Sex and Species Differences in Tyrosine Hydroxylase-Synthesizing Cells of the Rodent Olfactory Extended Amygdala

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ABSTRACT

The bed nucleus of the stria terminalis (BST) and the medial amygdala (MeA) are anatomically connected sites necessary for chemosensory regulation of social behaviors in rodents. Prairie voles (*Microtus ochrogaster*) are a valuable model for studying the neural regulation of social behaviors because, unlike many other rodents, they are gregarious, pair bond after copulating, and are biparental. We herein describe sex and species differences in immunoreactivity for tyrosine hydroxylase (TH), the rate-limiting enzyme for catecholamine synthesis, in the BST and MeA. Virgin male prairie voles had a large number of TH-immunoreactive cells in areas analogous to the rat principal nucleus of the BST (pBST) and the posterodorsal medial amygdala (MeAPd). Virgin female prairie voles had far fewer TH-immunoreactive cells in these sites (~17% of the number of cells as males in the pBST, ~35% of the number of cells in the MeAPd). A few TH-immunoreactive cells were found in the BST of male and female hamsters and meadow voles, but not in rats. The MeApd also contained a few TH-immunoreactive cells in male and female hamsters and male meadow voles, but not rats. Castration greatly reduced the number of TH-immunoreactive cells in the male prairie vole pBST and MeAPd, an effect that could be reversed with testosterone. Furthermore, treating ovariectomized females with testosterone substantially increased TH-immunoreactive cells in both sites. Therefore, a species-specific sex difference in TH expression is found in a chemosensory pathway in prairie voles. Expression of TH in these sites is influenced by circulating gonadal hormones in adults, which may be related to changes in their display of social behaviors across the reproductive cycle. J. Comp. Neurol. 500:103–115, 2007. © 2006 Wiley-Liss, Inc.

Indexing terms: chemosensory; dopamine; monogamy; paternal behavior; social behavior; voles

Neurons expressing tyrosine hydroxylase (TH), the rate-limiting enzyme for catecholamine synthesis, are widespread throughout the vertebrate brain. In most species, cells producing epinephrine or norepinephrine are invariably found in the brainstem, whereas cells producing dopamine are located in both the brainstem and the forebrain (Smeets and Gonzalez, 2000). The specific distribution of these catecholaminergic cells, however, differs between vertebrates (Tillet and Kitahama, 1998; Smeets and Gonzalez, 2000). In fact, differences are even found within the order Rodentia. For example, the anterior and posterior medial amygdala (MeA) and posteromedial bed nucleus of the stria terminalis (BSTpm) of Syrian hamsters (*Mesocricetus auratus*) contain catecholaminergic cells not found in rats, mice, or Siberian hamsters (Asmus

et al., 1992; Asmus and Newman, 1993; Shi and Bartness, 2000; Wommack and Delville, 2002). Furthermore, species-specific TH-immunoreactive cells are found in the Syrian hamster diagonal band of Broca, lateral preoptic area, and cortex (Vincent, 1988). There also may be dif-

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ferences between laboratory mice and rats in the distribution and density of TH-expressing cells in the preoptic area of the hypothalamus (Ruggiero et al., 1984).

Not only are there differences between rodent species, but sex differences in forebrain TH expression can be found within a species. TH-expressing cells of the anteroventral preoptic area (AVPV), necessary for gonadotropin release and ovulation (Weigand and Terasawa, 1982), are two to four times more numerous in gonadally intact female rats and mice than in intact males (Simerly et al., 1985a,b, 1997; Simerly, 1989; Zup et al., 2003). A sex difference in TH immunoreactivity in the AVPV also exists in prairie voles (Microtus ochrogaster), although subjects must be gonadectomized for this sex difference to be revealed (Lansing and Lonstein, 2006).

Other areas of the rat, mouse, and prairie vole forebrain have not been reported to be sexually dimorphic in the number of TH-immunoreactive cells (Simerly et al., 1985a,b; Simerly, 1989; Lansing and Lonstein, 2006). During our previous examination of sex differences in the number of TH-immunoreactive cells in the hypothalamus of prairie voles (Lansing and Lonstein, 2006), however, we noticed (but did not report) two unexpected populations of TH-immunoreactive cells that appeared sexually dimorphic. One population was found in an area analogous to the principle bed nucleus of the stria terminalis (pBST) of the rat or the posteromedial bed nucleus of the stria terminalis (BSTpm) of the hamster (Swanson, 1998; Wood and Swann, 2005). The other population of TH-immunoreactive cells was found in the area analogous to the posterodorsal medial amygdala (MeAPd) of both rats and hamsters. In both cases, we noticed a large number of TH-immunoreactive cells in males, but relatively few in females.

The pBST/BSTpm and MeAPd have dense, reciprocal connections in male rats and hamsters (Canteras et al., 1995; Coolen and Wood, 1998; Wood and Swann, 2005) and are involved in chemosensory processing necessary for their sociosexual behaviors (for reviews see Wood, 1998; Newman, 1999; Hull et al., 2002). The presence of TH-immunoreactive cells in the pBST/BSTpm and MeAPd of male, but not female, prairie voles is intriguing given the complex and unique social structure of this species (for review see Carter et al., 1995). Unlike many male rodents, male prairie voles form life-long pair bonds with their mates and later display high levels of parental care toward their offspring. Pair bonding and paternal behavior in male prairie voles require processing of olfactory inputs, insofar as either olfactory bulbectomy or lesions encompassing the MeAPd disrupt these behaviors (Kirkpatrick et al., 1994a,b). Furthermore, impeding dopaminergic neurotransmission also impairs these social behaviors in male prairie voles (Lonstein, 2002; Wang and Aragona, 2004).

We herein describe populations of TH-immunoreactive cells in the pBST/BSTpm and MeAPd of male prairie voles and examine the effects of gonadal hormones on the TH-immunoreactive cells in these sites. It may be that these relatively unique populations of catecholaminergic cells in the male prairie vole brain are involved in the chemosensory control of their pair bonding, parental, or other social behaviors.

MATERIALS AND METHODS

Subjects

Male and female prairie voles (Microtus ochrogaster) were born and raised in our colony, which was established in 2002 at Michigan State University, from breeding stock that originated from offspring of voles captured in 1994 in Urbana, Illinois, provided by Dr. Betty McGuire (Smith College, Northampton, MA) and Dr. Zuoxin Wang (Florida State University, Tallahassee, FL). These voles were outbred in 2000 at the University of Massachusetts with voles of Illinois origin provided by Dr. C. Sue Carter (University of Illinois at Chicago) and brought to Michigan State University in 2002. Animals were mated by socially isolating adult virgin female and male prairie voles for 4 days, after which females were placed in the cage of an unfamiliar male. Animals were maintained on a 14:10-hour light: dark cycle with an ambient temperature maintained at ~21°C. At all ages, animals were housed in clear plastic cages (48 × 28 × 16 cm) containing wood chips, wood shavings, and a substantial hay covering. Water and a food mixture containing cracked corn, whole oats, sunflower seeds, and rabbit chow (Tekland rodent diet No. 2031) in a ratio of 1:1:2:2 were freely available. Pups were weaned from their parents at 20 days of age, placed in same-sex sibling groups of two or three animals/cage between 50 and 60 days of age, and housed in these groups until they were killed at least 6 weeks later. The comparative study used eight voles of each sex, whereas the hormone manipulation study (see below) used 24 voles of each sex.

