Published in final edited form as:
Neuroscience. 2008 June 26; 154(3): 911–921. doi:10.1016/j.neuroscience.2008.03.087.

Absence of progestin receptors alters distribution of vasopressin fibers but not sexual differentiation of vasopressin system in mice

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Abstract

Perinatal estrogens increase the number of vasopressin-expressing cells and the density of vasopressin-immunoreactive fibers observed in adult male rodents. The mechanism of action of estrogens on sexual differentiation of the extra-hypothalamic vasopressin system is unknown. We hypothesized that the sexually dimorphic expression of progestin receptors (PRs) during development would masculinize vasopressin expression in mice. We compared the number of vasopressin-expressing cells in the bed nucleus of the stria terminalis (BNST) and medial amygdala and the density of vasopressin-immunoreactive fibers in several brain regions of male and female wild type and PR knockout mice using in situ hybridization and immunohistochemistry. As expected, sex differences in vasopressin cell number were observed in the BNST and medial amygdaloid nucleus. Vasopressin-immunoreactive fiber density was sexually dimorphic in the lateral septum, lateral habenular nucleus, medial amygdaloid nucleus, and mediodorsal thalamus. Sex differences were also observed in the principal nucleus of the BNST and medial preoptic area but not in the dorsomedial hypothalamus, all of which are thought to receive vasopressin innervation from the suprachiasmatic nucleus. Deletion of PRs did not alter the sex difference in vasopressin mRNA expression and vasopressin fiber immunoreactivity in any area examined. However, deletion of PRs increased the density of vasopressin fiber immunoreactivity in the lateral habenular nucleus. Our data suggest that PRs modulate vasopressin levels, but not sexual differentiation of vasopressin innervation in mice.

Keywords

progesterone receptor; BNST; medial amygdala; medial preoptic area; mediodorsal thalamus; lateral habenular nucleus

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Section editor: Joan I. Morrell

The vasopressin innervation of the brain (or the homologous vasotocin innervation in non-mammalian vertebrates) is one of the most consistently found sex differences in the brain (De Vries and Panzica, 2006). Males typically have more vasopressin-expressing cells than females in the bed nucleus of the stria terminalis (BNST) and medial amygdaloid nucleus (MeA), and accordingly vasopressin-immunoreactive (-ir) projections emanating from these nuclei are denser in males in most species examined (De Vries and Panzica, 2006). Non-mammalian vertebrates show similar sex differences in homologous vasotocin projections (reviewed in Goodson and Bass, 2003; De Vries and Panzica, 2006). The differentiation of this system has been studied most extensively in rodents.

In adult rats, vasopressin-expressing cell number in the BNST and MeA is one and half to two times greater in males compared to females (Van Leeuwen et al., 1985; Miller et al., 1989; De Vries et al., 1994; Wang and De Vries, 1995). Further, vasopressin-ir fiber density is about two times greater in the lateral septum (De Vries et al., 1981; De Vries and Al-Shamma, 1990), a projection site of the BNST and MeA (De Vries and Buijs, 1983; Caffe et al., 1987). Other brain areas with vasopressin-ir fibers that presumably come from the BNST and MeA, such as the lateral habenular nucleus, ventral hippocampus, and periaqueductal grey, are also sexually dimorphic with males having more fibers than females (De Vries and Buijs, 1983; DeVries et al., 1985; De Vries and Al-Shamma, 1990). Vasopressin-ir projections from sources other than the BNST and MeA, such as the suprachiasmatic nucleus, typically do not show such sex differences (De Vries and Al-Shamma, 1990). For example, in rats there is no sex difference in the vasopressin-ir innervation of the dorsomedial nucleus of the hypothalamus (De Vries and Al-Shamma, 1990), which is derived from the suprachiasmatic nucleus (Hoorneman and Buijs, 1982).

The sex differences in vasopressin cell number and vasopressin-ir fiber density are regulated by gonadal steroids both during development and in adulthood (De Vries et al., 1984; Wang et al., 1993). The expression of vasopressin in adulthood depends on circulating gonadal steroid hormones (De Vries et al., 1984). Gonadectomy drastically reduces vasopressin mRNA production in cells within 24 hours and, by the third day following surgery, decreases the overall number of vasopressin mRNA-expressing cells observed (Miller et al., 1989; Miller et al., 1992). Changes in vasopressin content of BNST and MeA projections lag behind, as it takes 8 to 15 weeks before vasopressin immunostaining disappears (De Vries et al., 1984; Mayes et al., 1988). Subsequent treatment with testosterone or its estrogenic metabolite, estradiol, restores vasopressin mRNA expression and vasopressin immunostaining (De Vries et al., 1984; De Vries et al., 1994). Such strong effects of gonadal steroids in adulthood suggest that sex differences in circulating gonadal hormones contribute to the differences in vasopressin innervation. However, when males and females are gonadectomized and treated similarly with testosterone in adulthood, males have more vasopressin-ir fibers in the lateral septum and more vasopressin mRNA and peptide expressing cells in the BNST and MeA (De Vries and Al-Shamma, 1990; Wang e al., 1993; De Vries et al., 1994). These differences appear to be caused primarily by differences in gonadal hormone levels during perinatal development (Wang et al., 1993; Han and De Vries, 2003). Testosterone surges in males during the late prenatal and early postnatal period are thought to induce steroid-dependent sex differences (Weisz and Ward, 1980; Montelica-Heino et al., 1988). In contrast, ovaries do not become steroidogenic until the second week

of life (Gelety and Magoffin, 1997). Perinatal manipulations of gonadal steroid levels permanently alter the sex difference in vasopressin expression. Gonadectomizing neonatal males reduces the number of vasopressin-expressing cells in the BNST and vasopressin-ir fiber density in the lateral septum of males to levels found in control females, whereas treating perinatal females with testosterone reverses these effects (Wang et al., 1993). While evidence suggests that testosterone acts via estrogenic as well as androgenic mechanisms (Han and De Vries, 2003), the molecular and cellular mechanisms underlying the effects of steroids on sexual differentiation are unknown.

Estrogens may act indirectly via the estrogen receptor-mediated induction of progestin receptors (PRs) during development. PRs are more prevalent in the developing than adult brain (Shughrue et al., 1992) and are present in the BNST on the day of birth (Wagner et al., 1998). There is a near absolute sex difference in PR expression during late embryonic and early postnatal life in brain areas, such as the medial preoptic area, anterior ventricular periventricular nucleus, and BNST, where males have a high level of PR expression and females have almost none (Wagner et al., 1998; Quadros et al., 2002a; G.J. De Vries, unpublished observations). The sex difference in PRs is greatest during the first week of life (Quadros et al., 2002b), suggesting that PRs may have a role in sexual differentiation (Wagner, 2006). Indeed, treatment with the PR antagonist RU486 alters the structural development of the medial preoptic nucleus (Quadros et al., 2002c).

