded to exposure group to confirm that the measurement methodology was reproducible. For the statistical analysis, the data from both investigators was averaged. The remaining subregions were then quantified by a single trained investigator, blinded to exposure groups. Only animals for which every section within the brain region of interest were perfectly intact were included in the analysis. Because this study used available tissue remaining from a prior published study, not all ideally optimal tissue was available (Arambula et al., 2017). Consequently, because of insufficient tissue or damaged sections, some material could not be analyzed. Only brains for which we had all required sections were used for the analysis thus 22 brains were excluded for the BNSTld, 17 for LSv, 12 for CeA, 24 for Hipp, 22 for PVT, 46 for PVN, 18 for VMH and 49 for BNSTp. Final animal numbers are provided in the figures and tables.

2.8. Statistical analysis

Statistical analysis for all of the data was performed using SPSS (IBM software, Inc., Armonk,NY) and graphed using Prism version 8 (GraphPad Software, Inc., La Jolla, CA). The statistical approach was designed to be consistent with our prior CLAIRITY-BPA studies and published guidelines for low dose EDC studies (Haseman et al., 2001). The litter was the statistical unit and each exposure group contained only one pup per sex per litter.

The average of three consecutive slices per region quantified in OTR autoradiography was used for analysis. For each ROI, all data were first analyzed to test for effects of sex (expected and unknown) by comparing the male and female controls via *t*-test. As in our prior CLARITY-BPA work, detection of known sex differences was considered confirmation that the experiment had sufficient power and rigor to identify known relationships. Because sex differences were confirmed, and OTR expression levels in many regions is known to be sexually dimorphic, to test for effects of exposure, we performed a one-way ANOVA within sex for each ROI. A Fisher's exact least significant difference (LSD) post-hoc test was then performed only when the ANOVA was significant. In the four regions where sexually dimorphic expression was found in the controls, *t*-tests were used to determine if these sex differences were preserved in each of the exposed groups (all doses of BPA and EE). Hence, we set out not only to determine whether exposure altered OTR density within each region, but also if there were instances where

Table 1
Summary of exposure main effects and effects on sex differences in the four regions where sex differences in OTR binding were observed in the controls. A main effect of exposure was only found for one region (BNSTld), and only in males, which eliminated the sex difference in OTR binding in all exposure groups. In two other regions (VMN and PVN) there was no main effect of exposure but sex differences in OTR binding was lost. Significant sex differences (two tailed t-test, ($p \leq 0.05$) are bolded with a (*) symbol. Regions of interest (ROI) examined are dorso lateral and posterior bed nucleus of stria terminalis (BNSTld and BNSTp, respectively), paraventricular hypothalamic nucleus (PVN), and ventromedial hypothalamus (VMH).

ROI	Main Effect of BPA Exposure	Control	.05 EE	2.5 BPA	25 BPA	2500 BPA
BNSTld	↑♂only	**T16, $p \leq 0.01$***(10♀8♂)	T17, $p \leq 0.42$(10♀9♂)	T18, $p \leq 0.33$(11♀9♂)	T17, $p \leq 0.61$(10♀9♂)	T18, $p \leq 0.63$(11♀9♂)
BNSTp	NO	**T13, $p \leq 0.001$***(7♀8♂)	**T14, $p \leq 0.001$***(6♀10♂)	**T9, $p \leq 0.001$***(4♀7♂)	**T13, $p \leq 0.001$***(6♀9♂)	**T11, $p \leq 0.001$***(5♀8♂)
VMH	NO	**T17, $p \leq 0.001$***(9♀10♂)	T19, $p \leq 0.07$(11♀10♂)	T18, $p \leq 0.22$(10♀10♂)	T18, $p \leq 0.20$(10♀10♂)	T18, $p \leq 0.3$(9♀11♂)
PVN	NO	**T11, $p \leq 0.02$***(6♀7♂)	T9, $p \leq 0.07$(7♀4♂)	T15, $p \leq 0.13$(9♀8♂)	T13, $p \leq 0.6$(9♀6♂)	T12, $p \leq 0.19$(7♀7♂)

exposure altered sex differences in density. A p-value less than 0.05 was considered statistically significant. Animals were considered outliers and removed if equal to or greater than two standard deviations from the group mean. Additionally, two animals (one 25 BPA female and one vehicle control male) were completely removed from the study because they were identified as outliers in three different regions.

