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Quantitative mapping reveals age and sex differences in vasopressin, but not oxytocin, immunoreactivity in the rat social behavior neural network

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Abstract

The neuropeptides vasopressin (AVP) and oxytocin (OT) have been implicated in the regulation of numerous social behaviors in adult and juvenile animals. AVP and OT signaling predominantly occur within a circuit of interconnected brain regions known collectively as the “social behavior neural network” (SBNN). Importantly, AVP and OT signaling within the SBNN has been shown to differentially regulate diverse social behaviors, depending on the age and/or sex of the animal. We hypothesized that variation in the display of these behaviors is due in part to age and sex differences in AVP and OT synthesis within the SBNN. However, a thorough characterization of AVP and OT-immunoreactive (ir) fibers and cell bodies across age and sex within the SBNN has been lacking in rats. We therefore quantified AVP- and OT-ir fibers and cell bodies in 22 subregions of the fore-brain SBNN in juvenile and adult, male and female rats. We found numerous age (16 subregions) and sex (10 subregions) differences in AVP-ir fiber fractional areas, and AVP-ir cell body numbers, which were mainly observed in the medial amygdala/bed nucleus of the stria terminalis to lateral septum circuit. In contrast to AVP, we observed no age or sex differences in OT-ir fiber fractional areas or cell bodies in any of the 22 subregions of the forebrain SBNN. Thus, unlike the static pattern observed for OT, AVP innervation of the forebrain SBNN appears to undergo developmental changes, and is highly sexually dimorphic, which likely has significant functional consequences for the regulation of social behavior.

Keywords

age differences; juvenile; oxytocin; sex differences; social behavior; vasopressin; RRID: AB_2313960; RRID: AB_2315026; RRID: AB_2336170

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AUTHOR CONTRIBUTIONS

All authors had full access to all the data in the study and take responsibility for the integrity of the data and the accuracy of the data analysis. Study concept and design: BD and AV. Acquisition of data: BD, EN, and HC. Analysis and interpretation of data: BD and AV. Drafting of the manuscript: BD and AV. Critical revision of the manuscript for important intellectual content: BD and AV. Statistical analysis: BD and AV. Obtained funding: AV. Administrative, technical, and material support: AV. Study supervision: AV.

CONFLICT OF INTEREST

The authors declare no conflicts of interest.

1 | INTRODUCTION

Vasopressin (AVP) and oxytocin (OT) have garnered considerable attention for their essential role in the regulation of various social behaviors in humans, rodents, and other animals (reviewed in Veenema & Neumann, 2008; Ross & Young, 2009; Heinrichs, von Dawans, & Domes, 2009; Albers, 2015; Caldwell & Albers, 2015). In rodents, both neuropeptides have been implicated in the regulation of social recognition, pair bonding, and aggression in adults, as well as play behavior among juveniles (Albers, Dean, Karom, Smith, & Huhman, 2006; Bredewold, Smith, Dumais, & Veenema, 2014; Dantzer, Koob, Bluthé, & Le Moal, 1988; Dumais, Alonso, Immormino, Bredewold, & Veenema, 2016; Engelmann & Landgraf, 1994; Lim & Young, 2004; Ross et al., 2009; Veenema, Bredewold, & De Vries, 2012, 2013). Importantly, AVP and OT systems appear to differentially regulate the expression of various social behaviors depending on the age and/or sex of the animal (Bluthé & Dantzer, 1990; Bredewold et al., 2014; Dantzer, Bluthé, Koob, & Le Moal, 1987; Dumais & Veenema, 2016a,b; Dumais et al., 2016; Engelmann, Ebner, Wotjak, & Landgraf, 1998; Gutzler, Karom, Erwin, & Albers, 2010; Harmon, Huhman, Moore, & Albers, 2002; Lukas & Neumann, 2014; Veenema et al., 2013). The juvenile period (synonymous with the early adolescent period, postnatal days 28–42 in rats) is characterized by heightened levels of social exploration and increased time spent engaging in peer interactions (Doremus-Fitzwater, Varlinskaya, & Spear, 2010), yet despite this critical period in social development, a direct comparison of adult and juvenile AVP and OT synthesis and fiber projections in regions implicated in social behavior has been lacking. Likewise, less is known about potential sex differences in AVP and OT-ir during the juvenile period. Such knowledge may contribute to the understanding of age- and sex-specific behavioral functions of AVP and OT systems.

AVP and OT are primarily synthesized in the paraventricular (PVN) and supraoptic nuclei (SON) of the hypothalamus. In addition, AVP-synthesizing neurons have been found in the medial amygdala (MeA), the posterior division of the bed nucleus of the stria terminalis (pBNST), and the suprachiasmatic nucleus (SCN) of the hypothalamus (Buijs, 1978, 1980). OT synthesizing neurons have also been observed in the accessory neurosecretory nucleus (AN) of the hypothalamus (Fliers, De Vries, & Swaab, 1985; Rhodes, Morrell, & Pfaff, 1981). Previous qualitative anatomical work has shown that AVP- and OT-synthesizing cell bodies send axonal projections to many forebrain regions (Knobloch et al., 2012; Otero-García, Agustín-Pavón, Lanuza, & Martínez-García, 2016; Rood et al., 2013). Moreover, sex differences in the AVP system have been well documented in rodents (reviewed in De Vries, 2008). In rats, males have more AVP-expressing neurons in the BNST and MeA (Szot & Dorsa, 1993; van Leeuwen, Caffé, & De Vries, 1985) and have a higher density of AVP-immunoreactive (AVP-ir) fibers in the lateral septum (LS) and lateral habenular nucleus (De Vries, Buijs, & Swaab, 1981) compared to females.

AVP-ir fibers, and, to a lesser extent, OT-ir fibers and their receptors have been observed in several forebrain constituents of the “social behavior neural network” (SBNN) in rodents (Bakker et al., 2006; Haussler, Jirikowski, & Caldwell, 1990; Hermes, Buijs, Masson-Pévet, & Pévet, 1990; Knobloch et al., 2012; Rood et al., 2013; Smith et al., 2017; Tribollet, Barberis, Jard, Dubois-Dauphin, & Dreifuss, 1988; Wang, Zhou, Hulihan, & Insel, 1996).

The forebrain SBNN consists of neural cell groups in the MeA, pBNST, LS, medial preoptic area (MPOA), anterior hypothalamus (AH), and ventromedial hypothalamus (VMH). All of these regions are interconnected, express gonadal hormone receptors, and have been individually identified as important sites of regulation or activation of a multitude of social behaviors, from pair bonding, to sexual and maternal behaviors (Newman, 1999). In this model, the specific pattern of activity across this interconnected network is thought to underlie the expression of a particular social behavior. Forebrain nuclei comprising the SBNN may therefore serve as important neural substrates on which AVP and OT systems regulate diverse social behaviors. We propose that age and sex differences in AVP and OT fractional areas in the SBNN may reflect differential availability of AVP and OT for activity-dependent neurotransmission in this network, which (through binding to their cognate receptors) may mediate the observed age and sex differences in the regulation of social behavior by AVP and OT systems. However, a thorough, quantitative analysis of age and sex differences in AVP and OT-ir in most regions of the SBNN is lacking in rats.

