RESEARCH ARTICLE

Neonatal Androgen-Dependent Sex Differences in Lumbar Spinal Cord Dopamine Concentrations and the Number of A_{11} Diencephalospinal Dopamine Neurons

Samuel S. Pappas,[1] Chelsea T. Tiernan,[2] Bahareh Behrouz,[3] Cynthia L. Jordan,[2,4] S. Marc Breedlove,[2,4] John L. Goudreau,[2,5,6] and Keith J. Lookingland[1,2,6]*

[1]Department of Physiology, Michigan State University, East Lansing, Michigan 48824
[2]Neuroscience Program, Michigan State University, East Lansing, Michigan 48824
[3]Department of Neuroscience, Mayo Clinic, Jacksonville, Florida 32224
[4]Department of Psychology, Michigan State University, East Lansing, Michigan 48824
[5]Department of Neurology and Ophthalmology, Michigan State University, East Lansing, Michigan 48824
[6]Department of Pharmacology and Toxicology, Michigan State University, East Lansing, Michigan 48824

ABSTRACT

A_{11} diencephalospinal dopamine (DA) neurons provide the major source of DA innervation to the spinal cord. DA in the dorsal and ventral horns modulates sensory, motor, nociceptive, and sexual functions. Previous studies from our laboratory revealed a sex difference in the density of DA innervation in the lumbar spinal cord. The purpose of this study was to determine whether sex differences in spinal cord DA are androgen dependent, influenced by adult or perinatal androgens, and whether a sex difference in the number of lumbar-projecting A_{11} neurons exists. Adult male mice have significantly higher DA concentrations in the lumbar spinal cord than either females or males carrying the testicular feminization mutation (tfm) in the androgen receptor (AR) gene, suggesting an AR-dependent origin. Spinal cord DA concentrations are not changed following orchidectomy in adult male mice or testosterone administration to ovariectomized adult female mice. Administration of exogenous testosterone to postnatal day 2 female mice results in DA concentrations in the adult lumbar spinal cord comparable to those of males. Male mice display significantly more lumbar-projecting A_{11} DA neurons than females, particularly in the caudal portion of the A_{11} cell body region, as determined by retrograde tract tracing and immunohistochemistry directed toward tyrosine hydroxylase. These results reveal an AR-dependent sex difference in both the number of lumbar-projecting A_{11} DA neurons and the lumbar spinal cord DA concentrations, organized by the presence of androgens early in life. The AR-dependent sex difference suggests thyat this system serves a sexually dimorphic function in the lumbar spinal cord. J. Comp. Neurol. 518:2423–2436, 2010.

© 2010 Wiley-Liss, Inc.

INDEXING TERMS: sex difference; spinal cord; dopamine; androgens; A_{11}; diencephalospinal

Dopamine (DA) neurons in the diencephalon are distributed in four distinct populations, including the A_{11}, A_{12}, A_{13}, and A_{14} cell groups (Lookingland and Moore, 2005). Among these, A_{11} diencephalospinal neurons are unique in providing the major source of DA innervation to the spinal cord. The A_{11} cell group consists of approximately 450 relatively large neurons that are 15–20 μm in diameter and clustered in the dorsocaudal region of the diencephalon (Ondo et al., 2000). A_{11} neurons project primarily to the dorsal horn, with minor projections to the intermediolateral cell column and ventral horn along the entire length of the spinal cord (Skagerberg et al., 1982; Ridet et al., 1992; Holstege et al., 1996).

An inhibitory role in the processing of nociceptive stimuli has been suggested for A_{11} diencephalospinal DA

Grant sponsor: National Institutes of Health; Grant number: RO1 NS28421 (to S.M.B.); Grant number: RO1 NS045195 (to C.L.J.); Grant number: F31 NS064783 (S.S.P.).