The eight male and five female meadow voles were born in the breeding colony of the Wang laboratory. Voles were weaned at 21 days of age and placed in same-sex sibling pairs in clear plastic cages (29 × 18 × 13 cm) containing cedar chip bedding until the time of death during adulthood. Food (Purina rabbit chow No. 5326), sunflower seeds, and water were provided ad libitum. All cages were maintained in a 14:10-hour light:dark cycle while the ambient temperature was maintained at about 21°C.

Six male and six female Long-Evans rats (Rattus norvegicus) were purchased from Harlan Laboratories (Indianapolis, IN) and sent to our laboratory when approximately 75 days of age. Rats were housed in same-sex groups of two or three animals per cage in clear polypropylene cages (48 × 28 × 16 cm) with wood shavings for bedding. Food (Tekland rodent diet No. 8640) and water were continuously available, lights were on a 12:12-hour light:dark cycle with onset at 0800 hours daily, and the ambient temperature was maintained at ~21°C. Rats were maintained in our laboratory for at least 3 weeks before death.

Seven male and seven female Syrian hamsters (Mesocricetus auratus) were also purchased from Harlan Laboratories and sent to our laboratory when approximately 75 days of age. Hamsters were singly housed in clear polycarbonate cages (12 × 4 × 8 in.) with food (Tekland rodent diet No. 8640) and water freely available. Animals were exposed to a 14:10-hour light:dark cycle, and the ambient temperature was maintained at ~21°C. Hamsters were maintained in our laboratory for at least 2 weeks prior to death.

Gonadectomy and hormone replacement

To determine the effects of circulating gonadal hormones on TH expression in prairie voles, voles of both sexes were weighed and anesthetized with an IP injection of an anesthetic cocktail containing ketamine (62.5 mg/kg), xylazine (7.5 mg/kg), and acepromazine (0.8 mg/kg). Males were either castrated or received a sham surgery in which an incision was made in the scrotum, but the testes were not removed. Similarly, female prairie voles were anesthetized and either were ovariectomized through a single midline ventral incision or received a sham surgery in which the ovaries were visualized but not removed. Half of the castrated males and ovariectomized females were subcutaneously implanted with a 2.5-cm-long Silastic capsule containing crystalline testosterone (Sigma, St. Louis, MO). This capsule provides supraphysiological levels of testosterone that maintain masculine behavior and neurochemistry in male prairie voles (e.g., Wang and DeVries, 1993; Lonstein et al., 2005). The other half of the castrated males and ovariectomized females, and the sham surgery controls of both sexes, received an empty Silastic capsule. Four weeks after surgery, subjects were killed and their brains collected as described below. Each of the six groups of prairie voles contained eight animals. All procedures were in accordance with the Institutional Animal Care and Use Committees at Michigan State University and Florida State University.

Perfusion, tissue collection, and immunocytochemistry

All voles, rats, and hamsters were weighed, overdosed with sodium pentobarbital, and perfused through the heart with 0.9% saline (100 ml for voles, 150 ml for hamsters and rats), followed by 4% paraformaldehyde in sodium phosphate buffer (NaPB; pH 7.6, 100 ml for voles, 150 ml for hamsters and rats). Brains were removed, postfixed for 1–4 hours (species comparison) or overnight (sex and hormonal condition comparison) in 4% paraformaldehyde in NaPB, and then submerged in a 20% sucrose/NaPB solution for at least 3 days before sectioning into 40-μm coronal sections with a freezing microtome. Immunocytochemistry for TH was performed on every other section through the brain for the prairie voles, meadow voles, and hamsters and on every third section through the rat brains. Immunocytochemical procedures were identical to a procedure described previously (Lansing and Lonstein, 2006). Briefly, sections were rinsed three times for 5 minutes each in Trisma-buffered saline (TBS; pH 7.6), incubated in 0.1% sodium borohydride for 15 min, rinsed three times in TBS, incubated in 0.3% Triton X-100 and 1% hydrogen peroxide in TBS for 10 min, rinsed three times in TBS, blocked with 20% normal goat serum in 0.3% Triton X in TBS for 15 min, and then incubated with a rabbit antityrosine hydroxylase polyclonal primary antiserum (AB152; 1:2,000; Chemicon, Temecula, CA) in 0.3% Triton X and 2% NGS in TBS at room temperature for approximately 18 hours. According to the manufacturer, the immunogen for this primary antibody is denatured TH from rat pheochromocytoma and has been shown by Western blot analysis not to recognize other monoaminergic synthetic enzymes. Indeed, we found labeling for all four species in the sites previously described to express TH in rodents, including the periventricular hypothalamus, zona incerta, substantia nigra,

and ventral tegmental area. Furthermore, in rats and hamsters, we did not see labeling in any site not previously described to express TH in these species. Sections were then rinsed three times in TBS, incubated in a biotinylated goat anti-rabbit secondary antibody (1:500; Vector Laboratories, Burlingame, CA) in 0.3% Triton-Y and 2% NGS, rinsed three times in TBS for 5 minutes each, and incubated with avidin-biotin complex (Vectastain Elite, Vector Laboratories) for 60 minutes. After rinsing three times with TBS, visualization of TH immunoreactivity occurred with the use of Vector SG chromagen (Vector Laboratories) according to the manufacturer's instructions, which provided a light blue cytoplasmic label. Sections were mounted on microscope slides, dehydrated, and coverslipped. Immunocyotchemical control procedures included omission of the primary or secondary antisera, which abolished any specific labeling. A single immunocytochemical run including the tissue from all four species was used for the species comparison, and a separate immunocytochemical run was used for the gonadectomy and hormone-replacement study in prairie voles.

To determine whether TH-immunoreactive cells in the pBST and MeAPd of prairie voles might be dopaminergic or noradrenergic, we examined whether cells in these sites expressed dopamine-β-hydroxylase (DBH), the enzyme that converts dopamine into norepinephrine. An additional series of brain sections that included the forebrain and brainstem of two gonadally intact male and two intact female prairie voles was processed immunocytochemically as described above but incubated for 3 days at 4°C in a rabbit polyclonal anti-DBH primary antiserum (AB1585; 1:500; Chemicon). According to the manufacturer, the immunogen for this primary antibody is DBH obtained from bovine adrenal glands and has been shown by Western blot analysis to be specific for DBH. Indeed, we found many DBH-immunoreactive cells in the locus coeruleus of both the male and the female prairie vole brainstem, but none were found in pBST or MeApd, or in the hypothalamus and zona incerta on these same sections. Omission of the primary or secondary antiserum abolished all specific labeling.

Analyses

To compare the presence of TH-immunoreactivity in the BST and MeA of prairie voles, meadow voles, hamsters, and rats of both sexes, the entire rostrocaudal extents of the BST and MeA were examined for each subject. The presence or absence of TH-immunoreactive cells at any rostrocaudal level within these areas was noted.