Here, we sought to fill this knowledge gap by quantifying the fractional areas encompassed by AVP and OT-ir fibers throughout the fore-brain SBNN in juvenile and adult, male and female rats. We further assessed age and sex differences in AVP and OT synthesis by quantifying the number of AVP-ir neurons in the MeA and pBNST as well as the number of OT-ir neurons in a region that includes the anteroventral portion of the BNST and the dorsal aspect of the MPOA (aBNST/MPOA). These results provide a quantitative map of the expression of AVP-ir and OT-ir neurons and their accompanying fiber innervation patterns across age and sex in forebrain SBNN regions, and demonstrate vastly different effects of age and sex between two structurally similar neuropeptides.

2 | MATERIALS AND METHODS

2.1 | Animals

Wistar rats were obtained from Charles River Laboratories (Wilmington, MA) and housed under standard laboratory conditions (12-hr light/dark cycle, 22°C, 60% humidity, with food and water available ad libitum). Rats were housed in same sex groups of 2–4 for 5 days following arrival to the animal care facility and were subsequently housed individually for 4 days prior to sacrifice. Rats were sexually naïve, and adult females were sacrificed at random stages of their estrous cycle. Experiments were conducted in accordance with the NIH *Guide to the Care and Use of Laboratory Animals* and approved by the Boston College Institutional Animal Care and Use Committee (IACUC).

2.2 | Tissue collection and immunohistochemistry

Juvenile (36 day old) and adult (70 day old) male and female rats ($n=7$ per group) were deeply anesthetized under continuous 3% isoflurane vapor received transcardiac perfusions with 4% paraformaldehyde dissolved in 0.1 M borate buffer (pH 9.5). Following perfusion, brains were removed and post-fixed in 4% paraformaldehyde in 0.1M borate buffer (pH 9.5) for 24 h before cryoprotection in 30% sucrose (dissolved in basic physiologic saline; 0.9% NaCl) for 48 hr. Following cryo-protection, brains were flash-frozen in cold methylbutane and stored at -45°C . Coronal (30 μm) sections were collected using a cryostat and stored

free-floating in antifreeze cryoprotectant at -20°C . Tissue sections from each subject were immunolabeled for AVP, with alternate sections immunolabeled for OT.

AVP and OT-ir were visualized using monoclonal primary antibodies provided by Dr. Harold Gainer (NINDS; Table 1). These highly specific antibodies were raised against mammalian AVP and OT-associated neurophysins and exhibit no cross-reactivity (Ben-Barak, Russel, Whitnall, Ozato, & Gainer, 1985). Briefly, tissue sections were first washed in tris buffered saline (TBS; pH 7.4) for 3–4 hr to remove residual antifreeze solution. Following this washing period, sections were subjected to an antigen retrieval step (0.05 M sodium citrate in TBS), blocked in blocking solution (20% normal goat serum [NGS], 0.3% Triton-X, 1% H_2O_2 in TBS), and incubated overnight at 4°C in mouse anti-AVP (PS41; 1:100, 2% NGS, 0.3% Triton-X) or mouse anti-OT (PS38; 1:100, 2% NGS, 0.3% Triton-X). Sections were rinsed in TBS and incubated in biotinylated secondary antibody solution (goat anti-mouse [1:500; Vector, Burlingame, CA], 2% NGS, 0.3% Triton-X in TBS) for 1 hr. Tissue sections were next incubated in avidin-biotin complex (ABC Elite Kit; Vector) for 1 hr and visualized using diaminobenzadine (DAB peroxidase substrate kit; Vector). Sections were mounted on gelatin-coated slides, rinsed in 50% ethanol, air-dried, and coverslipped using Permount (Fisher Scientific, Pittsburgh, PA). Slides were coded prior to image acquisition and, in all cases, analyzed by an experimenter blind to treatment groups.

2.3 | Antibody characterization

Both primary antibodies (PS41 and PS38) were characterized by Gainer and colleagues (NINDS). The monoclonal AVP antibody (PS41) was produced against rat AVP-associated neurophysin, while the monoclonal OT antibody (PS38) was produced against rat OT-associated neurophysin. Specificities for AVP- and OT-associated neurophysins were determined using liquid phase assays, immunoblot, and immunoprecipitation experiments, which demonstrated that each antibody brought down the appropriate neurophysin with no detectable cross-reactivity (Ben-Barak et al., 1985). Furthermore, Brattelboro rat tissue (which do not contain AVP-associated neurophysins) did not react with PS41, but reacted with high affinity to PS38 (Ben-Barak et al., 1985). Finally, epitope analysis indicated that the antigenic determinants were located near position 80–81 for OT neurophysin and 75–86 for AVP neurophysin (Ben-Barak et al., 1985).

2.4 | Quantitative fiber fractional area analysis

AVP and OT fiber fractional area (FFA) analysis was conducted in a manner similar to Rood et al. (2013). AVP-ir and OT-ir FFA was measured by manually thresholding gray-scale images of AVP-ir and OT-ir fibers using ImageJ (NIH; imagej.nih.gov/ij). AVP-ir and OT-ir cell bodies were removed from the FFA calculation in the MeA, pBNST, and aBNST/MPOA by excising the pixels attributed to these cell bodies using the polygon draw tool. Brain section images were acquired under a $20\times$ objective, cropped to $500 \times 500 \mu\text{m}^2$, and pixel density was averaged from three nonoverlapping sections in an evenly spaced series (90 μm apart) within each of the 22 regions of interest (ROI; Figure 1a), corresponding to the six forebrain nodes of the SBNN. This analysis revealed an estimate of the fractional area occupied by AVP or OT immunolabeled elements, which reflects both fiber densities and fiber caliber/thickness, since fiber caliber covaries with neuropeptide content. Background

staining levels were sufficient to discern tissue features, and thus forebrain images were acquired based on various anatomical landmarks specific to each ROI (e.g., size, location, and morphology of ventricles and white matter tracts, including the corpus callosum, anterior commissure, optic tract, and fornix) using the Rat Brain Atlas (Paxinos & Watson, 2007) as a guide. Where necessary, sections were compared against reference thionin stained tissues from different animals for each subject type (adult male, adult female, juvenile male, juvenile female) to ensure that regional sampling was comparable across subjects. Fiber fractional area (FFA) data are reported in number of pixels, with a greater number of pixels indicating a larger proportion of the area occupied by AVP-ir or OT-ir elements.

2.5 | Quantitative cell counting analysis

Cell bodies were quantified from images acquired under a 10X objective using the cell counter plugin in ImageJ, and averaged across three nonoverlapping sections in an evenly spaced series (90 μm apart) per animal within each of the three ROIs (AVP: MeP, pBNST; OT: aBNST/MPOA; Figure 1b). Sections were compared against reference thionin stained tissues from different animals for each subject type (adult male, adult female, juvenile male, juvenile female) to ensure that regional sampling was comparable across subjects, where necessary. Data are reported as the mean number of AVP-ir or OT-ir cell bodies per ROI, calculated by averaging cell body numbers across three nonoverlapping (90 μm apart) sections in an evenly spaced series within each ROI for each animal. An Abercrombie (1946) correction was applied to all cell counts in order to correct for overcounting in profile sections (see Guillery, 2002). For AVP-ir cells (pBNST, MePD), the average cell count was multiplied by a factor of 0.75, assuming an average diameter of 10 μm for AVP-ir cell bodies. For larger OT-ir cells (aBNST/MPOA), the average cell count was multiplied by a factor of 0.545, assuming an average diameter of 25 μm for OT-ir cell bodies in 30 μm sections.