*CORRESPONDENCE TO: Keith J. Lookingland, PhD, B-432 Life Sciences Building, Michigan State University, East Lansing, MI 48824.
E-mail: lookingl@msu.edu

Received July 16, 2009; Revised November 13, 2009; Accepted December 29, 2009
DOI 10.1002/cne.22340
Published online January 20, 2010 in Wiley InterScience (www.interscience.wiley.com)

© 2010 Wiley-Liss, Inc.

neurons terminating in the spinal cord (Jensen and Yaksh, 1984; Barasi and Duggal, 1985; Fleetwood-Walker et al, 1988; Gao et al, 2001; Wei et al, 2009). The presence of inhibitory D2/3 receptors in the dorsal horn of the cervical and lumbar spinal cord (Yokoyama et al., 1994; Levant and McCarson, 2001; Clemens et al., 2006; Zhu et al., 2007) further supports an antinociceptive role for descending A_{11} diencephalospinal DA tracts. In addition to sensory functions, spinal cord DA is neuromodulatory to motor networks in the ventral horn of the isolated rat spinal cord (Barriere et al, 2004) and chronic spinal cat (Barbeau and Rossignol, 1991) and likely regulates lower limb movements through a direct action on postsynaptic D2 receptors on α-motor neurons (Zhu et al., 2007). The presence of DA receptors on sexually dimorphic motor neurons in the ventral horn of male rodents (Van Dijken et al., 1996) suggests a sex-specific role for A_{11} DA neurons.

Several sex differences in spinal cord structure and function have been described. Among these, the best characterized is the spinal nucleus of the bulbocavernosus (SNB; Breedlove and Arnold, 1981), a motor neuron nucleus in the fifth and sixth lumbar segments innervating the sexually dimorphic bulbocavernosus and levator ani muscles in male rats. This nucleus is severely diminished in females (Breedlove and Arnold, 1980), and a similar sex difference has been described for humans (Forger and Breedlove, 1986). Male rats with the testicular feminization mutation (tfm) in the androgen receptor (AR) gene, which renders a dysfunctional protein, resemble normal females, with few SNB motor neurons (Breedlove and Arnold, 1981). This indicates that AR plays a critical role in the organization of this dimorphism. Moreover, the dimorphism is dependent on the presence of neonatal androgens (Nordeen et al., 1985). Evidence suggests that activation of AR in target muscles of the SNB spares these motor neurons from cell death early in life in males (Fishman et al., 1990; Freeman et al., 1996), implicating AR-expressing muscle fibers in mediating this effect (Monks et al., 2004). However, recent studies strongly suggest a more complex AR-dependent interaction between several cell types, insofar as AR expression in muscle fibers of tfm rats does not rescue the SNB system from death (Niel et al., 2009).

Previous studies in our laboratory suggest a sex difference in spinal cord DA concentrations; i.e., male mice have a higher concentration of DA in the gray matter of the spinal cord than do females (Pappas et al., 2008). The observed sex-related difference in spinal cord DA is most prominent in the lumbar regions and most likely is due to higher axon terminal network density in the male lumbar spinal cord arising from the A_{11} diencephalospinal system in mice. The purpose of these studies is to determine whether sex differences in spinal cord DA concentrations are due to an action of androgens on AR and to determine whether a sex difference in the number of lumbar-projecting A_{11} neurons exists. To this end, we examined lumbar spinal cord DA concentrations in female and male mice and in genetic male mice carrying a mutation of the AR gene, the tfm allele. The tfm allele of the AR gene produces a dysfunctional protein and can therefore be utilized as a tool to reveal AR-dependent sex differences. We also manipulated androgens in adult and newborn mice to examine the role of androgens in the sex difference. A_{11} neuron number, distribution, and cell soma size were examined in adult male, female, and tfm male mice, revealing an androgen-dependent sex difference in cell number within the caudal A_{11} region. Injection of the retrograde tracer fluorogold into the lumbar enlargement of the spinal cord and subsequent immunocolocalization with tyrosine hydroxylase (TH) demonstrate a sex difference in the number of lumbar-projecting A_{11} DA neurons. Our findings are consistent with the conclusion that the sex difference in spinal cord DA concentration is dependent on a neonatal action of androgens on AR in male mice and arises from a sexually dimorphic pattern of spinal cord-projecting A_{11} DA neurons in the diencephalon.

MATERIALS AND METHODS

Animals

Age-matched 8–10-week-old wild-type male, female, and tfm male littermates on a C57BL/6(J) background from our colony (as described by Zuloaga et al., 2008) or commercially purchased male and female C57BL/6 mice (Jackson Laboratories, Bar Harbor, ME) were used in all studies (8–10 mice per group in neurochemical studies and 3–4 mice per group in immunhistochemical studies). Animals were housed four per cage, maintained in a temperature-controlled (22°C ± 1°C) and light-controlled (12-hour light-dark cycle) room, and provided with food and tap water ad libitum. Tissue harvesting was performed between 8 AM and 10 AM for all experiments to control for possible circadian variations. The Michigan State University Institutional Animal Care and Use Committee approved all experiments using live animals. All experiments were conducted following the U.S. National Institutes of Health *Guidelines for the Care and Use of Laboratory Animals* and were compliant with the provisions of the Animal Welfare Act. The minimal number of animals required to achieve sufficient statistical power was used.