Slides for the gonadectomy and hormone-replacement study in prairie voles were masked and coded for analysis, which was performed by only one observer (K.V.N.). The three areas examined were analyzed bilaterally from consecutive sections in a one-in-two series, with the number of TH-immunoreactive cells totaled from these sections. The number of cells containing detectable TH immunoreactivity was counted by eye with the use of a Nikon E400 microscope at ×200 magnification with the aid of a reticle placed in one of the ocular lenses. The area of the BST analyzed included the area of the prairie vole brain that had the densest cluster of TH-immunoreactive cells and corresponded to the dorsal principal BST of the rat or the dorsal posteromedial BST of the hamster. The total number of TH-immunoreactive cells was quantified from four consecutive sections in a one-in-two series through the

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middle and caudal pBST, roughly corresponding to plates 20–22 of Swanson's (1998) atlas of the rat brain. Males rarely had TH-immunoreactive cells in the dorsal pBST rostral or caudal to these four sections. The area of the MeApd analyzed began with the section roughly corresponding to plate 28 of Swanson's rat atlas, where TH-immunoreactive cells first appeared for most subjects, and continued for six consecutive sections in a one-in-two series through the MeApd, ending approximately at the level corresponding to Swanson's atlas plate 30. Subjects reliably had six MeApd sections represented, but not necessarily more, because the amygdala was often lost in the most caudal sections. In addition, we also noticed a small cluster of TH-immunoreactive cells in the dorsal reticular nucleus of the thalamus, at the level roughly corresponding to Swanson's atlas plate 27. These cells are also found in the hamster brain (Asmus et al., 1992). These thalamic cells were found only in two sections of the one-in-two series for each subject and did not appear to differ between the sexes, so this was used as a control site not likely to be affected by the hormone manipulations. Average soma size of TH-immunoreactive cells in the pBST and MeApd was evaluated for each subject by nonsystematically choosing for each subject one hemisection through dorsal pBST and MeApd and then tracing the perimeter of every TH-immunoreactive soma under ×200 magnification with the use of an image analysis system (Image Pro Plus; Media Cybernetics). Stereology was not used to analyze these TH-immunoreactive cells for numerous reasons. First, these TH-immunoreactive populations were found on only a small number of sections through each brain region. Second, we were able to analyze every other section through these sites. Third, the TH-immunoreactive cells were not very densely packed, and, fourth, the total number of TH-immunoreactive cells per section was relatively small and easily quantified. Cell counts were subjected to an Abercrombie correction factor of 0.83 based on our finding of a z-plane average soma size of ~8 μm and are presented as corrected counts. Photomicrographs were arranged with Adobe Photoshop 6.0, with the Dodge tool used to reduce any inconsistencies in illumination within a given panel, and the brightness and contrast were adjusted to maintain consistency across panels.

Data from the sex and hormone treatment comparison in prairie voles were analyzed for each site with a 2 (sex) × 3 (treatment) ANOVA, followed by Fisher LSD post-hoc tests on main effects. Separate Fisher LSD post-hoc tests were then used as planned comparisons to compare treatment groups within each sex. Because a single hemisection was analyzed for somal area, the number of subjects included in this analysis was greater for some groups than the analysis of the total number of TH-immunoreactive cells, which required many intact sections for us to include a subject in the analysis. Statistical significance was indicated at $P = 0.05$.

RESULTS

Species comparison

All male prairie voles had a distinct cluster of intensely TH-immunoreactive cells in the dorsal pBST, with the majority of cells appearing at the rostrocaudal level where the third ventricle appears both dorsal and ventral to the anterior commissure. Many fewer cells were found in sec-
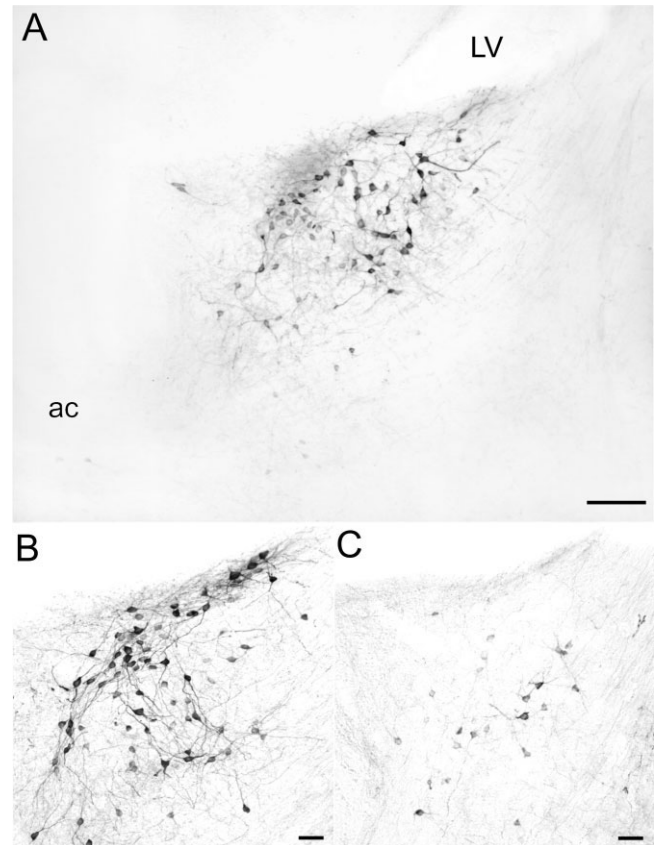

Fig. 1. Photomicrographs of TH-immunoreactive cells in the pBST of representative prairie voles. **A:** Male prairie vole at ×100 magnification. **B:** Male at ×200 magnification. **C:** Female at ×200 magnification. LV, lateral ventricle; ac, anterior commissure. Scale bars = 100 μm in A; 50 μm in B,C.

tions of the male dorsal pBST anterior or posterior to this dense cluster, although some TH-immunoreactive cells were found adjacent to the stria medullaris and fornix in more caudal sections, as the pBST extended ventromedially. In contrast to males, female prairie voles had many fewer TH-immunoreactive cells in the dorsal pBST (Fig. 1), and the intensity of immunoreactive labeling in most of these cells was relatively weak. Some females also had a few weakly immunoreactive cells in the ventromedial pBST more caudally. Small numbers (one to eight cells) of very weakly TH-immunoreactive cells were also found in each section of the dorsal pBST of most male and female meadow voles, and one to four moderately immunoreactive cells per section were found in the dorsal pBST of male and female hamsters (Fig. 2). No TH-immunoreactive cells were found in the dorsal pBST of any male or female rat (Fig. 2). Although relatively few or no TH-immunoreactive cells were found in the dorsal pBST of female prairie voles or meadow voles, hamsters, and rats of both sexes, the periventricular hypothalamus on these same sections contained a large number of intensely TH-immunoreactive cells, as previously described (e.g., Chan-Paley et al., 1984; Vincent, 1988; Lansing and Lonstein, 2006).

All male prairie voles showed a large number of highly TH-immunoreactive cells throughout the entire rostrocau-

Fig. 2. Photomicrographs ($\times 200$ magnification) of TH-immunoreactive cells in the dorsal pBST of representative male meadow voles, hamsters, and rats. Males of these species were similar to females. Scale bars = 50 μm.

dal extent of the MeApd, and some males also had a few TH-immunoreactive cells in the posteroventral MeA (MeApv; Fig. 3). The MeApd of all female prairie voles also contained TH-immunoreactive cells, but there were far fewer compared with males, and many of these cells were only weakly immunoreactive (Fig. 3). Some (one to 12 cells/section) very weakly TH-immunoreactive cells were also found in the MeApd of most male and female meadow voles. The MeApd of all hamsters contained a smaller

number (one to four cells/section) of moderately TH-immunoreactive cells, with no obvious difference between the sexes in the number of cells. No TH-immunoreactive cells were found in the MeApd of male or female rats (Fig. 4).