PRs may play a direct role in the sexual differentiation of vasopressin expression. In adult rats, vasopressin containing cells in the BNST and MeA co-express estrogen and androgen receptors (Axelson and van Leeuwen, 1990; Zhou et al., 1994), suggesting that gonadal steroids act directly on these cells. These same neurons also express PRs in adulthood (Auger & De Vries, 2002). In addition, PRs may influence vasopressin expression directly as subcutaneous injections of progesterone reduce vasopressin-ir fiber density in the lateral septum and lateral habenular nucleus and the number of cells expressing vasopressin mRNA in the BNST and MeA in rats (Auger & Vanzo, 2006). To determine if PRs regulate development of the sex difference in the vasopressin system, we examined vasopressin fiber density and vasopressin cell numbers in several brain regions of male and female wild type (WT) and progestin receptor knockout (PRKO) mice.

Experimental Procedures

Animals

PR^{lacZ} reporter mice in which a LacZ reporter gene was “knocked in” to the PR gene using gene-targeting methods (Ismail et al., 2002) were used in all experiments described. The lacZ “knock-in” results in a functional disruption of the PR gene, so these mice are also referred to as progestin receptor knock-outs (PRKOs). The PR^{lacZ} reporter mice had been backcrossed to a C57Bl/6 background for at least ten generations. Heterozygous animals were bred in our facilities to produce PRKO and wild type (WT) animals. Pups were weaned at 21 days. At this time, animals were tail clipped for genotyping analysis and ear-tagged for identification. Tail tissue was digested using the “Hot Shot” method (Truett et al., 2000). PCR was used to determine the presence or absence of the lacZ gene, which is found in heterozygous or PRKO animals, using the following primers: forward P1 5’-

TAGACAGTGTCTTAGACTCGTTGTTG-3' and P3 5'-GATGGGCACATGGATGAAATC-3' and reverse L1 5'-CTTCACCCACCGGTACCTTACGCTTC-3'. A 700 base pair band was observed for wild-type mice and a 200 base pair band for homozygous knockouts.

Animals were group-housed (~3/cage) with same-sex siblings, until they were used in experiments. Animals had *ad libitum* access to a low phytoestrogen rodent chow (Purina 2014) and water, and they were kept on a 14:10 reversed light cycle with lights off at 11h00. All procedures conformed to NIH guidelines and were in accordance with a protocol approved by the University of Massachusetts.

Surgeries and T implants

At approximately 6 months of age, animals underwent bilateral gonadectomy under Avertin anesthesia (0.02 ml / 1 g of body weight). All animals also received subcutaneous testosterone in Silastic capsules (Helix Medical, Carpinteria, CA; 0.062" ID / 0.125" OD, 7 mm total length and loaded with 3mm testosterone, Sigma-Aldrich, St. Louis, MO). Following surgery animals had access to water containing acetaminophen (3 ml cherry flavored children's tylenol / 100 ml total volume). Four weeks later, animals were euthanized with CO_2, and blood samples and brains were collected. Blood samples were allowed to clot for at least 1.5 hours. Serum was then extracted from blood samples for hormone analysis following a 15 minute spin at 20,000 RPM. Brains were bisected. One hemisphere was frozen in isopentane and stored at −70° C; the other was immersion-fixed in 5% acrolein for 4 hours and stored in 30% sucrose in 0.1 M phosphate buffered saline at 4° C until sectioning.

In situ hybridization

Transverse sections were cut at 20 μm and mounted on "Colorfrost Plus" slides (Fisher Scientific, Waltham, MA). Slides were stored at −70° C until processing for vasopressin mRNA. Prior to hybridization, slides were soaked in the following rinses under gentle agitation: 1) 4% paraformaldehyde in 0.1M sodium phosphate buffer at 4° C for 15 min; 2) 0.1M phosphate buffered saline at 4° C for 2 min; 3) 0.1M triethanolamine buffer (pH 8.0) for 1 min at room temperature (remainder of rinses were done at room temperature also); 4) 0.1M triethanolamine buffer (pH 8.0) with 2.5μl / ml acetic anhydride for 10 min; 5) 2x standard sodium citrate buffer (SSC, 0.3 M sodium chloride and 0.03 M sodium citrate in distilled water, pH 7.0) for 1 min; 6) 70% ethanol for 2 min; 7) 100% ethanol for 2 min; 8) chloroform for 5 min; 9) 100% ethanol for 2 min Hybridization was carried out by adding 60 μl of [^{35}S] labeled probe in hybridization buffer (50% formamide, 10% dextran sulfate, 0.3M NaCl, 10mM Tris, 1mM EDTA, 1× Denhardt's solution, 10 mM dithiothreitol) to each slide, covering the mixture with parafilm, and incubating it at 37° C for 18 hours. All solutions for pre-hybridization steps were made with DEPC-treated water. Following hybridization, slides were dipped in 1× SSC to remove parafilm and hybridization buffer. Sections were rinsed in 1× SSC for 4 × 15 min at 55° C. Subsequent rinses were done at room temperature in the following order: 1) 1x SSC for 3 × 35 min; 2) 70% ethanol for 2 min; 3) 95% ethanol for 2 min; 4) 100% ethanol for 2 min.

After verifying the hybridization by placing test slides against film, slides were dipped in emulsion (NTB2, Kodak, Rochester, NY) and preheated for 1 hour in a 42° C water bath. Once slides were dry, they were stored at 4° C in light tight boxes containing dessicant capsules. Twenty-four days later, slides were developed in the following solutions at 14° C: 1) Dektol-19 developer (Kodak, Rochester, NY) for 2 min; 2) distilled water for 30 seconds; 3) fixer (Kodak, Rochester, NY) for 5 min; 4) distilled water for 5 min. Brain sections were then counterstained with methyl green: 1) slides were rinsed under running water for 3 min; 2) 2% methyl green for 30 seconds; 3) running water for 1 min; and 4) 2 rapid dips into 50% ethanol. Finally, slides were coverslipped using Cytoseal (Richard Allen Scientific, Kalamazoo, MI).

Analysis of vasopressin mRNA (cell counts and silver grain analysis)

Cells were counted by an observer blind to treatment conditions. Cells with a density of silver grains above background were counted in every third section through the entire rostral to caudal extent of the BNST and MeA, using a 20x objective.

Silver grain analysis was done on ten cells from the BNST and ten from the MeA for each animal. Two cells from the sections containing the five highest numbers of cells were chosen for analysis. Cells were assigned consecutive numbers from right to left and top to bottom; then, a random number generator was used to choose which cells to analyze. Pictures of each cell were taken with a SPOT camera and software (Diagnostic Instruments, Sterling Heights, MI) using 2×2 binning and a 100x objective. These images were subsequently sharpened a single time in ImageJ (NIH), and gray level thresholding was used to determine the relative silver grain density in a circle (160 pixels in diameter) centered on the cell. The average silver grain density from the BNST and MeA were calculated for each subject and used in statistical analyses.