Hormone manipulations

Adult gonadectomy studies

Male and female mice were anesthetized with ketamine:xylazine (13.3 mg/kg:2 mg/kg, i.p.) and stabilized

under aseptic conditions. Male mice were orchidectomized via a midline ventral incision; female mice were ovariectomized via lateral incisions; abdominal muscles were sutured, and wounds were closed with surgical wound clips. Mice immediately received a midline dorsal subcutaneous implant of either an empty Silastic capsule or one containing crystalline testosterone (inner diameter 1.575 mm, outer diameter 3.175 mm, 6 mm effective release length; Daan et al., 1975). After surgical procedures, mice were maintained on supplemental heat and monitored thereafter for return of normal postures and behavior. One week after surgery, mice were killed by decapitation, and neurochemical analysis with high-performance liquid chromatography coupled with electrochemical detection (HPLC-ED) was performed as described below.

Neonatal androgen studies

On the second day of life (P2), male and female mouse pups received a single subcutaneous 50-µl injection of either peanut oil vehicle or testosterone (100 µg; Nwagwu et al, 2005). On day 56 of life (8 weeks), mice were killed by decapitation, and the concentrations of DA in the spinal cord were determined by HPLC-ED.

Tissue dissection and HPLC analyses

Mice were killed by decapitation, and brains and spinal columns were removed;m then, fresh tissue dissection of the median eminence was performed. Brains and spinal columns were then quickly frozen over dry ice. Coronal brain section (500 µm) were prepared on a cryostat (–10°C) and the striatum, nucleus accumbens, and periventricular nucleus were microdissected using a modification of the Palkovits technique (Palkovits, 1973). Samples containing approximately 25 µg total protein were placed into ice-cold 0.1 M phosphate-citrate buffer with 15% methanol. Samples were assayed for neurochemical content using HPLC-ED (Lindley et al, 1990).

Consecutive spinal cord segments delimited by their vertebral widths were dissected with a scalpel, thaw mounted on a frozen glass slide, and refrozen over dry ice as previously described (Pappas et al., 2008). Spinal column segments within vertebrae L1–L3 (correcting for the misalignment between vertebrae and spinal cord, this encompasses lower lumbar and sacral spinal cord segments containing the SNB motor nucleus) were viewed under a dissecting microscope, and bilateral micropunches of gray matter were taken from each segment using a modified 24-gauge needle, placed into 0.1 M phosphate-citrate buffer containing 15% methanol, and assayed with HPLC-ED. Brain and spinal cord concentrations of DA were determined by normalizing samples to protein content determined by Lowry protein assay (Lowry et al., 1951).

Immunohistochemistry and cell counts

Untreated age-matched 8-week-old littermate male and female mice or 12-week-old littermate WT male and Tfm male mice were anesthetized with a lethal dose of ketamine:xylazine and transcardially perfused with phosphate-buffered saline (PBS; 0.05 M, pH 7.4), followed by 4% paraformaldehyde in phosphate buffer. The brain was removed and postfixed in 4% paraformaldehyde overnight and then cryoprotected in 20% sucrose in phosphate buffer. Forty-micrometer consecutive serial coronal sections through the diencephalon (1.5–3.5 mm posterior to Bregma; Franklin and Paxinos, 1997) were prepared with a cryostat (–19°C), placed in PBS, and stored at 4°C.