Sex difference and effects of gonadal hormones on TH immunoreactivity in prairie voles

In the dorsal pBST, there were significant main effects of sex [$F_{(1,33)} = 11.63$, $P < 0.002$] and treatment [$F_{(2,33)} = 18.79$, $P < 0.0001$] on the number of TH-immunoreactive cells. The interaction between sex and treatment was not significant [$F_{(2,33)} = 1.69$, $P > 0.2$]. Post-hoc analysis on the main effects revealed that males had significantly more TH-immunoreactive cells than females and that testosterone-treated animals had significantly more TH-immunoreactive cells than gonadally intact and gonadectomized animals, which did not significantly differ from each other (Figs. 5, 6). Planned comparisons within each sex revealed that castration of males significantly reduced the number of TH-immunoreactive cells, whereas testosterone treatment maintained the number of cells in castrated males at a level similar to that of sham males. Ovariectomy had no significant effect on the already low number of TH-immunoreactive cells in the dorsal pBST of female prairie voles, whereas treating ovariectomized females with testosterone significantly increased the number of cells compared with the other two groups of females (Fig. 5). The average cross-sectional area of these TH-immunoreactive somata did not differ between the sexes [$F_{(1,39} = 0.35$, $P > 0.8$] or by treatment [$F_{(2,39)} = 2.05$, $P > 0.1$], and there was no significant interaction between these factors [$F_{(2,39)} = 2.40$, $P > 0.1$; group averages $= 109 \pm 4$ to 135 ± 5 μm^2].

In the MeApd, there were significant main effects of sex [$F_{(1,35)} = 25.35$, $P < 0.0001$] and treatment [$F_{(2,35)} = 22.47$, $P < 0.0001$], but no significant interaction between them [$F_{(2,35)} = 2.17$, $P > 0.12$]. Post-hoc analysis on the main effects revealed that the number of TH-immunoreactive cells was greater in males than females and that all three hormone treatments significantly differed from each other, with the number of TH-immunoreactive cells lowest in gonadectomized animals, intermediate in intact animals, and highest in testosterone-treated animals (Figs. 7, 8). Similar to the case in the dorsal pBST, planned comparisons of the treatment groups within each sex revealed that castration of males significantly reduced TH-immunoreactive cells in the MeApd, whereas testosterone treatment maintained a high number of these cells. Ovariectomy had no significant effect on the number of TH-immunoreactive cells in the the MeApd of females, whereas treating ovariectomized females with testosterone significantly increased it (Fig. 7). The average cross-sectional area of the TH-immunoreactive somata did not differ between the sexes [$F_{(1,37)} = 0.22$, $P > 0.64$] or by treatment [$F_{(2,37)} = 0.42$, $P > 0.65$], and there was no significant interaction between these factors [$F_{(2,37)} = 0.01$, $P > 0.99$; group averages $= 132 \pm 7$ to 143 ± 9 μm^2].

In the small cluster of TH-immunoreactive cells in the dorsal reticular nucleus of the thalamus, there were no

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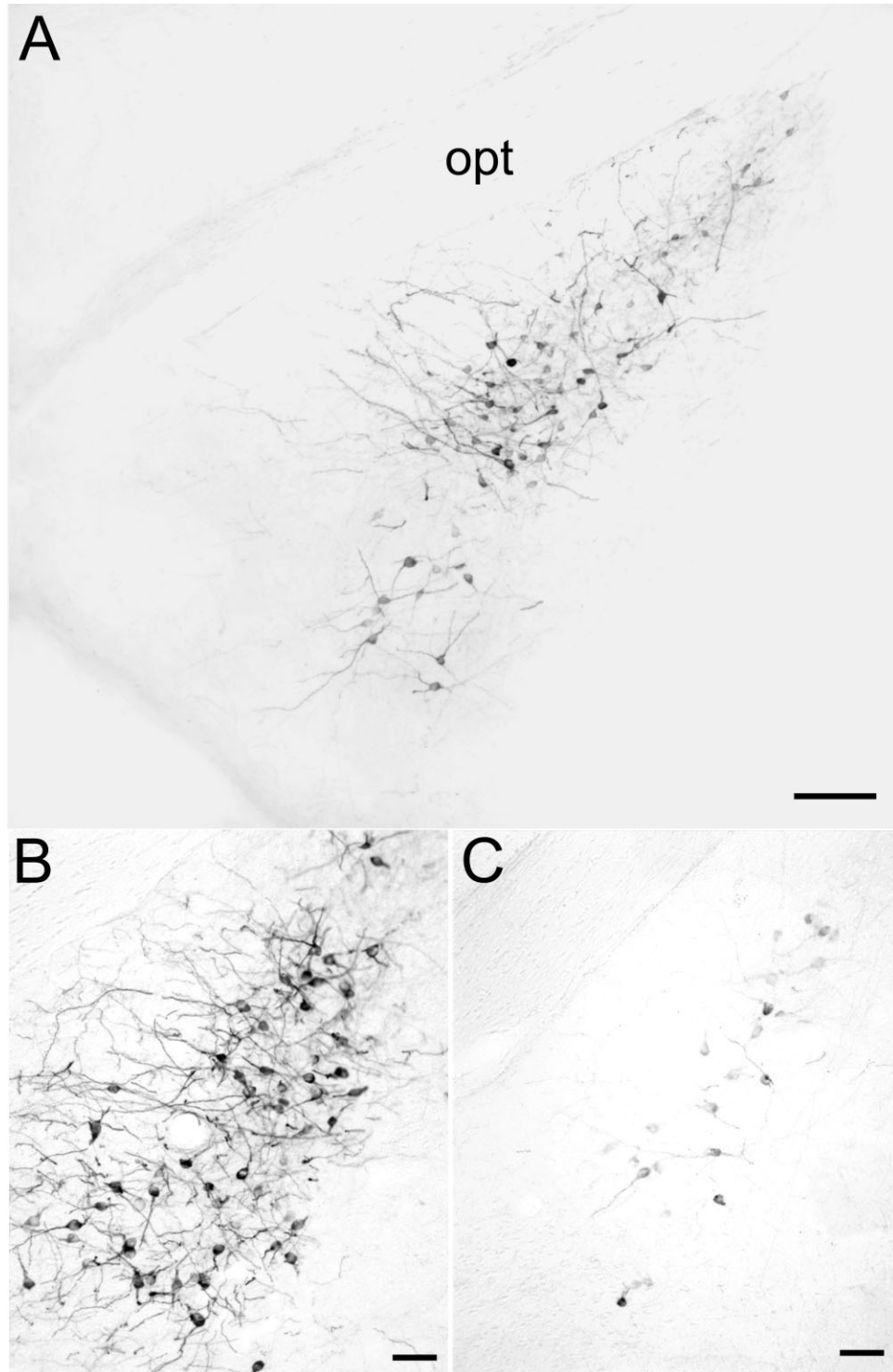

Fig. 3. Photomicrographs of TH-immunoreactive cells in the MeApd of representative prairie voles. **A:** Male prairie vole at ×100 magnification. **B:** Male at ×200 magnification. **C:** Female at ×200 magnification. opt, Optic tract. Scale bars = 100 μm in A; 50 μm in B,C.

significant main effects of sex [$F(1,34) = 1.31, P > 0.26$] or treatment [$F(2,34) = 0.33, P > 0.71$], and no significant interaction between them [$F(2,34) = 0.38, P > 0.69$; Fig. 9].