3. Results

As expected, labeling was highly selective and appreciable in several brain regions previously reported to have OTR binding in rats of similar age (Lukas et al., 2010; Shapiro and Insel, 1989) (Fig. 1). As expected, in the control groups OTR binding was sexually dimorphic in the BNSTp ($t(13) = 5.41$, $p \leq 0.001$), PVN ($t(11) = 2.86$, $p \leq 0.02$), and VMH ($t(17) = 3.93$, $p \leq 0.001$) with males having higher OTR binding (Table 1). Additionally, there was a significant effect of sex in the BNSTld ($t(16) = -2.826$, $p \leq 0.01$) with control females having greater OTR binding than control males (Table 1 and Fig. 2). No other sex differences were found within the controls for any other regions examined (Table 1). One-way ANOVA revealed a significant main effect of exposure only in the male BNSTld ($F_{4,43} = 5.579$, $p \leq 0.001$). Fisher's LSD revealed significant effects of EE ($p \leq 0.009$), 2.5 BPA ($p \leq 0.001$), and 25 BPA ($p \leq 0.001$), with each exposure increasing OTR binding compared to controls (Fig. 3). This elevation eliminated the sex difference in OTR binding in all of the exposed groups. No significant main effects of exposure were observed in any other regions examined, and no effects of exposure were found in females. Independent analysis by *t*-test revealed loss of sex differences in OTR binding for EE and all doses of BPA in the PVN, VMH, and BNSTld (Table 1).