2.6 | Statistics

PASW/SPSS Statistics software (Version 22; IMB, Armonk, NY) was used for all statistical analyses. Comparisons of fiber fractional areas and cell body averages for AVP and OT were made using a two-factor (age $\times$ sex) analysis of variance (ANOVA). We did not control for possible effects of estrous cycle phase on AVP-ir and OT-ir. However, variability (as interpreted by the average standard deviation) in fiber fractional area measurements was no greater in females than in males, suggesting that estrous cycle phase does not have a significant impact on AVP-ir and OT-ir. The false discovery rate (FDR) procedure was used to correct for multiple comparisons based on main effects, and were calculated separately for AVP and OT fiber fractional area measurements (AVP: $\alpha < 0.024$; OT: $\alpha < 0.004$; Benjamini & Hochberg, 1995). Because there were fewer comparisons, statistical significance for cell number measurements was set at $p < .05$. Where significant main effects were observed, Bonferroni post hoc tests were used to test for differences among groups based on $p < .05$.

3 | RESULTS

3.1 | Age and sex differences in AVP-ir fiber fractional area in the forebrain SBNN

Age differences in AVP-ir fiber fractional area were found in 16 of the 22 SBNN regions analyzed (Figure 2; see Table 2 for statistics). In detail, adult males and females displayed a greater fractional area occupied by AVP-ir fibers compared to juvenile males and females in 8 subregions of the LS: the dorsal (LSD), intermediate (LSI), and ventral (LSV) portions of the rostral (rLS) and medial (mLS) LS and the dorsal (cLSD) and intermediate (cLSI) portions of the caudal LS. Furthermore, adult males had a greater fiber fractional area occupied by AVP-ir fibers compared to juvenile males in 1 subregion of the LS [the ventral portion of the caudal lateral septum (cLSV)], 2 subregions of the BNST [the medial division of the anteromedial BNST (STMAM), and the lateral division of the intermediate BNST (STLI)], 1 subregion of the anterior hypothalamus (AH) [the anterior division of the AH (AHA)], and all four subregions of the MeA [the anterodorsal (MeAD), anteroventral (MeAV), posterodorsal (MePD) and posteroventral (MePV)]. There were no age differences between adult and juvenile females in these regions.

Sex differences in AVP-ir fiber fractional areas were found in 12 of the 22 SBNN regions analyzed (Figure 2, see Table 2 for statistics). Specifically, adult males showed a greater fractional area occupied by AVP-ir fibers compared to adult females in 5 subregions of the LS (mLSD, mLSI, mLSV, cLSI, and cLSV), 2 subregions of the BNST (STMAM, STLI), the MPOA, and all four subregions of the MeA (MeAD, MeAV, MePD, and MePV). In addition, juvenile males showed a greater fractional area occupied by AVP-ir fibers compared to juvenile females in the MPOA. No age or sex differences in AVP-ir fiber fractional areas were observed in the pBNST or VMH.

3.2 | Age and sex differences in AVP-ir cell body number in the pBNST and MeP

Adult males had significantly more AVP-ir cell bodies in the pBNST and MeP compared to juvenile males and adult females. Moreover, adult females had significantly more AVP-ir cell bodies in the pBNST and MeP compared to juvenile females (Figure 3a–j; see Table 2 for statistics).

3.3 | No age or sex differences in OT-ir fiber fractional areas in the forebrain SBNN, nor in OT-ir cell body numbers in the aBNST/MPOA

After correcting for multiple comparisons, no age or sex differences in OT-ir fiber fractional areas were found in any of the 22 SBNN regions analyzed (Figure 4; See Table 3 for statistics). Additionally, no effects of age or sex on the number of OT-ir cell bodies were observed in the aBNST/MPOA (Figure 5; see Table 3 for statistics).

4 | DISCUSSION

To our knowledge, this is the first comprehensive study to investigate both age (juvenile vs. adult) and sex differences in AVP-ir and OT-ir fiber innervation throughout the forebrain SBNN as well as AVP-ir and OT-ir cell body expression in the extended amygdala (aBNST/MPOA, pBNST, MeP). Overall, we observed a high density of AVP-ir fibers occupying a

large fractional area in key SBNN nodes, including the LS, BNST, and MeA in adult males (Figure 6a–c), whereas OT-ir in these regions was relatively sparse (Figure 6a–c'). Moreover, abundant age and sex differences in AVP-ir fiber fractional area were found, with 16 of 22 forebrain SBNN subregions analyzed showing robust age differences (higher in adults, with the exception of the AHA) and 10 of 22 SBNN subregions showing significant sex differences (higher in males). This corresponds with similar age (higher in adults) and sex (higher in males) differences in the number of AVP-ir cells in the pBNST and MeP. In contrast, we observed no age or sex differences in OT-ir throughout the forebrain SBNN (Figure 7a–b). Thus, unlike OT, AVP innervation of the forebrain SBNN appears to undergo developmental changes and is highly sexually dimorphic.

4.1 | Experimental considerations

An important consideration to address in the current study is the nature of our fiber fractional area measurements. While this terminology may appear to be loosely synonymous with “fiber density,” there are key differences. Our method of analysis quantifies the pixels attributed to AVP-ir and OT-ir elements. Importantly, these measurements may vary with neuropeptide content, such that higher caliber/thicker fibers will result in greater fiber fractional area measurements compared to thinner fibers of similar densities. Thus, while a greater fiber density in a given region contributes to a larger fiber fractional area measurement, it is not the only contributing factor. In qualitative terms, there does not appear to be obvious differences in fiber thicknesses reflective of neuropeptide content across ages and sexes. However, there are rather striking differences in fiber thickness when comparing AVP-ir to OT-ir fibers. Specifically, OT-ir fibers in most regions analyzed, although usually quite sparse, appear to be significantly thicker than AVP-ir fibers. For this reason, no quantitative comparisons between AVP-ir and OT-ir fibers were made, though differences in fiber innervation patterns are qualitatively compared in the LS, pBNST, and MeP (see Figure 6). Thus, interpretations of data in the following discussion come with the caveat that fiber fractional area measurements potentially reflect both differences in fiber density as well fiber thickness, reflective of peptide content. In order to obtain true fiber density measurements, a stereological approach using a systematic-random sampling scheme must be employed. This approach would be ideal for future studies aimed at examining the local spatial structure/densities of neuropeptide-immunoreactive fiber populations in discrete SBNN nodes.

4.2 | Age and sex differences in AVP-ir in the MeP/pBNST→LS circuit

4.2.1 | Age differences in AVP-ir in the MeP/pBNST→LS circuit—Nearly all age differences in AVP-ir were observed within the well-established MeP/pBNST→LS AVP circuit (De Vries, Buijs, & Sluiter, 1984; De Vries, Wang, Bullock, & Numan, 1994; Miller, De Vries, al-Shamma, & Dorsa, 1992; Rood et al., 2013; Wang & De Vries, 1995). In detail, adult male and female rats exhibited a greater fractional area of AVP-ir fibers throughout the LS (with the exception of the cLSV) and a greater number of AVP-ir cell bodies in the pBNST and MeP compared to juvenile male and female rats. In addition, adult males had a greater fractional area occupied by AVP-ir fibers in the anterior BNST (STMAM, STLI) and in the MeA compared to juvenile males.