DISCUSSION

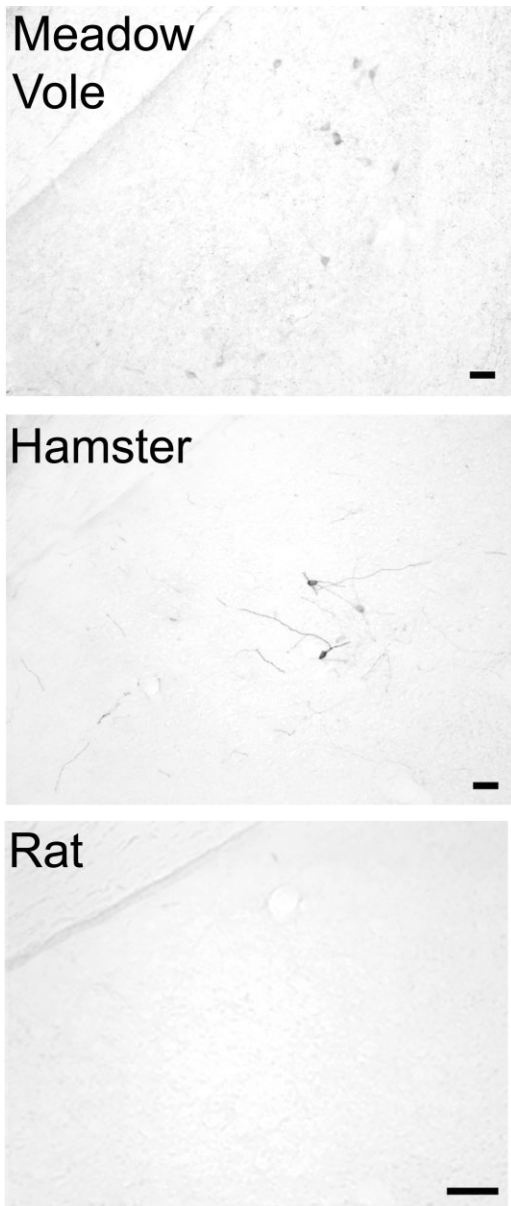We herein demonstrate that large numbers of TH-immunoreactive cells were found in the dorsal pBST and

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Fig. 4. Photomicrographs (×200 magnification) of the MeApd of representative male meadow voles, hamsters, and rats. Males of these species were similar to females. Scale bars = 50 μm.

Fig. 5. Number (Mean ± SEM) of TH-immunoreactive cells in every other section of the dorsal pBST of male and female prairie voles that were gonadally intact (Sham), gonadectomized (GDX), or gonadectomized and implanted with a capsule filled with testosterone (GDX + T). *Significant difference between the sexes, collapsed across treatment. α, GDX males significantly different from other groups of males; β, GDX + T females significantly different from other groups of females.

MeAPd of male prairie voles. Female prairie voles and meadow voles, hamsters, and rats of both sexes had relatively few or no TH-immunoreactive cells in these sites. Furthermore, these populations of cells were sensitive to manipulations in gonadal hormones in adult prairie voles, in that castration reduced the number of TH-immunoreactive cells in males, an effect that could be prevented by chronic treatment with testosterone. Females treated with testosterone during adulthood also showed a sharp increase in the number of TH-immunoreactive cells. Differences between the sexes in TH-immunoreactive cells within a known chemosensory p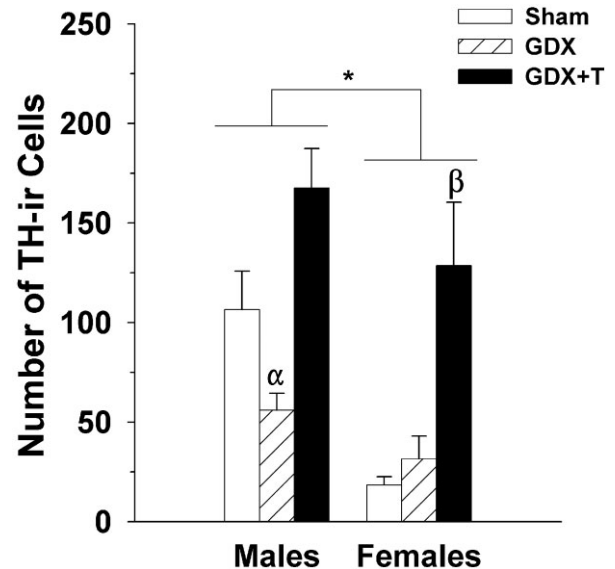athway, and the sensitivity of these cells to circulating hormones, indicate that catecholamine release from these sites may be involved in regulating sex differences in social behaviors in prairie voles as well as changes in these social behaviors across the reproductive cycle at times when hormones dramatically fluctuate.