4. Discussion

Here we showed that developmental BPA exposure alters OTR

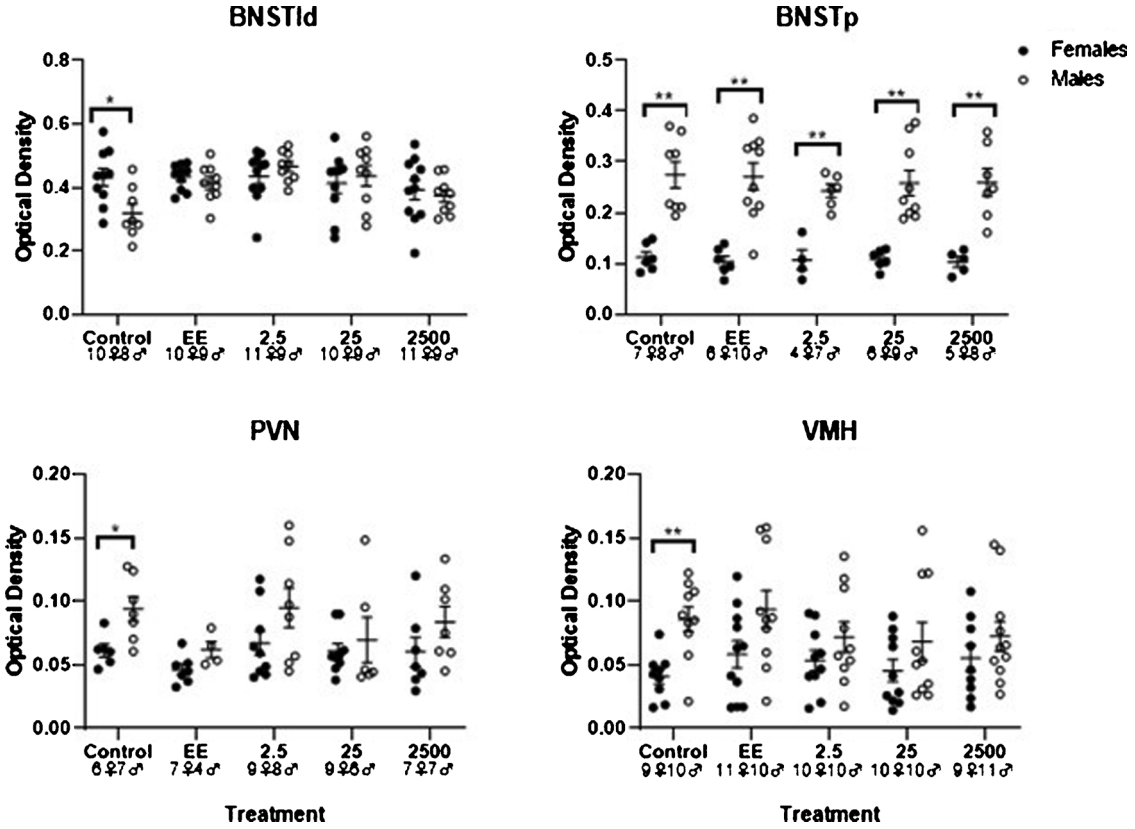

Fig. 2. Expression intensity of OTR, measured by optical density, in the four regions, BNSTld, BNSTp, PVN and VMH, where density was found to be sexually dimorphic. Male controls had significantly more OTR expression in the BNSTp, PVN and VMH. These sex differences were not statistically significant in the BNSTld, PVN or VMH in any of the exposed groups. No effect of exposure was found in females. The sample size for each region of interest is indicated below the exposure groups. Each dot represents a data point and bars are mean ± SEM. (*) $p \leq 0.05$ and (**) $p \leq 0.01$.

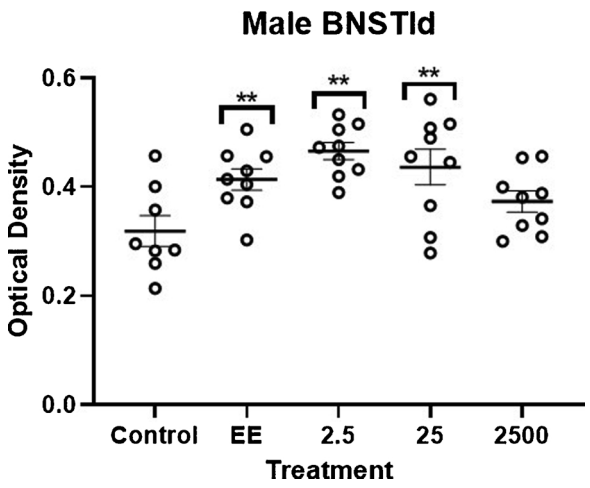

Fig. 3. Expression intensity of OTR, measured by optical density, in the BNSTld of males. OTR density was significantly higher in the EE (n = 9), 2.5 BPA (n = 9), and 25 BPA (n = 9) groups, compared to controls (n = 8). Each dot represents a data point and bars are mean ± SEM. (**) $p \leq 0.01$.

binding in a sex- and region-specific manner. Significantly, sexually dimorphic binding previously reported in juvenile Wistar rats was also reproduced here in the NCTR-SD rat, with males having greater OTR binding in BNSTp, VMH and PVN (Smith et al., 2017). These results confirm that we were sufficiently powered to successfully detect known sex differences, and that these sex differences are preserved across rat strains. Additionally, we identified sexually dimorphic OTR binding in the juvenile BNSTld, an observation that has not been previously reported. In this region, females have greater OTR expression then males. Developmental BPA exposure, below the current FDA NOAEL, eliminated sex differences in OTR binding in 3 of the 4 regions identified to have sexual dimorphisms: the BNSTld, VMH, and PVN. Additionally, a main effect of exposure was found for OTR binding in the male BNSTld, demonstrating that this region is particularly sensitive. The three impacted regions coordinate a wide range of sexually dimorphic reproductive and socioemotional behaviors and thus localized disruption of OT signaling pathways by BPA could be a mechanism by which later in life behavioral deficits attributed to BPA emerge. This study provides further evidence that early life BPA exposure disrupts brain sexual differentiation.