Immunocytochemistry

Transverse sections were cut at 30 μm on a freezing microtome. Immunocytochemistry was carried out in mesh-bottomed cups and all incubations and rinses were done at room temperature except where noted. Sections were incubated in the following solutions: 3 × 5 min in Tris-buffered saline (TBS; 0.05M Tris, 0.9% NaCl, pH 7.6); 0.05M Sodium Citrate in TBS for 30 min; 3 × 5 min in TBS; 0.1M Glycine in TBS for 30 min; 3 × 5 min in TBS. Finally, sections were incubated in blocking solution (20% normal goat serum (NGS), 0.3% Triton-X (Labchem, Inc., Pittsburgh, PA) and 1% H_2O_2 in TBS) for 30 min before being transferred to 2 ml eppendorf tubes containing primary antibody (2% NGS, 1% BSA, 0.3% Triton-X, 0.1% thyroglobulin (Sigma-Aldrich, St. Louis, MO) and 1:20,000 guinea pig anti-vasopressin antiserum (T-5048, Peninsula Labs, San Carlos, CA).

After incubating overnight (~18 hours), sections were returned to wells and rinsed with 37°C TBS containing 1% NGS and 0.02% Triton-X for 3 × 10 min. Next, sections were incubated in secondary antiserum (1:250 biotynilated goat anti-guinea pig IgG, Vector Labs, Burlingame, CA in TBS with 2% NGS and 0.32% Triton-X) for one hour followed by 3 × 10 min rinses in TBS containing 0.2% Triton-X. Sections were then incubated in avidin-biotin complex in TBS (ABC elite kit, Vector Labs) followed by 3 × 15 min rinses in TBS.

Finally, sections were incubated in DAB (0.05% 3, 3'- diaminobenzidine tetrachloride, 0.07% β-D glucose, 0.1% Ni-ammonium sulfate, 7.5 mM ammonium chloride, and 0.46% glucose oxidase in TBS) for 25 min followed by 4 × 5 min rinses in TBS.

Sections were mounted on "Superfrost" slides (Fisher Scientific) and coverslipped with Permount (Fisher Scientific) following dehydration in an ethanol series as follows: 2 × 2 min in distilled water; 1 × 2 min in 50% ethanol; 1 × 2 min in 70% ethanol; 1 × 2 min in 95% ethanol; 2 × 2 min in 100% ethanol; and 2 × 2 min in Hemo-D (Scientific Safety Solvents, Keller, TX).

Analysis of immunoreactive fibers

Fiber density was determined by gray level thresholding of digital images using NIH image 1.47. The images were captured under brightfield illumination using a CCD camera mounted on a microscope with a 20× objective. Previous studies of vasopressin fiber density report only those sections containing the densest innervation within each brain region of interest. In the current study, we analyzed brain regions by examining multiple sections through the region based on anatomical features (Figure 1). Brain regions included the lateral septum (Bregma 0.74, 0.50, and 0.38mm; Paxinos and Franklin, 2001), lateral habenula (Bregma −1.22, −1.46, and −1.70mm), medial preoptic area (Bregma 0.02 and −0.10mm), principal nucleus of the bed nucleus of the stria terminalis (Bregma −0.10mm), posterodorsal medial amygdala (Bregma −1.46 and − 1.70mm), mediodorsal thalamic nucleus (Bregma −0.70 and −0.82mm), and the dorsomedial nucleus of the hypothalamus (Bregma −1.58).

Steroid hormone levels

Serum levels of testosterone and progesterone were measured by radioimmunoassay at University of Virginia Center for Research in Reproduction Ligand Assay and Analysis Core, which is supported by NICHD (SCCPRR) Grant U54- HD28934. Assays were conducted with commercially available kits for testosterone (TKTT2, Diagnostic Product Corporation, Los Angeles, CA) and progesterone (TKPG2, Diagnostic Product Corporation). Samples were analyzed in a single assay (*i.e.*, one assay for testosterone and one for progesterone) to limit inter-assay variability. Published intra-assay variability were 3.5% and 4% for testosterone and progesterone assays respectively.

Statistics

All fiber and silver grain densities and cell counts were analyzed using two-way ANOVAs with sex (male or female) and genotype (WT or PRKO) as factors. Significance was set at $p < 0.05$.

Results

Vasopressin mRNA expressing cells

Male mice had significantly more vasopressin mRNA-expressing cells than females in the BNST (Figure 2A; $F(1, 24) = 21.56$, $p = 0.0001$) and MeA (Figure 2A; $F(1, 27) = 45.34$, $p <$

0.0001). There were no differences between WT and PRKO mice in either area. On average, males had approximately 1.4 times more cells than females in both the BNST and MeA.

In the BNST, female mice had greater silver grain density per cell than males (Figure 2B; $F(1, 24) = 14.42$, $p < 0.001$), but there were no differences between WT and PRKO mice. In the MeA (Figure 2B), there were no sex or genotype differences in silver grain density per cell. There were no significant interactions in any of the above analyses.

Vasopressin-ir fibers

Vasopressin-ir fiber density was analyzed in a number of brain regions, which are thought to receive their vasopressin innervation from either the sexually dimorphic BNST or MeA (*e.g.*, lateral septum, lateral habenular nucleus, MeA, and mediodorsal thalamus (Figure 3A–D); De Vries and Buijs, 1983; DeVries et al., 1985) or the non-sexually dimorphic suprachiasmatic nucleus (*e.g.*, principal nucleus of the BNST, medial preoptic area, and dorsomedial hypothalamus (Figure 5A–C); Hoorneman and Buijs, 1982; Delville et al., 1998).

In the lateral septum (Figure 3A), male mice had greater vasopressin-ir fiber density than females in each of the rostral to caudal sections analyzed. Males had 1.94, 1.89, and 1.77 times more vasopressin-ir fiber density in the rostral ($F(1, 28) = 15.66$, $p < 0.0005$), medial ($F(1, 28) = 46.30$, $p < 0.0001$), and caudal ($F(1, 28) = 25.22$, $p < 0.0001$) sections of the lateral septum respectively. There were no differences between WT and PRKO animals at any level of the lateral septum.

In the lateral habenular nucleus (Figure 3B), there were no sex or genotype differences in the rostral or caudal sections. However, in the medial section of the lateral habenular nucleus, males had significantly greater, about 1.3 times greater, vasopressin-ir fiber density than females ($F(1, 28) = 12.44$, $p < 0.002$) and PRKO mice had greater vasopressin-ir fiber density than WT animals ($F(1, 28) = 4.93$, $p < 0.05$).

The MeA (Figure 3C), which shares reciprocal projections with the BNST (Cooke and Simerly, 2005), had sexually dimorphic vasopressin-ir fiber density in both rostral ($F(1, 28) = 11.00$, $p < 0.005$) and caudal ($F(1, 28) = 12.21$, $p < 0.002$) sections; males had 1.75 and 1.62 times more vasopressin-immunoreactivity than females in the rostral and caudal sections respectively. PRKO mice did not differ from WT mice in either area of the MeA examined.