Species differences in TH immunoreactivity

There seems to be notable species differences in TH expression in the pBST and MeApd. However, the absence of large numbers of TH-immunoreactive cells in the BST and MeA of meadow voles, hamsters, and rats of both sexes does not necessarily indicate that TH-producing cells do not exist in the BST and MeA of these species. In fact, Syrian hamsters of both sexes can express a large number of TH-immunoreactive cells in these sites, apparently as many as we found for male prairie voles, if treated with colchicine 48 hours prior to sacrifice (Asmus et al., 1992; Asmus and Newman, 1993). The "appearance" of these immunocytochemically identifiable cells in hamsters likely is due to increased accumulation of TH in the somata after colchicine treatment, rather than a de novo increase in TH synthesis that can occur as a result of colchicine (Cortes et al., 1990). This is suggested by the finding that these cells are present in high numbers when TH mRNA is visualized with in situ hybridization in hamsters not treated with colchicine (Asmus and Newman, 1993). Nonetheless, under normal conditions, only male prairie voles express high enough levels of TH in the dorsal pBST and MeAPd to be visualized with immunocytochemistry, although other rodents may express relatively low levels of TH in these sites. The comparison between species in TH immunoreactivity in the pBST and MeApd also revealed that male prairie voles, meadow
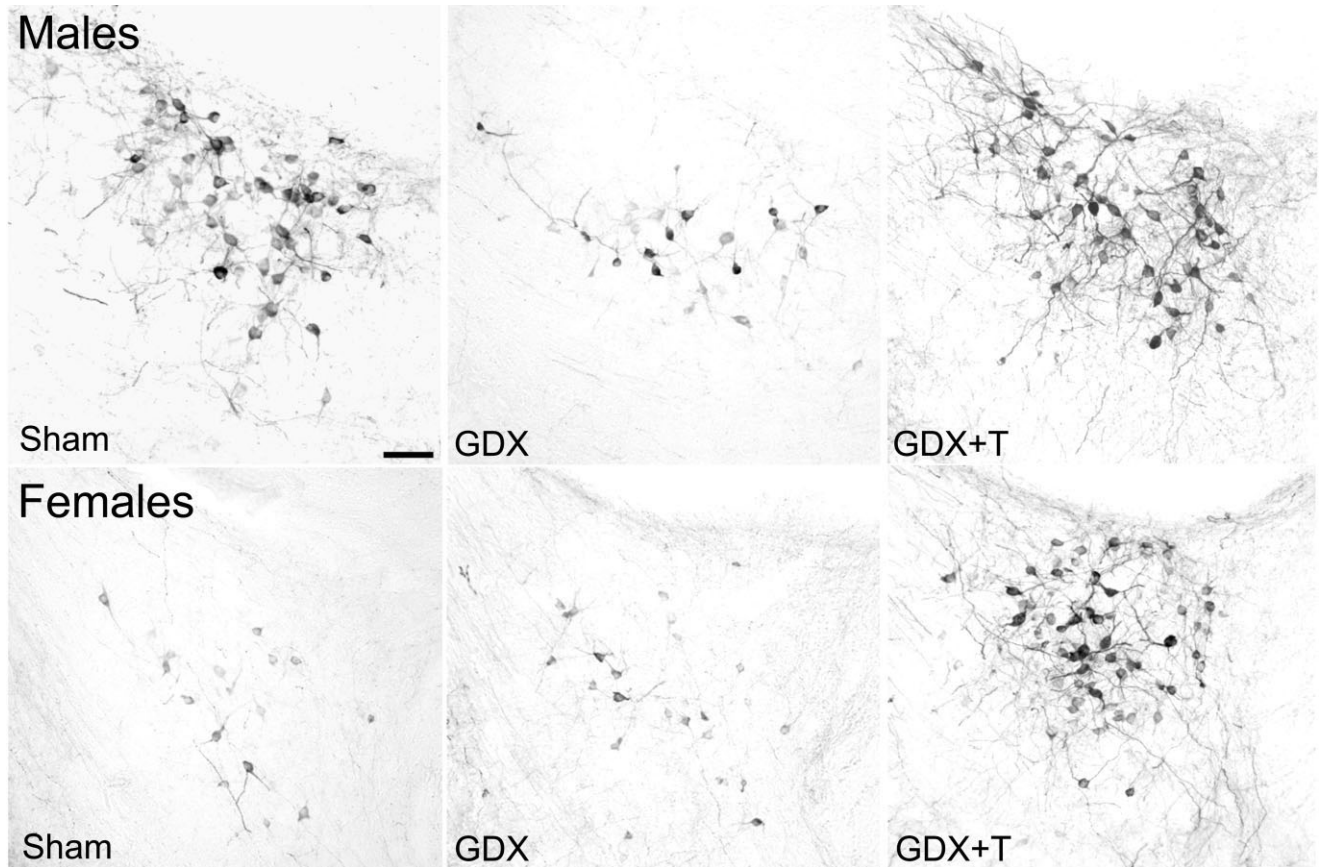
Fig. 6. Photomicrographs (×200 magnification) of TH-immunoreactive cells in the dorsal pBST of male (top) and female (bottom) prairie voles that were gonadally intact (Sham), gonadectomized (GDX), or gonadectomized and implanted with a capsule filled with testosterone (GDX + T). Scale bar = 50 μm.

voles, and hamsters have at least some TH-immunoreactive cells in both sites, whereas rats do not. All of the rodents examined herein are within the superfamily Muroidea. However, based on recent genetic examination, voles and hamsters are both members of the family Cricetidae, whereas Old World (laboratory) rats are within the family Muridae (Steppan et al., 2004). Hamsters and voles are more phylogenetically related to each other than either species is to laboratory rats, and cells expressing TH in these regions of the brain may be relatively specific to Cricetidae. Nonetheless, there are some notable differences between voles and hamsters. We found that TH-immunoreactive cells were rare in the anterior MeA of male voles but are quite dense in both the anterior and the posterior MeA of colchicine-treated male hamsters (Asmus et al., 1992), indicating that the distribution of TH-immunoreactive cells in these sites is similar, but not identical, among relatively closely related species.

Possibly more surprising, based on degree of relatedness, is the difference in the level of TH expression between male prairie and male meadow voles. The brains of these two very closely related species differ in numerous ways, including expression of receptors for oxytocin (Insel and Shapiro, 1992), vasopressin (Insel et al., 1994), corticotropin-releasing factor (Lim et al., 2005), estrogen (Fowler et al., 2005), and dopamine (Curtis, Fowler, Lon-

stein, and Wang, in preparation). Male prairie and meadow voles also differ in how social interactions alter neurochemistry (Bamshad et al., 1993; Wang et al., 1994; Curtis et al., 2003). This divergent neurochemistry has often been suggested to be related to differences between these species in their social organization, with prairie voles highly gregarious and socially monogamous and meadow voles nongregarious and polygamous (Young et al., 2001). An examination of TH expression in the pBST and MeApd of males from other monogamous species, such as California mice (*Peromyscus californicus*), Djungarian hamsters (*Phodopus campbelli*), or Mongolian gerbils (*Meriones unguiculatus*), would be useful to determine whether very high TH expression in these sites is typical of monogamous male rodents.

Sex differences and hormone sensitivity of TH-immunoreactive cells

There was a large sex difference in the number of TH-immunoreactive cells in the prairie vole dorsal pBST and MeApd. As noted above with regard to differences between species, the absence of TH-immunoreactive cells in untreated female prairie voles does not necessarily mean that TH-synthesizing cells do not exist, but rather that levels of TH are below the threshold of immunocytochemical detection. It is valuable to note that males do not have
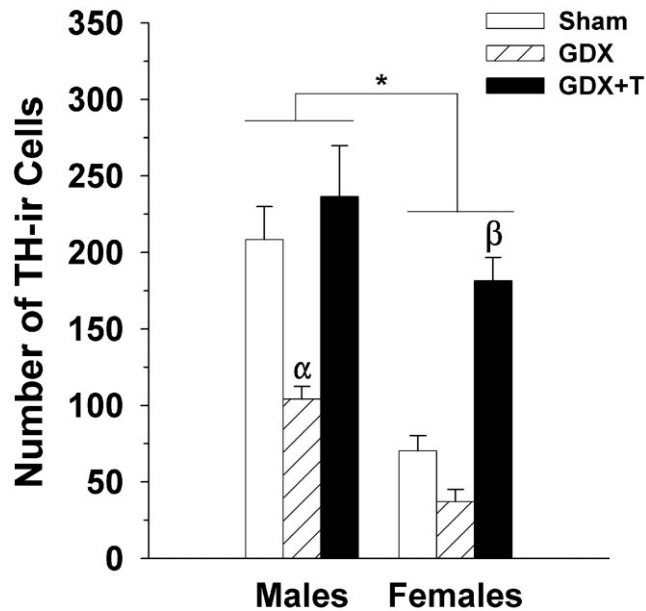
Fig. 7. Number (Mean ± SEM) of TH-immunoreactive cells in every other section of the MeApd of male and female prairie voles that were gonadally intact (Sham), gonadectomized (GDX), or gonadectomized and implanted with a capsule filled with testosterone (GDX + T). *Significant difference between the sexes, collapsed across treatment. α, GDX males significantly different from other groups of males; β, GDX + T females significantly different from other groups of females.

greater levels of TH-immunoreactive cells everywhere in the brain; sex differences in detectable cells are not found in the dorsal reticular thalamus (present results) or hypothalamus and zona incerta (Lansing and Lonstein, 2006). Furthermore, we previously found that the sex difference in TH expression in the AVPV is the reverse of that found in the pBST or MeApd (Lansing and Lonstein, 2006). The sex differences in the dorsal pBST and MeApd are, therefore, quite unique.