AVP-ir in the MeP/pBNST→LS circuit is dependent on gonadal steroid hormones. For example, previous work has shown that removing circulating gonadal steroid hormones in adult males and females via gonadectomy leads to a near complete depletion of AVP-ir cell bodies and fibers in the MeP and pBNST, as well as AVP-ir fibers in the LS (De Vries et al., 1984, 1994; Rood et al., 2013; Wang & De Vries, 1995). Albeit lower than in adults, here we show that AVP-ir is clearly present in this circuit in juveniles. This suggests that low levels of circulating gonadal steroid hormones, such as those observed in juveniles (Arteaga-Silva et al., 2013) are sufficient to maintain detectable levels of AVP-ir. In support, gonadectomizing 26-day-old male rats resulted in a complete loss of AVP-ir cells in both the pBNST and MeA when measured 1 week later (Pak, Chung, Hinds, & Handa, 2009). Thus, the age-dependent increase in AVP-ir in this circuit likely corresponds with a puberty-induced rise in circulating gonadal steroid hormones.

Although the molecular mechanisms through which gonadal steroid hormones regulate AVP synthesis in the MeP/pBNST→LS circuit have not been fully elucidated, research points to the likely involvement of both the estrogen receptor (ER) and androgen receptor (AR). In detail, treatment with 17 β -estradiol (E₂), which activates ERs, restores ~90% of AVP mRNA expression in the MeP and pBNST following castration in adult rats, but complete restoration of AVP expression is only achieved after treatment with both E₂ and 5 α -dihydrotestosterone (DHT), the latter activating the AR (De Vries et al., 1994). Furthermore, adult rats lacking a functional AR show decreased AVP-ir in the BNST and MeA compared to their control littermates (Allieri et al., 2013). Interestingly, it appears that circulating gonadal steroid hormones play both an activational and organizational role in adult AVP expression, since adult rats gonadectomized at birth and treated neonatally with E₂ or DHT had significantly more AVP-ir cells in the BNST compared to oil-treated controls despite receiving testosterone in adulthood (Han & De Vries, 2003). Finally, recent work has shown that AVP mRNA expression in the BNST is maintained in the adult rat brain through steroid hormone-dependent epigenetic modifications of the AVP gene promoter (Auger, Coss, Auger, & Forbes-Lorman, 2011). Whether similar epigenetic mechanisms are involved in the maintenance of AVP expression in the juvenile rat brain remains unclear.

In rodents, olfactory signals guide many aspects of social behavior and both the MeA and BNST are critical sites for the processing of social olfactory cues (Baum, Kang, Martel, & Cherry, 2009). Furthermore, the MeA and BNST project to areas implicated in sexual motivation, such as the ventral striatum (DiBenedictis, Helfand, Baum, & Cherry, 2014; Novejarque, Gutiérrez-Castellanos, Lanuza, & Martínez-García, 2011; Pardo-Bellver, Cádiz-Moretti, Novejarque, Martínez-García, & Lanuza, 2012). Lesions of the MeA, BNST, or ventral striatum result in a reduction in the display of both courtship and sexual behaviors in adult rodent species of both sexes, including rats, mice, and hamsters (for review, see Petrulis, 2013). Importantly, AVP signaling in the MeA and BNST appears to play a specific role in the regulation of adult sociosexual behaviors in rats (Hari Dass & Vyas, 2014) and mice (Ho, Murray, Demas, & Goodson, 2010). Furthermore, AVP signaling in the LS has also been implicated in the expression of adult behaviors such as partner preference in prairie voles (Liu, Curtis, & Wang, 2001) as well as intermale aggression in rats (Veenema, Beiderbeck, Lukas, & Neuman, 2010) and zebra finches (Goodson & Adkins-Regan, 1999). Based on these findings, it is intuitive to surmise that age differences in AVP-ir in the MeP/

pBNST→LS circuit serve to support an age-specific role for AVP in these regions, such that higher AVP-ir in adult rats may reflect higher availability of AVP for activity-dependent neurotransmission, which in turn may be critical for the regulation of adult-specific social behaviors, such as reproductive and territorial behaviors.

AVP signaling in the adult LS is also important for social behaviors outside of a sexual/aggressive context, such as the recognition of juvenile conspecifics. Here, blockade of the AVP V1A receptor (V1AR) in the LS impaired social recognition in adult rats, while AVP infusions into the LS improved it (Dantzer et al., 1988; Engelmann & Landgraf, 1994; Everts & Koolhaas, 1997; Lukas, Toth, Veenema, & Neumann, 2013; Veenema et al., 2012). Surprisingly, LS-V1AR blockade did not impair social recognition in juvenile rats, but rather induced a preference to investigate familiar over novel conspecifics (Veenema et al., 2012). Moreover, AVP signaling in the LS regulates social play behavior in juvenile rats (Bredewold et al., 2014; Veenema et al., 2013). Together, these findings clearly demonstrate that, irrespective of expression levels, AVP in the MeP/pBNST→LS circuit plays a role in regulating social behavior at both adult and juvenile ages.

4.2.2 | Sex differences in AVP-ir in the MeP/pBNST→LS circuit—Robust sex differences were observed in the MeP/pBNST→LS circuit, with adult male rats having more AVP-ir cell bodies in the pBNST and MeP and a greater fractional area occupied by AVP-ir fibers in the MeA and LS compared to adult female rats. This is in line with previous studies showing that AVP fiber density in the LS and AVP mRNA expression in the pBNST and MeA is higher in adult male versus adult female rats (De Vries et al., 1981; Miller et al., 1992; Szot & Dorsa, 1993) as well as in several other species, including mice and prairie voles (Bamshad, Novak, & De Vries, 1993; Rood et al., 2013).

In contrast to adults, we show that sex differences in AVP-ir among juveniles are apparent in only two subregions of the LS, the mLSd and cLSv. The general lack of sex differences in AVP-ir in juveniles may partly be due to the overall lower levels of AVP-ir compared to adults. Taken together, this may indicate that sex differences in AVP-ir in the MeP/pBNST→LS circuit likely have the greatest functional consequences after puberty.

Higher levels of AVP-ir in the adult male rat MeP/pBNST→LS circuit compared to females are likely the result of sex differences in the regulation of AVP expression via direct gonadal steroid hormone action. In support, AVP-ir cells in the adult BNST and MeA express ER alpha and AR (Axelson, Shannon, & Van Leeuwen, 1992; Zhou, Blaustein, & De Vries, 1994). However, sex differences in circulating gonadal hormone levels may be necessary, but are less likely to be sufficient to induce sex-specific AVP expression in this circuit. For example, gona-dectomized adult male and female rats exposed to similar levels of testosterone still differ, with adult males having higher AVP expression than adult females (De Vries & al-Shamma, 1990). This suggests that additional sex differences such as in AR and aromatase expression are likely essential in mediating gonadal steroid-induced sex-specific AVP expression (De Vries & al-Shamma, 1990). Furthermore, in addition to the activational and organizational effects of gonadal steroid hormones, the male Y chromosome also contributes to the masculinized expression of AVP-ir fibers in the LS (De Vries et al., 2002).

Sex differences in AVP-ir in the MeP/pBNST→LS circuit of adult rats may indicate the sex-specific involvement of AVP in the regulation of adult social behaviors, such as reproductive and aggressive behaviors. For example, AVP mRNA expression in BNST neurons increased following mating in male, but not female, prairie voles (Wang, Ferris, & De Vries, 1994). In line with this, copulation with a female augments Fos expression in AVP-ir neurons in the MeP and BNST of adult male rats (Hari Dass & Vyas, 2014) and mice (Ho et al., 2010). Interestingly, intracerebroventricular administration of AVP decreases hop-dart proceptive behaviors as well as lordosis responses in female rats, an effect that is rescued by central infusion of a V1AR antagonist (Pedersen & Boccia, 2006). While more studies are needed to determine whether the effects of AVP observed in females are specific to the MeP/pBNST→LS circuit, the possibility emerges that mating behavior is facilitated by higher levels of AVP signaling in males, while it requires lower levels of AVP signaling in females.