Similar to the lateral septum, the mediodorsal thalamus (Figure 3D) was sexually dimorphic in vasopressin-ir fiber density in both rostral ($F(1, 25) = 29.29$, $p < 0.0001$) and caudal ($F(1, 25) = 22.33$, $p < 0.0001$) sections, but PRKO mice did not differ from WT. This sex difference in the mediodorsal thalamus, which had not been reported previously, was the largest difference observed in the current study with males having approximately 2.09 and 2.44 times as much vasopressin-immunoreactivity as females in the rostral and caudal sections of the mediodorsal thalamus (Figure 4). Because vasopressin-ir fiber density was measured within a traced region around the mediodorsal thalamus as opposed to a standard size box as in other brain regions, it is possible that these sex differences were due to a

difference in the size of the analyzed area. There was no sex difference in the size of area analyzed in the rostral mediodorsal thalamus, but the area analyzed was larger in males than females in the caudal mediodorsal thalamus ($F(1, 28) = 5.17$, $p < 0.05$). However, when we analyzed the percent of area covered (e.g., pixels covered / total pixels examined), males still had significantly greater vasopressin-ir fiber density than females ($F(1, 28) = 15.29$, $p < 0.001$), suggesting that the difference in size of the MDthal did not account for the difference in vasopressin innervation.

Males also had significantly more vasopressin-immunoreactivity than females in the principal nucleus of the BNST (figure 5A; $F(1, 27) = 4.50$, $p < 0.05$). The magnitude of the sex difference (*i.e.*, 1.32 times more vasopressin-immunoreactivity) was smaller than that observed in the lateral septum and mediodorsal thalamus, but similar to that in the lateral habenular nucleus.

Despite low numbers of fibers, density of vasopressin-ir fibers in the medial preoptic area was highly sexually dimorphic (figure 5B; rostral, $F(1, 27) = 28.36$, $p < 0.0001$; caudal, $F(1, 26) = 12.04$, $p < 0.002$). Males had 2.15 and 1.78 times denser innervation than females in the rostral and caudal sections of the medial preoptic area respectively. This difference was surprising given that vasopressin fibers in the medial preoptic area are thought to originate from the suprachiasmatic nucleus, which does not contain obvious sex differences in vasopressin expression (De Vries et al., 1981; Crenshaw et al., 1992; Delville et al., 1998).

Finally, there were no significant sex or genotype differences observed in the vasopressin-immunoreactivity of the dorsomedial hypothalamus (figure 5C), which has been shown to receive vasopressin projections from the suprachiasmatic nucleus (Hoorneman and Buijs, 1982).

Testosterone and Progesterone RIA

Similar testosterone implants were given to all subjects in order to maintain vasopressin expression in the BNST and MeA and to allow observation of the effects of PR on the sexual differentiation of vasopressin expression independent of the activational effects of testosterone. Serum testosterone levels did not differ between males and females or between PRKO and WT animals (table 1). However, there was a significant interaction ($F(1, 27) = 4.62$, $p < 0.05$); PRKO males had lower levels of testosterone overall than WT males as shown by Fisher's LSD test ($p < 0.02$), whereas female PRKO and WT animals did not differ from each other. Examination of individual values revealed one male PRKO mouse that had roughly one-third the levels of testosterone of other animals. When this animal was removed from the analysis, the interaction failed to reach significance ($F(1, 26) = 3.12$, $p = 0.09$). However, this subject had a similar amount of vasopressin immunoreactivity in the lateral septum and similar numbers of vasopressin mRNA containing cells in the BNST and MeA compared to other PRKO males, suggesting that testosterone levels were high enough to maintain vasopressin expression. Thus, we chose to leave the outlier in all analyses despite lower testosterone levels.

Serum progesterone concentration was determined for most subjects (not enough serum remained after the testosterone radioimmunoassay for some subjects). Serum progesterone

levels did not differ between groups (table 1). Because all animals had been gonadectomized, circulating progesterone was most likely secreted by the adrenal glands (Corpechot et al., 1993)

Discussion

Influence of progestin receptors on AVP expression

Our results demonstrate that the absence of progestin receptors (PRs) does not have a major impact on sex differences in vasopressin expression in the mouse brain. Males had more vasopressin-expressing cells than females in the BNST and MeA and higher densities of vasopressin-ir fibers in areas presumed to receive projections from these cells in WT as well as PRKO mice. Although, PRs did not seem to influence sexual differentiation of vasopressin expression, they may influence vasopressin fiber density in some brain areas. Specifically, PRKO males and females had a greater vasopressin-ir fiber density than WT animals in the lateral habenular nucleus. The physiological significance of this finding is unclear. The lateral habenular nucleus forms a major relay point between the forebrain and midbrain (reviewed in Sutherland, 1982 and Lecourtier and Kelly, 2007) and plays an important role in learning, memory and attention, functions that are modulated by vasopressin (Caldwell et al., 2008). Because the lateral habenular nucleus does not appear to contain PRs (Quadros et al., 2007), these differences likely originate in cells in which vasopressin is produced, the BNST or MeA.

In this study, mice were gonadectomized and received supplemental testosterone but not progesterone, resulting in similar levels of testosterone and low levels of progesterone, presumably secreted by the adrenal glands (Corpechot et al., 1993). Administration of testosterone was necessary to maintain steroid dependent vasopressin expression in the BNST and MeA, and the similar levels of testosterone across groups normalized activational effects of testosterone. In addition, the low levels of progesterone should have minimized, but perhaps not eliminated, potential activational effects of progesterone. Therefore, differences among groups most likely arise due to developmental processes rather than circulating levels of steroid hormones in adulthood. However, because progesterone was still present, the greater vasopressin-ir fiber density observed in the lateral habenular nucleus of PRKO mice could be due to the loss of inhibition by progesterone in adulthood or by the absence of PR during development, a difference which the methods used in this study were not designed to resolve.

We also expected to see greater vasopressin-ir fiber density in the lateral septum of PRKO mice because progesterone treatment reduces the number of vasopressin-ir cells in the BNST and the density of vasopressin-ir fibers in the lateral septum and lateral habenular nucleus in rats (Auger and Vanzo, 2006). The lack of differences between PRKO and WT mice in these areas, as compared to rats, may be due to species differences in progesterone function. Alternatively, we might have seen a difference between genotypes, if we had treated animals with supplemental progesterone. However, differences observed due to administration of progesterone would be related to the activational effects of progesterone rather than the developmental impact of PRs.

Sex differences in AVP cell number and vasopressin mRNA expression

In the current study, male mice had more cells expressing vasopressin mRNA in the BNST and MeA than females, similar to what has been reported for rats (De Vries et al., 1994; Wang et al., 1995), suggesting that in mice as in rats sex differences in vasopressin-ir fiber density in BNST and MeA projection sites are caused primarily by a sex difference in vasopressin cell number. We found, however, that females expressed significantly more vasopressin mRNA per cell than males in the BNST, but levels did not differ in the MeA. The higher levels of vasopressin mRNA cannot be explained by differences in testosterone levels, because if anything, WT females had higher levels of testosterone than did WT males. The higher levels in females were surprising on two accounts. First, cells in the BNST and MeA are otherwise remarkably similar in morphology and neurochemistry (Alheid et al., 1995). Secondly, in rats, BNST cells express more vasopressin mRNA in males than in females (De Vries et al., 1994; Wang et al., 1995). Why this sex difference is reversed in the BNST of rats and mice is unclear. It suggests, however, that sexual differentiation of vasopressin expression differs between mice and rats.