Our data also demonstrate that these TH-immunoreactive populations are sensitive to gonadal hormones. When treatment groups were compared within each sex, we found that castration of male prairie voles demasculinized (i.e., reduced) the number of TH-immunoreactive cells in both sites, whereas exogenous testosterone maintained the numbers of these cells at a level similar to that of gonadally intact males. Conversely, the number of TH-immunoreactive cells was masculinized (i.e., increased) in females given testosterone. The lack of an effect of ovariectomy on these cells in females was expected, insofar as prairie voles are induced ovulators and, in the absence of sensory cues from males, have little circulating estradiol (Cohen-Parsons and Carter, 1987). This does not necessarily mean that estradiol is not responsible for modulating TH expression in these sites. Many effects of testosterone on sex differences in the rodent brain are mediated through its aromatization to estradiol and subsequent activity of the estradiol receptor (DeVries and Simerly, 2002; Wallin and Baum, 2002). The pBST and MeApd are extremely steroid sensitive and express high levels of estradiol receptors in prairie voles of both sexes (Hnatczuk et al., 1994; Cushing and Wynne-

Edwards, 2005; Fowler et al., 2005). Aromatization of testosterone into estradiol may be largely responsible for testosterone's effects on TH-immunoreactive cells in these sites, a detail that should be examined in future experiments.

The effects of hormone manipulations in male prairie voles were somewhat dissimilar to those found in colchicine-treated male hamsters. Castration of hamsters reduces TH immunoreactivity only in the anterior MeA, not the posterior MeA or BSTpm (Asmus and Newman, 1993). Furthermore, although exogenous testosterone fully maintained of the number of TH-containing cells in the MeApd of male prairie voles, it does not in the hamster anterior MeA. Asmus and Newman (1993) also found that the decrease in TH immunoreactivity in the hamster anterior MeA after castration was transient, insofar as high numbers of TH-immunoreactive cells were again found in males examined 12 weeks after castration. We do not know whether similar long-term castration effects occur in the MeApd of male prairie voles. It appears that differences in TH expression between members of Cricetidae are apparently not only in distribution but also in sensitivity to gonadal hormones.

Even though castration greatly reduced TH expression in males and testosterone greatly increased it in females, we do not claim that the sex difference was completely eliminated by equating adult circulating hormones. Indeed, adult female prairie voles treated with testosterone still had ~25% fewer TH-expressing cells in the dorsal pBST than testosterone-treated males. Conversely, castrated males still had almost twice as many cells as ovariectomized females. We can accurately state, though, that the *magnitude* of the sex difference is greatly reduced when gonadal hormones are equated in adult animals. In the dorsal pBST, the magnitude of the sex difference changed from an almost 600% difference in intact animals to a 25% difference when animals were similarly treated with testosterone. The sex difference in the MeApd went from almost 300% to 25%. A similar reduction in the magnitude of the sex difference in medial amygdala volume occurs when adult male and female rats are given the same testosterone treatment (Cooke et al., 1999), so prairie voles are not the only species in which adult gonadal hormones can greatly alter the magnitude of a neural sex difference.

Given that the sex differences in TH expression were not completely eliminated, gonadal hormones acting on these sites during perinatal development also probably influence this sex difference to some extent. Perinatal exposure to testicular hormones may render adult males more sensitive to a given amount of testosterone, leading to the persistence of a sex difference in TH immunoreactivity even when adult males and females receive the same hormone treatment. Adult female prairie voles have greater estrogen receptor expression in these sites than males (Hnatczuk et al., 1994), so reduced effectiveness of testosterone in adult females may instead be due to reduced intraneuronal aromatization of testosterone to estradiol (Roselli et al., 1985). We also do not exclude the possibility that testosterone actions on the androgen receptor, which is sexually dimorphic in these sites (Wood and Newman, 1999), also contributes to the sex differences (Morris et al., 2005).
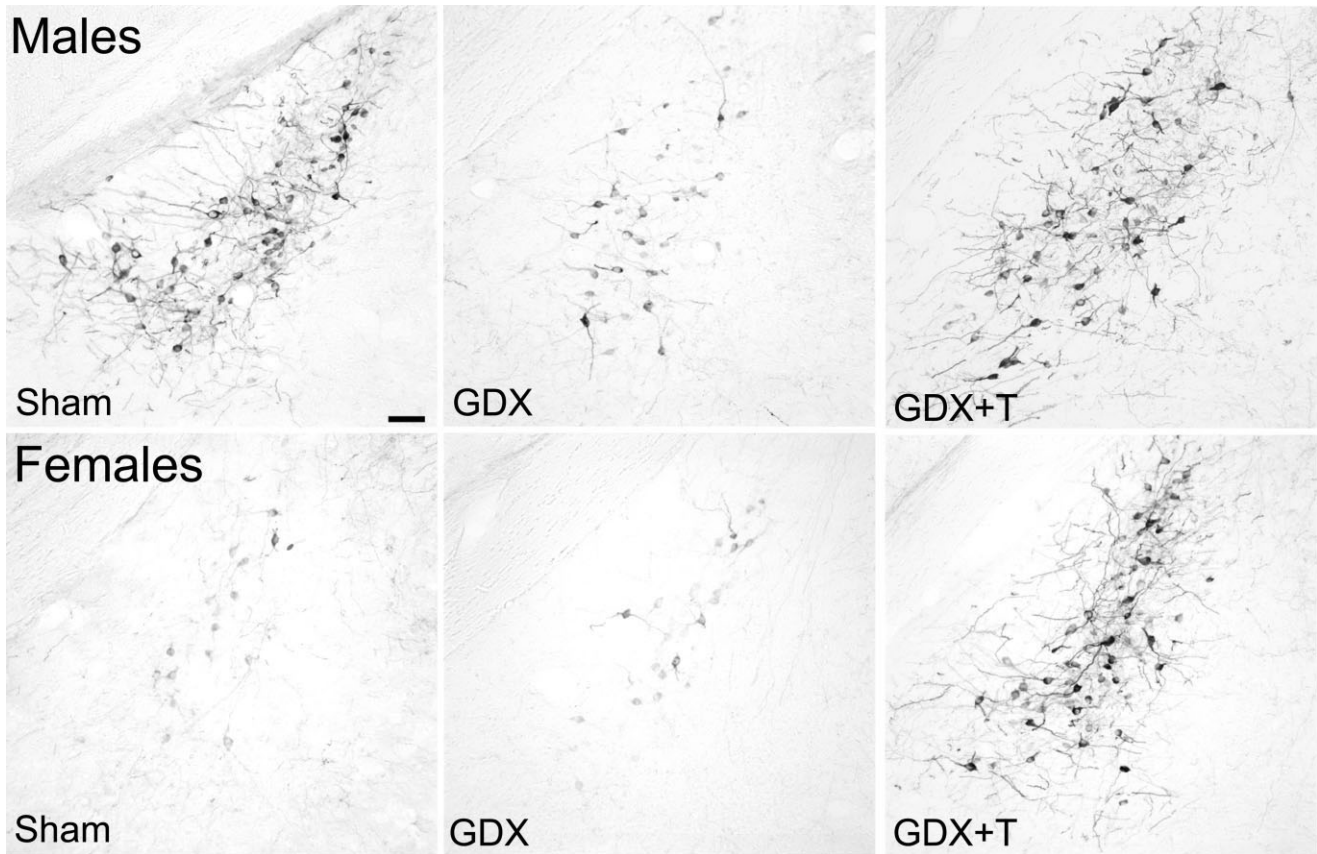
Fig. 8. Photomicrographs (×200 magnification) of TH-immunoreactive cells in the MeApd of male (top) and female (bottom) prairie voles that were gonadally intact (Sham), gonadectomized (GDX), or gonadectomized and implanted with a capsule filled with testosterone (GDX + T). Scale bar = 50 μm.