These data are consistent with the other published CLARITY-BPA studies (both the “core study” and the “grantee studies”) in multiple ways. Most significantly, we found effects below the current FDA reference dose, including the lowest dose of 2.5 µg/kg bw. Other CLARITY-BPA organs found to be adversely impacted in this low dose range include heart, ovary, prostate, and mammary gland (summarized in (Patisaul, 2019; Prins et al., 2018)). The animals used for this study also underwent behavioral testing prior to sacrifice, and their brains were examined to assess volumetric impacts on sexually dimorphic nuclei. Behavioral outcomes were less remarkable than predicted with statistically significant effects of 2.5 and 25 mg/kg bw/day BPA identified for only few anxiety-related traits (Rebuli et al., 2015). Expected volumetric sex differences in the AVPV, SDN and MePD were detected in the unexposed controls, and BPA did not eliminate those differences. However, all doses of BPA enlarged the female AVPV and a similar enlargement was observed in males at the 25 and 2,500 µg/kg BW dose levels (Arambula et al., 2017); outcomes consistent with our prior work in SD rats from Charles River (Patisaul et al., 2006).

Additional CLARITY-BPA brain and behavior outcomes (summarized in (Patisaul, 2019)) include some evidence that adult 2500 µg/kg bw BPA females were less capable than control females of locating the escape box of a Barnes Maze in the allotted time, suggesting impairments to spatial navigation (Johnson et al., 2016). Our transcriptomics data from neonatal (PND 1) animals exposed prenatally, found disrupted expression of OT in the hypothalamus and hippocampus, and OTR and vasopressin receptors in the amygdala (Arambula et al., 2016, 2018). In the amygdala, Oxtr mRNA expression was higher in 25 and 250 µg BPA males, and all exposed females (Arambula et al., 2018). In the current study, neither sex nor exposure affected OTR levels in the CeA or neighboring regions of the amygdala, suggesting effects in the amygdala may be transient. Evidence of disrupted hippocampal OT and AVP gene expression was also found in the 2,500 BPA µg/kg females tested as adults in the Barnes maze (Cheong et al., 2018). A final NTP report integrating and summarizing all available CLARITY-BPA data is expected in August, 2019 and all raw data from the project is publically available online (for a timeline and additional information see: https://ntp.niehs.nih.gov/results/areas/bpa/index.html).

Collectively, the CLARITY-BPA data are highly concordant with a robust literature by a multitude of laboratories showing that BPA impacts OT and AVP pathways throughout the brain of multiple species (Adewale et al., 2011; Cao et al., 2012; Patisaul, 2017; Patisaul et al., 2012; Sullivan et al., 2014; Wolstenholme et al., 2012); (Ottinger et al., 2008; Panzica et al., 2005; Patisaul, 2017; Patisaul et al., 2012; Sullivan et al., 2014). For example, neonatal exposure to BPA (50 µg/kg bw or 50 mg/kg bw sc injection) from PND 1 to PND 3 in female Long Evans rats significantly increased the number of OT neurons in the adult PVN (Adewale et al., 2011). OTR binding was not measured. Subsequent work in prairie voles also found that BPA exposure (5 µg/kg, 50 µg/kg or 50 mg/kg via oral administration to the pup by micropipette over PND 8–14) had sex- and dose- specific effects on OT and AVP neuron numbers in the PVN, as well as altered locomotor and anxiety behaviors in the females (Sullivan et al., 2014). Here we found loss of sexually dimorphic PVN OTR binding in all exposure groups suggesting BPA-related disruption of OT signaling in the PVN is likely multi-modal and can occur in multiple critical windows.