Sex differences in vasopressin fiber density

Vasopressin-ir fibers are found in discrete locations throughout the rodent brain and are derived from a small number of distinct nuclei: BNST, MeA, suprachiasmatic nucleus, paraventricular nucleus, and supraoptic nucleus (DeVries et al., 1983, 1985; Van Leeuwen et al., 1985; Castel and Morris, 1988). Many of the vasopressin-ir fibers and terminals observed in the brains of rats and mice are steroid-responsive (*i.e.*, they disappear following gonadectomy; DeVries et al., 1985; Mayes et al., 1988) and are sexually dimorphic with males having a greater density of fibers and terminals than females (De Vries and Al-Shamma, 1990). Such projections are thought to originate in the BNST and MeA because vasopressin expression is steroid-dependent and sexually dimorphic in these areas (Van Leeuwen et al., 1985; Wang et al., 1995). In contrast, the lack of steroid responsiveness of fibers in other areas suggests a different point of origin.

Our results confirm the finding that male mice have a greater vasopressin-ir fiber density than females in the lateral septum, lateral habenular nucleus, and MeA (Mayes et al., 1988; De Vries et al., 2002; Bakker et al., 2006). We also report for the first time a marked sex difference in the mediodorsal thalamus. Vasopressin-ir fibers in all these areas disappear after castration in rats (DeVries et al., 1985), suggesting that the BNST or MeA are the most likely sources of innervation (DeVries et al., 1985; De Vries and Al-Shamma, 1990). However, despite circumstantial evidence as to the origins of vasopressin fibers, only the vasopressin projections from the BNST to the lateral septum and from the MeA to the ventral hippocampus have been adequately confirmed through lesion and tract-tracing analysis (De Vries and Buijs, 1983; Caffe et al., 1987).

We did not observe a sex difference in vasopressin-ir fiber density in the dorsomedial hypothalamus, as predicted. Similarly, vasopressin innervation in the dorsomedial hypothalamus is not steroid-responsive or sexually dimorphic in rats (DeVries et al., 1985; De Vries and Al-Shamma, 1990). Retrograde tracer injections into the dorsomedial hypothalamus in rats (Watts and Swanson, 1987) and lesions of the suprachiasmatic nucleus

in rats and hamsters (Hoorneman and Buijs, 1982; Delville et al., 1998) indicate that this area receives vasopressin input from the suprachiasmatic nucleus. In general, current evidence suggests that brain areas with sexually dimorphic or steroid-dependent vasopressin-ir fibers receive that vasopressin innervation from the BNST or MeA, whereas the lack of steroid-responsiveness or sexual dimorphism of vasopressin-ir fibers in other brain areas suggests a different point of origin (*e.g.*, the suprachiasmatic nucleus; De Vries and Panzica, 2006). However, our results suggest that exceptions to this rule may exist.

Vasopressin-ir fiber density was sexually dimorphic in the principal nucleus of the BNST, which probably receives vasopressin input from the SCN, as lesions of the suprachiasmatic nucleus eliminate vasopressin innervation of the BNST in golden hamsters (Delville et al., 1998). One other study in mice reported a lack of sex differences in the BNST (Bakker et al., 2006); however, it is unclear if the area examined in that study corresponds to the area examined in the current study. It is possible that in mice the principal nucleus of the BNST receives vasopressin innervation from multiple vasopressin-producing areas. In rats, vasopressin fiber density of discrete areas in the principal nucleus of the BNST is responsive to steroids (DeVries et al., 1985) suggesting that steroid-sensitive vasopressin cells in the BNST contribute to the vasopressin innervation of the principal nucleus. The principal nucleus may also receive vasopressin input from the MeA because the BNST has reciprocal projections with the MeA, (Canteras et al., 1995; Cooke and Simerly, 2005). Receiving vasopressin innervation from the suprachiasmatic nucleus as well as the MeA and BNST could explain why the sex difference in vasopressin innervation of the principal nucleus of the BNST was less pronounced compared to the lateral septum, where innervation seems to be derived almost exclusively from the BNST and MeA.

The medial preoptic nucleus is also thought to receive vasopressin innervation from the suprachiasmatic nucleus (Castel and Morris, 1988; Crenshaw et al., 1992; Abrahamson and Moore, 2001). In support, in golden hamsters, lesions of the suprachiasmatic nucleus eliminate nearly all of the vasopressin-ir fiber staining in the medial preoptic nucleus (Delville et al., 1998). In accordance, the vasopressin-ir fibers in the medial preoptic nucleus of the mouse brain are not steroid-sensitive (Plumari et al., 2002), suggesting that contributions of the BNST and MeA to vasopressin innervation in this area are insignificant. Nevertheless, the present study showed a significant sex difference with males having nearly twice as much vasopressin-ir staining as females. While sex differences in vasopressin-ir fiber density have not been examined specifically in the medial preoptic nucleus of the rat, a sex difference has been described in the medial preoptic nucleus of gerbils (Crenshaw et al., 1992). In these animals, the medial sexually dimorphic area of the medial preoptic nucleus displays a sexually dimorphic, but not steroid-sensitive, vasopressin innervation, suggesting that in gerbils this area receives its vasopressin input from a source other than the BNST and MeA (Crenshaw et al., 1992).

In conclusion, although the present study did not find evidence for a role of PRs in sexual differentiation of vasopressin expression, it demonstrated that PR influences a subset of vasopressin fibers that are presumably derived from the BNST and MeA. All these fibers showed marked sex differences with males having more fibers than females. Such global differences were not found in fibers presumably derived from the SCN. Subsets of these

fibers, however, did differ between males and females, most notably in the MPOA. The differences in the extent of sexual dimorphism between presumed vasopressin-ir projections from the BNST and MeA on the one hand and the SCN on the other, suggest different mechanisms of sexual differentiation. The global differences found in BNST and MeA projections suggest that during development steroids may target the cells of origin. In the case of the SCN, however, sex differences in the targets, which have been reported for the BNST as well as the MPOA in mice (e.g., Forger et al., 2004; Shah et al., 2004) may impose sex differences on a subset of projections. As the evidence for many of the SCN projections and most of the BNST and MeA projections is mainly circumstantial (De Vries and Panzica, 2006), it is imperative to do a thorough analysis of vasopressin anatomy before the the sexual differentiation of vasopressin innervation of the brain can be fully understood.

Acknowledgments

We would like to thank Dr. John Lydon for providing the breeding pairs that generated mice used in these experiments, Dr. Dena Jacob for her assistance with manuscript editing, and Lynn Bengston for her assistance with the formatting of figures. The research was funded by KO2 MH01497, RO1 MH47538, and T32 MH47538 to GJD, NS 19327 and MH 067630 to JDB, and NICHD (SCCPRR) U54-HD28934 to University of Virginia CRRLAAC.