Functional considerations

Differences in TH immunoreactivity between individual cells within a given structure reflect functional and anatomical heterogeneity (Zigmond and Ben-Ari, 1977; Benno et al., 1982; Bayer and Pickel, 1990; Weiss-Wunder and Chesselet, 1990, 1991). It is reasonable to believe that differences between animals in TH immunoreactivity in a given site also reflects such heterogeneity. The presence of a large number of TH-immunoreactive cells in the pBST and MeAPd of male prairie voles is presumably associated with unique anatomy and neural processing compared with female prairie voles, as well as meadow voles, hamsters, and rats of both sexes. Indeed, these populations in male prairie voles do not contain an insignificant number of cells. Our quantification of all TH-immunoreactive cells in every other section suggests that the male prairie vole dorsal pBST contains over 200 TH-immunoreactive cells, and the MeApd contains at least twice that many. The absence of DBH immunoreactivity the pBST and MeAPd indicates that these cells are dopaminergic, rather than noradrenergic, which is consistent with what is found in colchicine-treated hamsters (Vincent, 1988; Asmus et al., 1992; Asmus and Newman, 1993).

In hamsters, lesions encompassing the BSTpm reduce olfactory investigation of estrous females, although many lesioned males continue to copulate, albeit at a reduced level (Powers et al., 1987). Lesions of the MeA that include the MeApd, particularly its rostral component, also severely reduce males' olfactory investigation of females and virtually eliminate copulation (Lehman et al., 1980; Powers et al., 1987). Various impairments in copulation are also found in male laboratory rats after pBST or MeApd lesions (Emery and Sachs, 1976; Valcourt and Sachs, 1979; Kondo, 1992; McGregor and Herbert, 1992; Claro et al., 1995). Not much is known about the prairie vole pBST and MeApd, but the anatomy and function of these areas in other rodents suggests that these TH-immunoreactive populations in male prairie voles could be related to the chemosensory control of copulation, as well as to the more unique social behaviors they display, such as pair bonding and paternal care.

Both male and female prairie voles pair bond and show parental care, though, which complicates the suggestion that pBST/MeApd TH expression in males is involved in their ability to display these behaviors, as one might expect that the sexes employ similar neural mechanisms to achieve these similar behavioral endpoints. De Vries (2004) has suggested that the opposite may be possible, such that sex differences in the brain, rather than similarities, produce similar behavioral or physiological endpoints by compensating for sex differences in circulating gonadal hormones. A sex difference in TH expression in
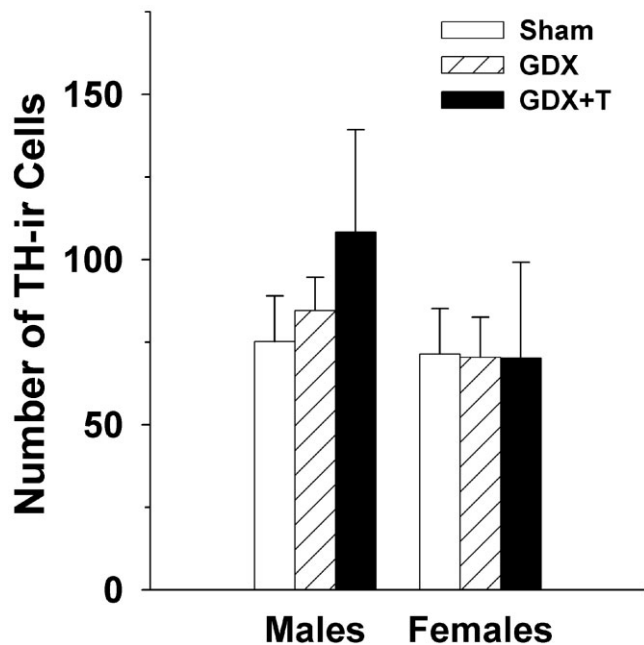
Fig. 9. Number (mean ± SEM) of TH-immunoreactive cells in every other section of the dorsolateral thalamus of male and female prairie voles that were gonadally intact (Sham), gonadectomized (GDX), or gonadectomized and implanted with a capsule filled with testosterone (GDX + T).

the pBST and MeApd may allow males to show monogamous behaviors even in light of their higher circulating androgens, which are sometimes regarded as inconsistent with monogamous behaviors (Wingfield et al., 2001).

Nonetheless, any function for the dorsal pBST and its catecholaminergic cells in the formation and expression of pair bonds in male prairie voles remains to be examined. The pBST and surrounding area show increased activity of the immediate early gene *c-fos* when male prairie voles copulate with females (Lim and Young, 2004), which normally is necessary for their pair bonding (see Carter et al., 1995). Furthermore, peripheral injection of the dopamine antagonist haloperidol eliminates males' social preferences for their mates (Aragona et al., 2003). Conversely, injection of the dopamine agonist apomorphine promotes males' social preferences for familiar females in the absence of mating (Aragona et al., 2003). Areas of the brain where changes in dopaminergic neurotransmission produce these effects include the mesolimbic system (Aragona et al., 2003, 2006; Curtis and Wang, 2005), but, as with other social behaviors, multiple catecholaminergic systems are likely to be involved (Dominguez and Hull, 2005; Miller and Lonstein, 2005). A function for the dorsal pBST in parental behavior in prairie voles is also unknown. It may be sex-specific, in that paternal behavior in prairie voles requires intact olfaction, whereas maternal behavior does not (Williams et al., 1992; Kirkpatrick et al., 1994a), suggesting that olfactory inputs to the pBST may be more important for paternal care than maternal care. In partial support, lesions of the entire dorsal BST (including the dorsal pBST) of lactating female rats do not significantly affect their maternal behavior (Numan and Numan, 1996).

In contrast to the pBST, the MeAPd has been investigated for roles in both pair bonding and paternal behavior in male prairie voles. Although large lesions of the corticomedial amygdala (which include the MeApd) reduce contact with a familiar female, lesions specifically of the medial amygdala do not (Kirkpatrick et al., 1994b). Such lesions do reduce their paternal behavior, though (Kirkpatrick et al., 1994b). Inhibiting dopaminergic neurotransmission with peripheral injection of haloperidol also impairs their paternal behavior (Lonstein, 2002), and the MeApd may be one source of critical dopaminergic neurotransmission.

In summary, species-specific catecholaminergic activity originating from cells in the pBST and MeApd of male prairie voles may be involved in the display of their unique social behaviors. Direct examination of how catecholamines originating from these two sites influence any behavior in male prairie voles remains to be determined.

ACKNOWLEDGMENTS

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