Higher AVP-ir in the MeP/pBNST→LS circuit of males versus females may parallel the differential involvement of AVP in male and female aggression. A potential role of AVP in male aggression is largely based on the observation that male rats castrated as adults show reduced AVP-ir in the MeP/pBNST→LS circuit as well as reduced levels of intermale aggression (Koolhaas, Moor, Hiemstra, & Bohus, 1991; Miller et al., 1992; Szot & Dorsa, 1993; van Leeuwen et al., 1985). Therefore, it was proposed that AVP in this circuit facilitates intermale aggression. While some studies provide support for this notion, others do not. For example, administration of AVP into the LS facilitated inter-male aggression in castrated rats (Koolhaas et al., 1991) and extracellular AVP release in the LS correlated positively with intermale aggression in testes intact rats (Veenema et al., 2010). However, low AVP fiber density in the LS corresponded with high levels of intermale aggression in wild type rats and wild house mice (Compaan, Buijs, Pool, De Ruiter, & Koolhaas, 1993; Everts & Koolhaas, 1997), and extracellular AVP release in the LS decreased during intermale aggression in rats showing high levels of aggression (Beiderbeck, Neumann, & Veenema, 2007). Taken together, these studies suggest that the role of AVP in this circuit largely depends on individual differences in innate levels of intermale aggression, and that higher AVP-ir may not directly translate to higher levels of aggression.

Unfortunately, no studies thus far have directly assessed the causal role of AVP in the MeP/pBNST→LS circuit in female aggression. However, V1AR binding density in the LS correlated positively with maternal aggression in rats (Caughey et al., 2011), suggesting a potential role of AVP in female aggression. Importantly, females in the current study were virgins, and it is unclear whether the sex differences in AVP in the MeP/pBNST→LS circuit still exists when comparing males with lactating females. Clearly, more work is needed to determine the role of AVP in the MeP/pBNST→LS circuit in male and female reproductive and aggressive behaviors, and whether sex differences in this circuit have implications for the sex-specific regulation of these behaviors.

Finally, it is important to consider that sex differences in AVP-ir in this circuit may not necessarily translate into a sex-specific behavioral role of AVP. Indeed, administration of AVP in the LS prolonged, while administration of V1AR antagonists in the LS impaired social recognition similarly in adult male and female rats (Veenema et al., 2012). These

findings are the first to demonstrate a functional role of AVP in the MeP/pBNST→LS circuit in adult female rats, despite relatively low levels of AVP-ir in this circuit.

4.3 | Age differences in AVP-ir in the AHA

The anterior portion of the AH (AHA) was the only region in the fore-brain SBNN in which AVP-ir was higher in juvenile males than in adult males. This is surprising given the extensive body of work showing that AVP signaling in the AH facilitates intermale aggression in diverse adult rodent species (Albers et al., 2006; Caldwell & Albers, 2004; Cooper, Karom, Huhman, & Albers, 2005; Gobrogge, Liu, Young, & Wang, 2009; Ferris et al., 1997; Potegal & Ferris, 1989). Since aggression levels are very low in juveniles, it would be interesting to determine the role of AH-AVP in juvenile social behaviors, such as social play behavior. Along these lines, Cheng and Delville (2009) showed that in juvenile golden hamsters, microinjections of a V1AR antagonist into the AH inhibited social play behavior. Given our findings, future work should focus on the behavioral relevance of AH-AVP in juvenile rats.

4.4 | Sex differences in AVP-ir in the MPOA

Sex differences in AVP fiber fractional area in the MPOA were already apparent at the juvenile age, with males having a higher density of AVP fibers than females, and this effect persisted into adulthood. Interestingly, the MPOA is one of a few regions that show abundant AVP fibers, but virtually no V1AR (Smith et al., 2017). However, the MPOA contains oxytocin receptors in both sexes and at both juvenile and adult ages (Smith et al., 2017), suggesting the possibility that AVP released in the MPOA acts through oxytocin receptors rather than V1AR, which has been suggested to occur in other brain regions (Chini, Manning, & Guillon, 2008; Song, Larkin, Malley, & Albers, 2016; Song et al., 2014).

Classically, the MPOA has been implicated in the expression of male sexual behavior. In adult male rats, lesions of the MPOA reduced, while electrical stimulation of the MPOA facilitated sexual behavior (Arendash & Gorski, 1983; Malsbury, 1971). This concurs with physiological evidence wherein male rats showed an augmented Fos response in the MPOA following copulation with an estrous female rat (Baum & Everitt, 1992). In contrast to males, MPOA lesions facilitated the display of sexual behavior in adult female rats (Hoshina, Takeo, Nakano, Sato, & Sakuma, 1994; Powers & Valenstein, 1972). Interestingly, MPOA lesions also disrupt the onset and the maintenance of maternal behavior (Numan, 1974; Numan, Rosenblatt, & Komisaruk, 1977), suggesting that activation of the MPOA inhibits pathways mediating female sexual behaviors, but activates pathways mediating maternal behaviors. Although it is unknown whether AVP signaling in the MPOA plays a role in the regulation of rat sexual behavior, the display of maternal behavior in lactating rats is associated with increased extra-cellular AVP release in the MPOA (Bosch et al., 2010). It would be interesting to determine whether lactating females have a higher density of AVP-ir fibers in the MPOA compared to virgin females, and whether this corresponds with greater AVP release in the MPOA.

Combining our AVP data with the aforementioned MPOA studies, we speculate that high AVP signaling in the MPOA facilitates male sexual behavior and female maternal behavior,

while low AVP signaling may allow for the expression of sexual behavior in females. Thus, high versus low AVP signaling in the MPOA may result in altered MPOA activity, producing a change in the relative contribution of the MPOA in the SBNN. This may in turn drive the expression of the appropriate behavior (sexual behavior in males, and either maternal or sexual behavior in females). In this way, quantitative changes in AVP signaling in one SBNN node might produce qualitative changes in social behavior by altering SBNN activity as a whole.

Finally, current data in adults do not provide an explanation for a possible functional role of the sex difference in AVP-ir fiber density in the MPOA of juvenile rats. The only study to investigate the role of the MPOA in juveniles showed that pup exposure-induced maternal behavior in juvenile female rats relies on the MPOA (Kalinichev, Rosenblatt, & Morrell, 2000). It would be interesting to determine whether this pup-induced maternal behavior requires MPOA-AVP signaling in juvenile females.

4.5 | No age or sex differences in AVP-ir in the VMH

Given the lack of age and sex differences in AVP-ir in the VMH, we speculate that AVP-ir fibers observed in the VMH likely originate from hypothalamic populations in the PVN and/or SON. While minor reciprocal connections between the PVN and VMH have been observed using anterograde tract tracing techniques in rats (Ter Horst & Luiten, 1987), the phenotype of these neuronal projections remains unknown. This putative AVPergic PVN→VMH pathway should be confirmed using genetically-guided, viral tract tracing techniques.