That effects might be different across brain region, sex, and age is not surprising given that the ontogeny of the OT system is highly dynamic, with regional OTR expression changing dramatically over the lifespan. In mice, OTR mRNA is detected as early as embryonic day (ED) 12 in whole head samples, with females having more OTR expression than males (Hammock and Levitt, 2013; Tamborski et al., 2016). Prenatal patterns of OTR expression are similar in other rodents, including rats (Snijdewint et al., 1989; Yoshimura et al., 1996), prairie voles (Ophir et al., 2013; Wang et al., 1997), and humans ((Kang et al., 2011) and http://hbatlas.org/ (for a comprehensive review see (Grinevich et al., 2014; Vaidyanathan and Hammock, 2017)). First detection at pubertal onset, or a more complex transient pattern with multiple maturation stages can also occur (Vaidyanathan and Hammock, 2017). For the latter, change from the “infant pattern” to the “adult pattern” largely occurs in 3 stages: the first between PND 16 and PND 22 (preweaning period), the second at PND 35 (onset of puberty) and the third between PND 35 and adulthood (PND 90) (Smith et al., 2017; Tribollet et al., 1989). Thus, by sampling just prior to pubertal onset we assessed OTR binding at a critical transition point for many brain regions.

Loss of sex differences in the PVN, VMH and BNSTld at the pubertal transition is likely biologically meaningful and possibly indicative of permanent disruptions. Prior work has shown that OTR binding density can be significantly higher at PND 35 compared to adults, with expression generally higher in juveniles; likely because it is playing an organizational role (Smith et al., 2017). In most cases adult sex differences in OTR binding are also found in peripubertal juveniles with differences in some regions, such as the VMNvl, increasing in magnitude with age (Dumais et al., 2013; Smith et al., 2017). Gonadectomy of adult rats decreases OTR binding in multiple sexually dimorphic regions including the VMH, BNSTld and CPUd in both males and females, demonstrating that steroid hormones are required to maintain robust expression in some regions (Tribollet et al., 1990). For the present studies, the animals were pre-pubertal and BPA dosing ceased on PND 21 thus suppression of circulating steroids by BPA is likely not the

mechanism by which OTR binding sex differences were lost. Because BPA exposure included pre- and post-natal windows, including adolescence, when OTR expression and binding levels can be dramatically changing, it is not possible to discern when BPA exposure induced OTR binding disruption. Going forward, it would be useful to further interrogate when and how long BPA alters OTR expression with the hypothesis that it might be the time around weaning.

In addition to OTR, estrogen and androgen receptors are also present in the BNSTp, BNSTld, VMH and PVN throughout the lifespan (Dong and Swanson, 2006; Ju and Swanson, 1989; Wu and Gore, 2010) and disruption of steroid hormone expression in these, and other regions, is a known action of developmental BPA exposure (Arambula et al., 2016, 2018; Cao et al., 2014, 2012; Cao et al., 2013; Kundakovic et al., 2013; Monje et al., 2007; Patisaul et al., 2012; Rebuli et al., 2014; Yu et al., 2015). Thus, disruption of steroid hormone receptor levels is a highly plausible a mechanism by which BPA disrupts OTR binding levels and, by extension, other aspects of OT signaling. For example, in the adult, ERα mediates estrogen-induced transcription of OTR in the medial amygdala (MeA) (Quinones-Jenab et al., 1997; Young et al., 1998) while ERβ is required for inducing OT release in the PVN (Hrabovszky et al., 1998; Shughrue et al., 2002). Prior work by us and others (Al-Bader et al., 2008; Cao et al., 2014; Cao and Patisaul, 2011, 2013; Kuhnemann et al., 1994; Kumar et al., 2014; Perez et al., 2003; Yokosuka et al., 1997) has clearly shown that, like OTR, expression patterns of nuclear estrogen receptors (ERα and ERβ) differ quite dramatically across region, sex and age; a phenomenon likely reflective of their different functional roles across development (Laflamme et al., 1998). These transient points where receptor expression is changing are likely critical windows for development and thus particularly vulnerable to endocrine disruption.