Abbreviations

3V	third ventricle
ac	anterior commissure
BNST	bed nucleus of the stria terminalis
cc	corpus callosum
LV	lateral ventricle
MeA	medial amygdaloid nucleus
PVT	paraventricular thalamus
opt	optic tract
sm	stria medullaris
-ir	immunoreactive
PR	progestin receptor
PRKO	progestin receptor knockout
WT	wild type

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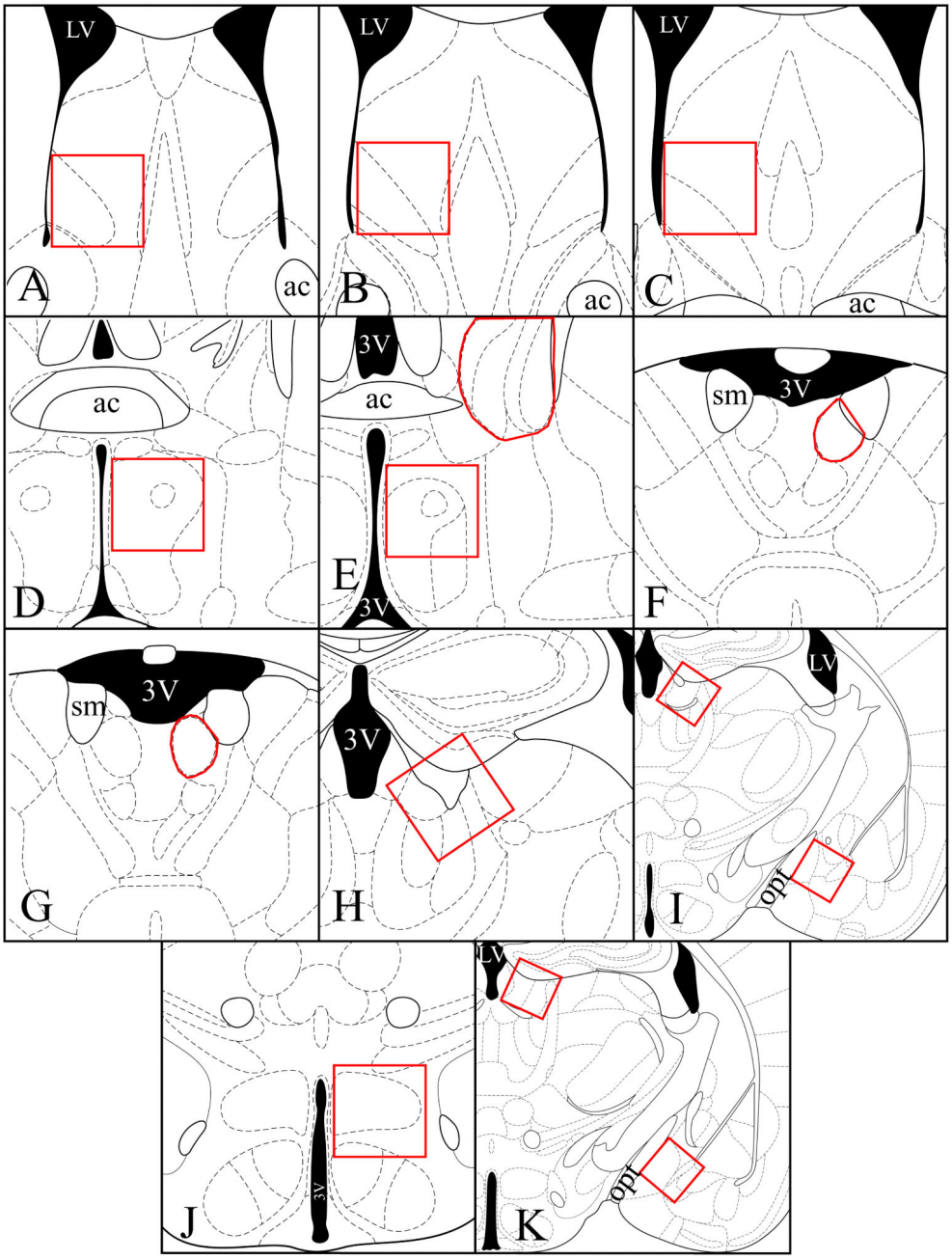

Figure 1.

Brain maps depicting the areas analyzed for vasopressin-ir fiber density were adapted from Paxinos and Franklin (2001). The area analyzed is shown in red. A) rostral lateral septum, B) medial lateral septum, C) caudal lateral septum, D) rostral medial preoptic area, E) caudal medial preoptic area (box) and principal nucleus of the BNST (traced region), F) rostral mediodorsal thalamus, G) caudal mediodorsal thalamus, H) rostral lateral habenular nucleus, I) medial lateral habenular nucleus (upper box) and rostral MeA (lower box), J) dorsomedial hypothalamus, and K) caudal lateral habenular nucleus (upper box) and caudal MeA (lower

box). 3V = third ventricle, ac = anterior commisure, LV = lateral ventricle, opt = optic tract, sm = stria medularis.

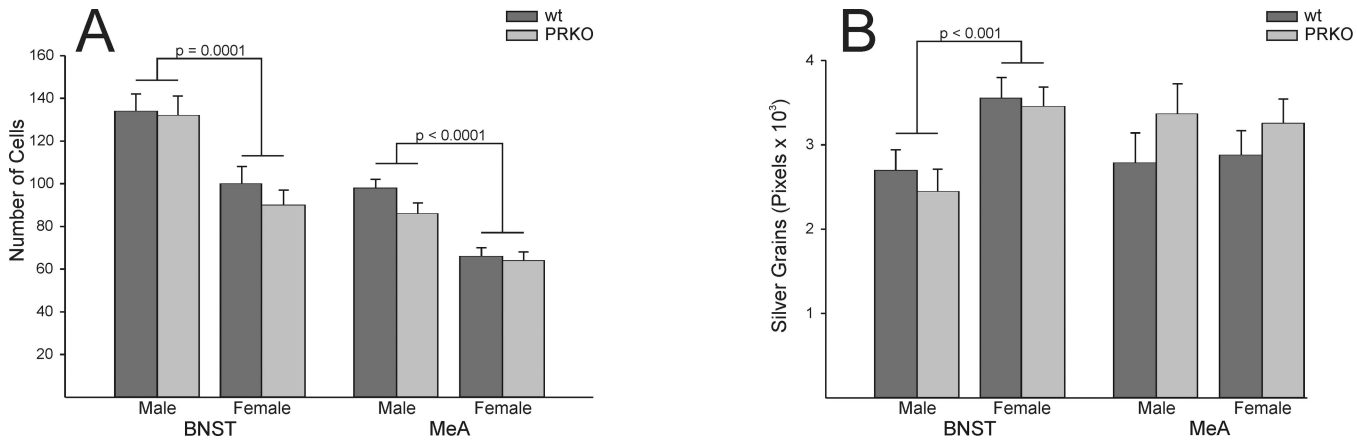

Figure 2.