4.6 | No age or sex differences in OT-ir in the forebrain SBNN

In stark contrast to AVP, we observed no age or sex differences in OT-ir cell bodies or fibers throughout the forebrain SBNN. Moreover, OT-ir fiber fractional areas were generally much lower than AVP-ir in most SBNN nodes with the exception of the BNST, AH and VMH. Although AVP and OT are structurally similar (both are nine amino acid peptides differing by only two amino acids), they are clearly regulated very differently. Interestingly, robust age and sex differences were found for the OTR across multiple nodes of the rat SBNN (Smith et al., 2017). Specifically, adult rats expressed higher OTR binding density compared to juvenile rats in the pBNST, MePD, MPOA, and VMH, while juveniles had higher OTR binding in the ventral portion of the LS and males had higher levels of OTR binding in these regions compared to females (Smith et al., 2017). Thus, the lack of age and sex differences in OT-ir in the SBNN may be compensated by age and sex differences in OTR. The OTR may therefore mediate the observed sex differences in behavioral and neural responses to OT system manipulations (for review see Dumais & Veenema, 2016a,b). Additionally, there may be age and sex differences in the mechanisms regulating OT release, particularly in response to social stimuli. In support, extracellular OT release in the pBNST was higher in adult male rats compared to adult female rats when exposed to a juvenile conspecific (Dumais et al., 2016). Finally, it is well known that the OT system undergoes dramatic changes during pregnancy and lactation to facilitate maternal behaviors (Jirikowski, Caldwell, Pilgrim, Stumpf, & Pedersen, 1989; Meddle, Bishop, Gkoumassi, van Leeuwen, & Douglas, 2007; Pedersen, Ascher, Monroe, & Prange, 1982; Theodosios, Montagnese, Rodriguez, Vincent, &

Poulain, 1986). It would therefore be interesting to determine whether sex differences in OT-ir in the forebrain SBNN emerge under these circumstances.

5 | CONCLUSION

In summary, we showed for the first time that there are many age (juveniles vs. adults) and sex differences in AVP-ir (but not OT-ir) throughout the forebrain SBNN, most notably the LS, BNST, and MeA (age and sex), as well as the AH (age only) and MPOA (sex only) in rats. We discussed that, despite having lower levels of AVP-ir in this network, AVP signaling is nevertheless crucial for the proper display of social behaviors in juveniles (social play, social recognition) and adult females (social recognition, sexual behavior), but that the function of AVP may differ depending on age (juvenile versus adult social recognition) or sex (male versus female sexual behavior). Because AVP, OT, and their homologs regulate social behavior across vertebrate taxa (for review, see Goodson 2008; Kelly & Ophir 2015), further research should explore whether there are similar age and sex differences in AVP-ir (or a similar lack of age and sex differences in OT-ir) in other species. Most importantly, the behavioral consequences of the numerous age and sex differences in SBNN-AVP-ir described here should be further elucidated.

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Abbreviations

aBNST	bed nucleus of the stria terminalis, anterior part
AH	anterior hypothalamus
AHA	anterior hypothalamus, anterior portion
AHC	anterior hypothalamus, central part
AHP	anterior hypothalamus, posterior part
AR	androgen receptor
AVP	arginine vasopressin
BNST	bed nucleus of the stria terminalis
cLSD	lateral septum, dorsal caudal part
cLSI	lateral septum, intermediate caudal part
cLSV	lateral septum, ventral caudal part
ER	estrogen receptor

FFA	fiber fractional area
ir	immunoreactive/immunoreactivity
LS	lateral septum
LSD	lateral septum, dorsal part
LSI	lateral septum, intermediate part
LSV	lateral septum, ventral part
MeA	medial amygdala
MeAD	medial amygdala, anterodorsal part
MeAV	medial amygdala, anteroventral part
MeP	medial amygdala, posterior part
MePD	medial amygdala, posterodorsal part
MePV	medial amygdala, posteroventral part
mLS	lateral septum, medial part
mLSD	lateral septum, dorsal medial part
mLSI	lateral septum, intermediate medial part
mLSV	lateral septum, ventral medial part
MPOA	medial preoptic area
OT	oxytocin
pBNST	bed nucleus of the stria terminalis, posterior part
PN	postnatal
PVN	paraventricular hypothalamic nucleus
rLS	lateral septum, rostral part
rLSD	lateral septum, dorsal rostral part
rLSI	lateral septum, intermediate rostral part
rLSV	lateral septum, ventral rostral part
SBNN	social behavior neural network
SCN	suprachiasmatic nucleus
SON	supraoptic nucleus
STLI	bed nucleus of the stria terminalis, intermediate part

STMAM	bed nucleus of the stria terminalis, anteromedial part
VMH	ventromedial hypothalamus
VMHdm	ventromedial hypothalamus, dorsomedial part
VMHvl	ventromedial hypothalamus, ventrolateral part
V1AR	arginine vasopressin receptor 1A.

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A. Quantitative Fiber Fractional Area Regions **B. Quantitative Cell Counting Regions** **FIGURE 1.**

Forebrain SBNN regions quantified for vasopressin-immunoreactive (AVP-ir) and oxytocin-immunoreactive (OT-ir) fiber fractional area and cell body number. Modified schematic from the rat brain atlas of Paxinos and Watson (2007) depicting regions in which AVP-ir and OT-ir fibers or cell bodies were quantified. (a) Forebrain regions in which AVP-ir and OT-ir fiber fractional area was analyzed. Regions (from left to right) included the dorsal (LSD), intermediate (LSI) and ventral (LSV) portions of the rostral (rLS), medial (mLS), and caudal (cLS) lateral septum; the medial division of the anteromedial bed nucleus of the stria terminalis (STMAM); the lateral division of the intermediate bed nucleus of the stria terminalis (STLI); the medial preoptic area (MPOA); the posterior bed nucleus of the stria terminalis (pBNST); the anterior (AHA), central (AHC), and posterior (AHP) regions of the anterior hypothalamus; the dorsomedial (VMHdm) and ventrolateral (VMHvl) divisions of the ventro-medial hypothalamus; and the anterodorsal (MeAD), anteroventral (MeAV), posterodorsal (MePD), and posteroventral (MePV) medial amygdala. (b) Forebrain regions in which AVP-ir and OT-ir cell bodies were quantified. Regions (from left to right) included an area encompassing the anteroventral BNST and medial preoptic area (aBNST/MPOA; OT-ir only); the posterior bed nucleus of the stria terminalis (pBNST; AVP-ir only) and the posterodorsal medial amygdala (MePD; AVP-ir only). Numbers shown at the bottom right represent the distance in mm relative to bregma for each section. cc = corpus callosum; aca = anterior commissure; LV = lateral ventricle; 3V = third ventricle; opt = optic tract

FIGURE 2.

Age and sex differences in vasopressin-immunoreactive (AVP-ir) fiber fractional areas were observed throughout the forebrain SBNN. Age differences were found in all subregions of the lateral septum (a–o) and medial amygdala (ee–ii) as well as in the STMAM (p), STLI (p), and AHA (z–dd), with adults having a greater fractional area occupied by AVP-ir fibers than juveniles in all regions, with the exception of the AHA (higher in juveniles). Sex differences were observed in 5 of the 9 subregions of the lateral septum (mLSD, mLSI, mLSV, cLSI, cLSV; f–o), MPOA (u–y), and all subregions of the medial amygdala (MeAD, MeAV, MePD, MePV; ee–ii), with adult males having a greater fractional area occupied by AVP-ir fibers than adult females. Juvenile males also had a greater fractional area occupied by AVP-ir fibers than juvenile females in the MPOA (ee–ii). Data are presented as mean $\pm$ SEM, * p < .05, ** p < .01, *** p < .001, # p < .05 versus corresponding juvenile group, two-way ANOVA followed by Bonferroni tests with FDR correction for multiple comparisons (α < 0.024). Insets on select photomicrographs depict higher magnification (40 $\times$ objective) images of boxed fibers within the adjoining lower magnification image set. FFA = fiber fractional area

FIGURE 3.