To our knowledge, we are the first to report a sex difference in OTR binding in the BNSTld. The adult BNST is a very diverse region consisting of up to 18 sub-nuclei; each with highly distinctive and sexually dimorphic features including hormone receptor subpopulations, neurotransmitters, transporters and proteins (Bota et al., 2012; Lebow and Chen, 2016). Studies in juveniles are more limited. In a prior study using identical methodology to map OTR expression in 35 day old Wistar rats, a sex specific difference was observed in 9 of 25 regions examined, but OTR in the BNSTld did not differ by sex (Smith et al., 2017). Other OTR mapping studies using autoradiography or *in situ* hybridization in juvenile rats either did not analyze the BNST by subregions, or only looked at males (Tribollet et al., 1989; Yoshimura et al., 1996). Thus, one possible reason a sex difference in BDSTld OTR binding has not been previously reported, is that we focused on prepubertal animals of both sexes, and levels may change post-puberty. Another important factor is the method by which the BNST is anatomically subdivided, with the number of sub nuclei defined differently across studies based on different defining criteria including regional cellular architecture, connectivity and other factors (Dong and Swanson, 2004; Kash et al., 2015). For example, an additional subnucleus of the highly sexually dimorphic BNSTp was recently reported in mice and defined as the ventral BNSTp, with prepubertal females having greater volume and neuron numbers compared to males (Morishita et al., 2017). The anatomical and functional complexity of the BNST demands that future work on the possible effects of BPA and other EDCs in this region use both sexes and well-defined criteria for defining the sub-nuclei.

The functional significance of region- and sex-specific disrupted OTR binding by BPA exposure remains to be established. However, it is consistent with, and likely contributes to, the frequently reported sex-specific behavioral changes attributed to BPA exposure in rodents, including heightened anxiety and disrupted exploratory behavior (Cox et al., 2010; Jasarevic et al., 2013; Nesan et al., 2018; Patisaul et al., 2012; Wolstenholme et al., 2012, 2013). OTR binding in the central amygdala, for example, negatively correlates with social interest in females, while OTR binding in the male MeA positively correlates with social interest (Dumais et al., 2013). These and other studies emphasize that different OTR binding densities across regions and sex coordinate different aspects of socioemotional behaviors including anxiety, alloparental behavior and pair-bond formation (Carter et al., 2009). OT signaling is also critical for the "GABA switch;" the postnatal transition of neuronal GABA neurotransmission from excitatory to inhibitory (Tyzio et al., 2006, 2014). There is some evidence that perinatal BPA may disrupt GABA levels and signaling during this critical transition period but a role for OTR has not been explored in this context (Franssen et al., 2016; Zalko et al., 2016). OT is also important for heart development, as well as autonomic regulation of the circulatory system. Overexpression of OTR in the PVN affects neurogenic control of circulation including baroreceptor reflex sensitivity and blood pressure variability (Lozic et al., 2014), demonstrating that disruption of this mechanism might contribute to risk of cardiovascular disease. Epidemiological studies have repeatedly linked higher urinary BPA levels with various types of cardiovascular diseases, including angina, hypertension, heart attack and coronary and peripheral arterial disease (Melzer et al., 2010; Ranciere et al., 2015). At least one study has associated urinary BPA with elevated blood pressure and decreased heart rate variability, both of which may result from disruption of autonomic control. While most experimental work has focused on disruption at the level of the heart itself, including calcium handling, which is particularly vulnerable in females (Gao and Wang, 2014), possible effects of BPA on autonomic regulation have not been as well studied (Belcher et al., 2015).

In conclusion, this study provides further evidence that perinatal BPA exposure can affect brain sexual differentiation and the organization of the OT/OTR system. How this outcome ultimately impacts physiology, behavior or the development of other, related neural systems such as GABA or AVP signaling pathways remains unclear, but is consistent with extensive prior work linking BPA with disrupted socioemotional behaviors and heightened risk of cardiovascular disease. Extensive description of the experimental design for CLARITY-BPA (Heindel et al., 2015; Schug et al., 2013), including critical analyses of its strengths and limitations, have been published elsewhere (Prins et al., 2018; Vandenberg et al., 2019; Vom Saal, 2018), and the final, integrated report is due from the NTP in August, 2019. To date, the published CLARITY-BPA studies, including our own (summarized in (Patisaul, 2019; Prins et al., 2018)), demonstrate that BPA exposure, even at doses below the NOAEL, impacts multiple organ systems including heart, prostate, mammary glands ovary and brain.

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