The number of vasopressin mRNA-expressing cells and silver grain density, a measure of mRNA production, in the BNST and MeA of male and female WT and PRKO mice. A) Male mice had more vasopressin expressing cells than females in the BNST and MeA, but WT mice did not differ from PRKO mice. B) Female mice had greater silver grain density in the BNST than males. There were no sex differences in the MeA or genotype differences in either area.

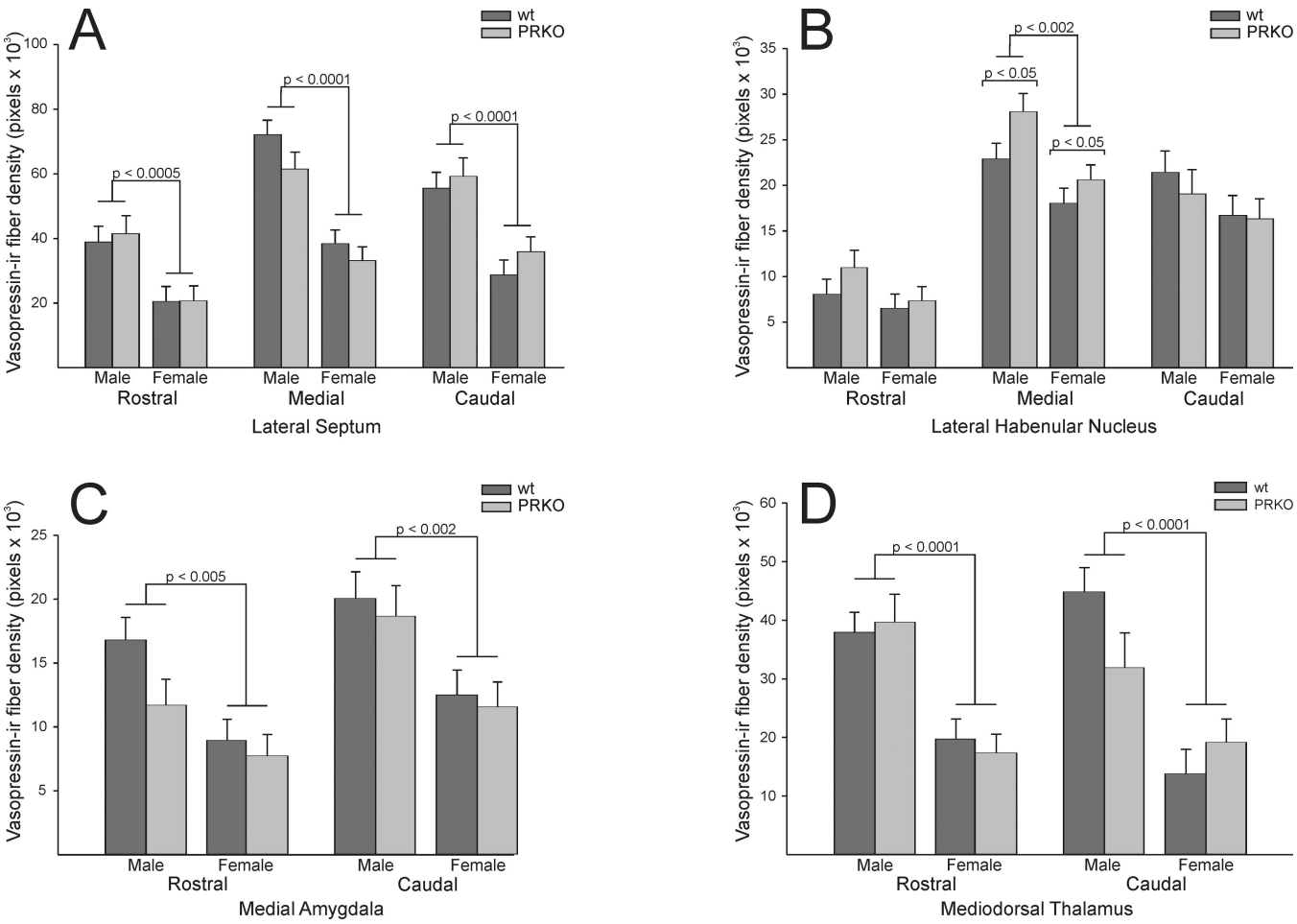

Figure 3.

Vasopressin-ir fiber density in A) lateral septum, B) lateral habenular nucleus, C) MeA, and D) mediodorsal thalamus of male and female WT and PRKO mice. Sex differences were observed in the lateral septum, lateral habenular nucleus, MeA, and mediodorsal thalamus. In addition, PRKO mice had greater vasopressin-ir fiber density than WT mice in the lateral habenular nucleus. There were no effects of genotype in any other area, and there were no sex by genotype effects in any area.