Age and sex differences in vasopressin-immunoreactive (AVP-ir) cell numbers in the pBNST and MeP (a, f) Quantification of AVP-ir cell bodies in the pBNST (b–e, top), and MePD (g–j, bottom), averaged over three 30 μm sections using a correction factor (average cell count was multiplied by a factor of 0.75 to correct for overcounting) revealed a significant effect of age and sex, with adult rats having a greater number of AVP-ir cell bodies than juvenile rats, and adult males having a greater number of AVP-ir cell bodies than adult females. Data are presented as mean $\pm$ SEM, * $p < .05$, *** $p < .001$, # $p < .05$ versus corresponding juvenile group, two-way ANOVA followed by Bonferroni post hoc tests. LV =lateral ventricle; opt =optic tract

FIGURE 4.

No age or sex differences in oxytocin immunoreactive (OT-ir) fiber fractional areas were observed throughout the forebrain SBNN. No age or sex differences in the fiber fractional area occupied by OT-ir fibers were found in the lateral septum (a–o), stria terminalis (p–t), medial preoptic area (u–y), anterior hypothalamus (z–dd), medial amygdala (ee–ii) or ventromedial hypothalamus (jj–nn). Data are presented as mean $\pm$ SEM, two-way ANOVA with FDR correction for multiple comparisons ($\alpha < 0.004$). Insets on select photomicrographs depict higher magnification (40 $\times$ objective) images of boxed fibers within the adjoining lower magnification image set. FFA = fiber fractional area

FIGURE 5.

No age or sex differences were observed in oxytocin-immunoreactive (OT-ir) cell numbers in the aBNST/MPOA. (a) Quantification of OT-ir cell bodies in the aBNST/MPOA, averaged over three 30-μm sections using a correction factor (average cell count was multiplied by a factor of 0.545 to correct for overcounting), revealed similar OT-ir cell numbers in juvenile and adult male and female rats (b–e). Data are presented as mean $\pm$ SEM, two-way ANOVA. LV =lateral ventricle; opt =optic tract

FIGURE 6.

Neuropeptide-specific fiber immunoreactivity patterns in three core nodes of the SBNN. Low (4×) magnification photomicrographs of AVP-ir (a–c) and OT-ir (a'–c') fibers in the lateral septum (a–a'), fibers and cell bodies in the posterior bed nucleus of the stria terminalis (b–b'), and fibers and cell bodies in the posterior medial amygdala (c–c'). AVP-ir and OT-ir images were taken from adjacent sections of the same adult male rat. cc = corpus callosum; LV = lateral ventricle, IVF = interventricular foramen; opt = optic tract. Numbers shown at the bottom right represent the distance in mm relative to bregma for each section

FIGURE 7.

Disparate patterns of age and sex differences in AVP and OT immunoreactivity in the rat forebrain SBNN. (a) Age and sex differences in AVP-ir were observed in the LS, BNST, and MeA, while age differences alone were found only in the AH, and sex differences alone were found only in the MPOA. The VMH displayed no age or sex differences. (b) No age or sex differences in OT-ir were observed in any of the forebrain SBNN nodes (LS, BNST, MPOA, AH, VMH, MeA). [Color figure can be viewed at wileyonlinelibrary.com]

TABLE 1

Antibodies used for immunohistochemistry

Antibody	Antigen	Species	RRID	Dilution	Manufacturer
Primary					
PS41	Anti-AVP associated neurophysin	Mouse, monoclonal	AB 2313960	1:100	Gainer, NINDS
PS38	Anti-OT associated neurophysin	Mouse, monoclonal	AB 2315026	1:100	Gainer, NINDS
Secondary					
Goat anti-mouse	Anti-mouse IgG	Goat	AB 2336170	1:500	Vector Laboratories

TABLE 2

Statistical details of main effects of age and sex for arginine vasopressin immunoreactivity presented in Figures 2 and 4 using two way ANOVAs

	Age differences	Sex differences	Age × Sex interaction	Age effect	Sex effect
Fiber fractional area					
<i>Lateral septum</i>					
rLSD	<i>$F_{(1,16)} = 11.2; p = .004$</i>	$F_{(1,16)} = 1.3; p = .270$	$F_{(1,16)} = 0.03; p = .864$	Adults >Juveniles	
rLSI	<i>$F_{(1,16)} = 20.7; p = .0003$</i>	$F_{(1,16)} = 0.53; p = .477$	$F_{(1,16)} = 11.1; p = .982$	Adults >Juveniles	
rLSV	<i>$F_{(1,16)} = 13.9; p = .002$</i>	$F_{(1,16)} = 4.54; p = .049$	$F_{(1,16)} = 0.03; p = .873$	Adults >Juveniles	
mLSD	<i>$F_{(1,23)} = 71.9; p < .0001$</i>	<i>$F_{(1,23)} = 45.5; p < .0001$</i>	<i>$F_{(1,23)} = 15.6; p = .001$</i>	Adults >Juveniles	Males >Females
mLSI	<i>$F_{(1,24)} = 81.1; p < .0001$</i>	<i>$F_{(1,24)} = 7.5; p = .012$</i>	$F_{(1,24)} = 1.14; p = .296$	Adults >Juveniles	Males >Females
mLSV	<i>$F_{(1,24)} = 23.8; p < .0001$</i>	<i>$F_{(1,24)} = 6.6; p = .017$</i>	$F_{(1,24)} = 2.99; p = .096$	Adults >Juveniles	Males >Females
cLSD	<i>$F_{(1,20)} = 29.9; p < .0001$</i>	$F_{(1,20)} = 3.15; p = .091$	$F_{(1,20)} = 0.77; p = .784$	Adults >Juveniles	
cLSI	<i>$F_{(1,24)} = 72.4; p < .0001$</i>	<i>$F_{(1,24)} = 15.7; p = .001$</i>	$F_{(1,24)} = 5.64; p = .026$	Adults >Juveniles	Males >Females
cLSV	<i>$F_{(1,23)} = 37.8; p < .0001$</i>	<i>$F_{(1,23)} = 33.4; p < .0001$</i>	<i>$F_{(1,23)} = 16.8; p = .0004$</i>	Adults >Juveniles	Males >Females
<i>Medial amygdala</i>					
MeAD	<i>$F_{(1,24)} = 23.3; p < .0001$</i>	<i>$F_{(1,24)} = 7.2; p = .013$</i>	$F_{(1,24)} = 2.66; p = .116$	Adults >Juveniles	Males >Females
MeAV	<i>$F_{(1,24)} = 7.4; p = .012$</i>	<i>$F_{(1,24)} = 6.1; p = .021$</i>	$F_{(1,24)} = 1.71; p = .203$	Adults >Juveniles	Males >Females
MePD	<i>$F_{(1,24)} = 13.9; p = .001$</i>	<i>$F_{(1,24)} = 7.3; p = .013$</i>	$F_{(1,24)} = 0.70; p = .412$	Adults >Juveniles	Males >Females
MePV	<i>$F_{(1,24)} = 20.0; p < .0001$</i>	<i>$F_{(1,24)} = 7.8; p = .010$</i>	$F_{(1,24)} = 4.40; p = .047$	Adults >Juveniles	Males >Females
<i>Stria terminalis</i>					
STMAM	<i>$F_{(1,23)} = 7.6; p = 0.011$</i>	$F_{(1,23)} = 1.98; p = 0.17$	$F_{(1,23)} = 2.76; p = .110$	Adults >Juveniles	
STLI	<i>$F_{(1,23)} = 6.0; p = 0.02$</i>	$F_{(1,23)} = 5.37; p = 0.03$	$F_{(1,23)} = 3.19; p = .087$	Adults >Juveniles	
pBNST	$F_{(1,23)} = 0.4; p = 0.519$	$F_{(1,23)} = 0.04; p = 0.843$	$F_{(1,23)} = 0.23; p = .639$		
<i>Hypothalamic nuclei</i>					
MPOA	$F_{(1,24)} = 3.78; p = .064$	<i>$F_{(1,24)} = 13.6; p = .001$</i>	$F_{(1,24)} = 0.41; p = .527$		Males >Females
AHA	<i>$F_{(1,23)} = 8.2; p = .009$</i>	$F_{(1,23)} = 0.94; p = .344$	$F_{(1,23)} = 2.22; p = .149$	Juveniles >Adults	
AHC	$F_{(1,23)} = 0.79; p = .382$	$F_{(1,23)} = 2.01; p = .169$	$F_{(1,23)} = 1.66; p = .211$		
AHP	$F_{(1,23)} = 3.22; p = .086$	$F_{(1,23)} = 0.80; p = .380$	$F_{(1,23)} = 1.03; p = .320$		
VMHdm	$F_{(1,23)} = 0.04; p = .848$	$F_{(1,23)} = 0.06; p = .811$	$F_{(1,23)} = 0.03; p = .860$		
VMHvl	$F_{(1,23)} = 0.02; p = .889$	$F_{(1,23)} = 0.11; p = .740$	$F_{(1,23)} = 0.54; p = .468$		
<i>Cell number</i>					
pBNST	<i>$F_{(1,20)} = 23.3; p < .0001$</i>	$F_{(1,20)} = 2.47; p = .132$	$F_{(1,20)} = 1.67; p = .211$	Adults >Juveniles	
MePD	<i>$F_{(1,23)} = 42.7; p < .0001$</i>	<i>$F_{(1,23)} = 14.7; p = .001$</i>	<i>$F_{(1,23)} = 7.50; p = .012$</i>	Adults >Juveniles	Males >Females