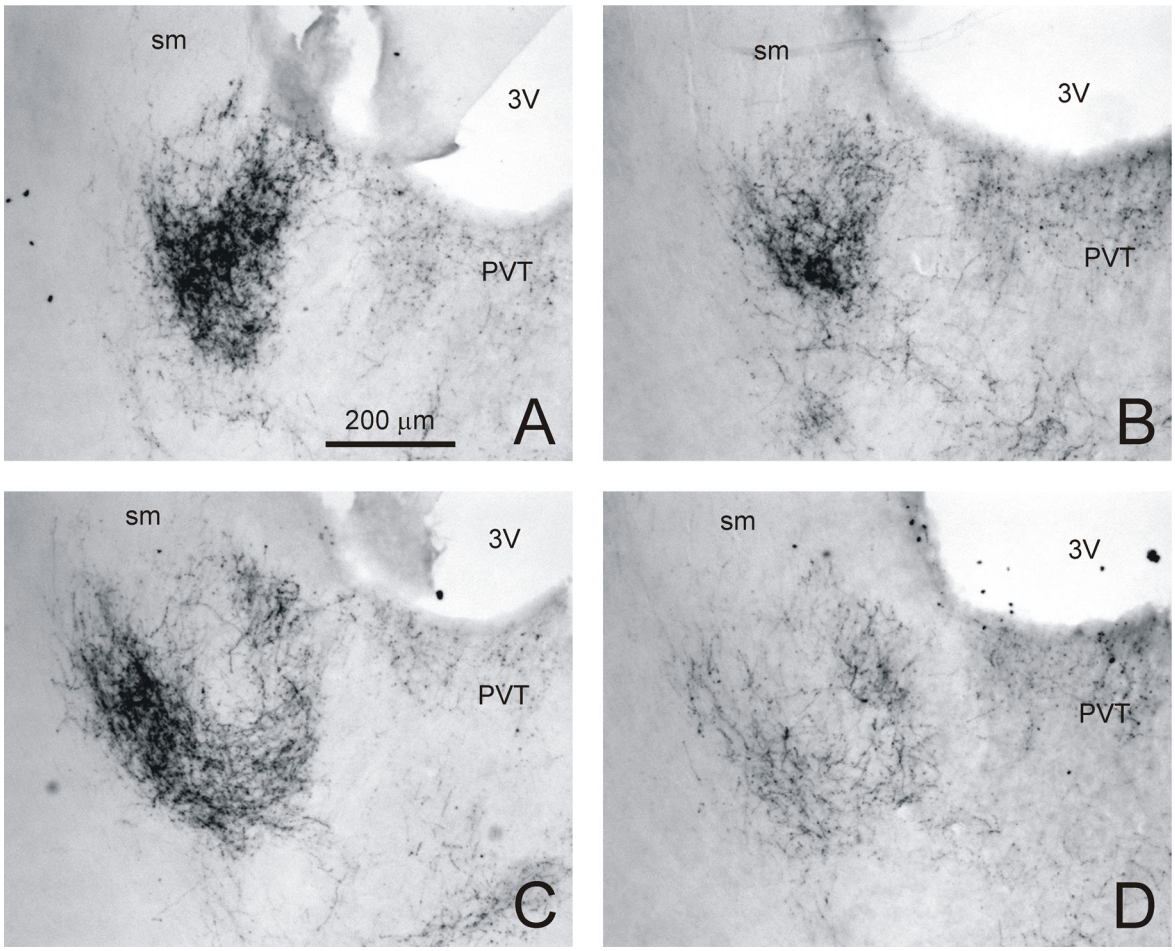

Figure 4.

Photomicrographs of vasopressin immunoreactivity in the mediodorsal thalamus. A) male, rostral mediodorsal thalamus; B) female, rostral mediodorsal thalamus; C) male, caudal mediodorsal thalamus; and D) female, caudal mediodorsal thalamus. Images were adjusted in Adobe Photoshop (*i.e.*, −15 brightness and +50 contrast). 3V = third ventricle, PVT = paraventricular thalamus, sm = stria medularis.

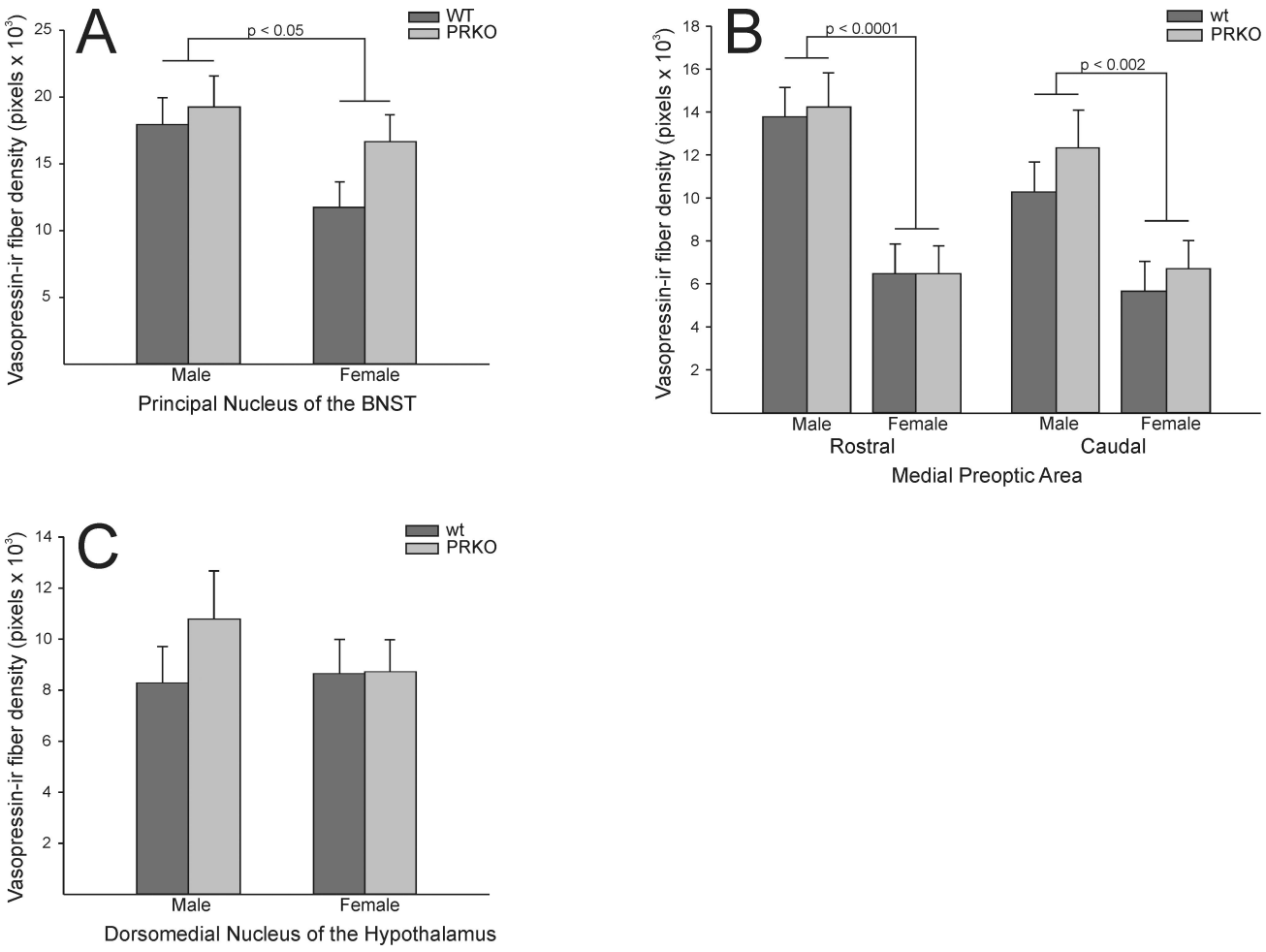

Figure 5.

Vasopressin-ir fiber density in A) principal nucleus of the BNST, B) rostral and caudal medial preoptic nucleus, and C) dorsomedial hypothalamus of male and female WT and PRKO mice. Sex differences were observed in the principal nucleus of the BNST and the rostral and caudal medial preoptic nucleus, but not the dorsomedial hypothalamus. There were no differences between WT and PRKO mice in any of these areas, and there were no sex by genotype interactions.

Table 1

Mean ± SEM serum testosterone (ng / dl) and progesterone (ng / ml) levels are shown. There were no significant sex (*i.e.*, male vs. female) or genotype (*i.e.*, WT vs. PRKO) differences. However, there was a significant sex by genotype interaction in testosterone results ($p < 0.05$; letters indicate group differences). A Fisher's LSD test indicated that PRKO males had significantly less testosterone than WT males, and females did not differ from either group.

	WT male	PRKO male	WT female	PRKO female
Testosterone	192 ± 17^{a}	127 ± 20^{b}	163 ± 19^{ab}	175 ± 17^{ab}
Progesterone	5.0 ± 1.8	5.9 ± 1.8	1.7 ± 1.6	4.8 ± 1.6