Note. Fiber fractional area statistics were corrected for multiple comparisons using an FDR correction ($\alpha < 0.024$). Statistically significant effects are highlighted in italics.

TABLE 3

Statistical details of main effects of age and sex for oxytocin immunoreactivity presented in Figures 3 and 5 using two-way ANOVAs

	Age differences	Sex differences	Age × sex interaction
Fiber fractional area			
<i>Lateral septum</i>			
rLSD	$F_{(1,14)} = 2.52; p = .135$	$F_{(1,14)} = 0.50; p = .826$	$F_{(1,14)} = 3.42; p = .086$
rLSI	$F_{(1,14)} = 1.96; p = .183$	$F_{(1,14)} = 0.67; p = .428$	$F_{(1,14)} = 2.58; p = .130$
rLSV	$F_{(1,14)} = 0.00; p = .994$	$F_{(1,14)} = 0.03; p = .859$	$F_{(1,14)} = 0.18; p = .680$
mLSD	$F_{(1,22)} = 0.00; p = .986$	$F_{(1,22)} = 0.01; p = .940$	$F_{(1,22)} = 0.37; p = .365$
mLSI	$F_{(1,23)} = 0.04; p = .847$	$F_{(1,23)} = 0.35; p = .558$	$F_{(1,23)} = 2.98; p = .098$
mLSV	$F_{(1,23)} = 0.00; p = .952$	$F_{(1,23)} = 2.13; p = .158$	$F_{(1,23)} = 8.11; p = .009$
cLSD	$F_{(1,22)} = 0.22; p = .641$	$F_{(1,22)} = 1.42; p = .247$	$F_{(1,22)} = 0.21; p = .653$
cLSI	$F_{(1,23)} = 0.32; p = .580$	$F_{(1,23)} = 0.06; p = .803$	$F_{(1,23)} = 1.35; p = .257$
cLSV	$F_{(1,24)} = 0.37; p = .551$	$F_{(1,24)} = 0.09; p = .765$	$F_{(1,24)} = 2.35; p = .139$
<i>Medial amygdala</i>			
MeAD	$F_{(1,24)} = 0.05; p = .817$	$F_{(1,24)} = 0.62; p = .438$	$F_{(1,24)} = 1.10; p = .306$
MeAV	$F_{(1,24)} = 7.18; p = .013$	$F_{(1,24)} = 0.28; p = .601$	$F_{(1,24)} = 0.79; p = .382$
MePD	$F_{(1,23)} = 0.11; p = .747$	$F_{(1,23)} = 0.82; p = .374$	$F_{(1,23)} = 0.05; p = .820$
MePV	$F_{(1,24)} = 0.86; p = .363$	$F_{(1,24)} = 0.29; p = .593$	$F_{(1,24)} = 0.07; p = .801$
<i>Stria terminalis</i>			
STMAM	$F_{(1,22)} = 0.05; p = .819$	$F_{(1,22)} = 0.85; p = .366$	$F_{(1,22)} = 2.17; p = .155$
STLI	$F_{(1,22)} = 1.46; p = .240$	$F_{(1,22)} = 5.38; p = .030$	$F_{(1,22)} = 0.00; p = .996$
pBNST	$F_{(1,24)} = 1.18; p = .289$	$F_{(1,24)} = 0.28; p = .599$	$F_{(1,24)} = 0.35; p = .558$
<i>Hypothalamic nuclei</i>			
MPOA	$F_{(1,23)} = 0.60; p = .445$	$F_{(1,23)} = 2.25; p = .147$	$F_{(1,23)} = 0.48; p = .493$
AHA	$F_{(1,23)} = 0.09; p = .764$	$F_{(1,23)} = 5.04; p = .035$	$F_{(1,23)} = 0.52; p = .480$
AHC	$F_{(1,23)} = 1.61; p = .217$	$F_{(1,23)} = 0.41; p = .530$	$F_{(1,23)} = 0.46; p = .504$
AHP	$F_{(1,22)} = 0.89; p = .536$	$F_{(1,22)} = 1.88; p = .185$	$F_{(1,22)} = 0.92; p = .349$
VMHdm	$F_{(1,23)} = 4.30; p = .050$	$F_{(1,23)} = 0.46; p = .503$	$F_{(1,23)} = 0.44; p = .514$
VMHvl	$F_{(1,23)} = 0.14; p = .715$	$F_{(1,23)} = 0.87; p = .362$	$F_{(1,23)} = 0.24; p = .626$
Cell number			
aBNST/MPOA	$F_{(1,22)} = 0.01; p = .942$	$F_{(1,22)} = 1.95; p = .176$	$F_{(1,22)} = 0.14; p = .717$

Note. Fiber fractional area statistics were corrected for multiple comparisons using an FDR correction ($\alpha < 0.004$)