Free-floating 40-µm coronal sections were rinsed in PBS containing 0.1% Triton X-100 (PBS-TX), incubated in 0.3% H_2O_2, and 5% normal goat serum (NGS) to reduce background. Sections were incubated in rabbit polyclonal antibody directed against the full-length tyrosine hydroxylase (TH) from rat pheochromocytoma (AB152; Chemicon, Temecula, CA. The antibody stains a single band of 60 kDa molecular weight on Western blot of PC12 lysates; manufacturer's information) at 1:2,000 dilution in PBS-TX/NGS for 24 hours, followed by incubation in biotinylated goat anti-rabbit antibody (BA-1000; Vector Laboratories, Burlingame, CA; 1:500 dilution in PBS-TX) for 1 hour. Omitting the primary or secondary antibody from incubation prevented all staining. Sections were exposed to the avidin-biotin-peroxidase complex using an ABC kit (PK-6100; Vector Laboratories) for 2 hours followed by 0.05% 3,3′-diaminobenzidine (D5905; Sigma, St. Louis, MO) and 0.01% H_2O_2. Sections were mounted on coated slides, dried overnight, dehydrated in successive incubations of ethanol followed by xylenes, and coverslipped with permount (Fisher Scientific, Fair Lawn, NJ).

Under brightfield optics, stained sections were viewed on a computer screen, and regions containing A_{11} TH-immunoreactive neurons were delineated. Photomicrographs were taken through a $\times 10$ objective, and TH^+ neurons were counted within the area of interest by an investigator blind to treatment and/or genotype. TH^+ neurons had to meet three criteria to be counted as part of the A_{11} population. First, neurons must be located between Bregma –1.9 and –3.0 mm, with a midregion landmark determined to be Bregma –2.4 mm. This landmark is where neurons begin to appear in more dorsal areas, close to the midline, surrounding the dorsal third ventricle and appearing medial to the fasciculus retroflexus. A_{11} neurons rostral to this landmark were classified as "rostral A_{11}" and neurons caudal to the midregion landmark were classified as "caudal A_{11}." Second,

neurons must be large, containing extensive dendrites and have a cell body $\geq$15 μm in diameter. Size discrimination is particularly important in the caudal A_{11} region, in that TH^+ neurons of the ventral tegmental area begin to appear but are smaller (see Fig. 4). Third, only TH^+ neurons containing a darkly stained cytoplasm and clear nucleus were counted. Cell counts were adjusted using the Abercrombie formula to correct for potential double counting of cell profiles. The ratio of "real" cells to our observed numbers was calculated using the equation $T/T + h$, where T equals the section thickness, and h equals the mean diameter of the clear neuronal nuclei counted (Guillery, 2002).

To assess the size of TH^+ neurons, 45–50 caudal-dorsal A_{11} neurons (observed as clusters of neurons from three consecutive sections) per animal were visualized and captured at high magnification, and cell soma profile area was measured by importing the images into an image analysis system (Image Pro Plus; Media Cybernetics, Bethesda, MD). The same three criteria were used to sample TH^+ neurons for soma area measurements. Upon viewing of cell clusters in a single focal plane, only cells in focus were outlined using a computer mouse, generating measures of soma area in square micrometers. Average soma area was then calculated for each animal. No digital manipulation was used in preparation of images.

Fluorogold injection

Male and female mice were given a single subcutaneous anesthetic injection (ketamine:xylazine 13.3 mg/ml:2 mg/ml) and received a small midline dorsal incision in the skin over the spinal column. Overlying muscles were cleared from the spinal column with a scalpel, and vertebral bone was exposed. A small laminectomy was made in vertebra L1, and a glass micropipette (20 μm tip) was lowered into the spinal cord (0.5 mm below dura); then, 0.5 μl of a 4% fluorogold solution was injected using a 5-μl Hamilton syringe. After a 5-minute delay to prevent tracer reflux, the micropipette was removed and the skin was closed with surgical wound clips. Mice were maintained postoperatively on supplemental heat and analgesia and were monitored for return of normal postures and behaviors.

Ten days following spinal cord injection, mice were killed, brains fixed, and coronal sections prepared as described above. Free-floating 40-μm sections were incubated in PBS-TX, followed by 5% normal goat serum to reduce nonspecific staining. Sections were immunostained as described above, but TH staining in this case was visualized by exposing sections to a goat anti-rabbit antibody conjugated to Cy3 (111-165-003; Jackson Imunoresearch, West Grove, PA; 1:400 dilution in PBS-TX) for 1 hour. Omitting the primary or secondary antibody from incubation prevented all TH immunoreactivity. Sections were mounted on slides and coverslipped with ProLong Gold mounting medium (P36930; Invitrogen, Carlsbad, CA). Sections were viewed under epifluorescence to delineate the A_{11} region, and images were captured. Fluorogold-traced neurons were visible as diffuse blue cell bodies under UV excitation (excitation wavelength range 340–380 nm, emission wavelength range 435–485 nm). TH^+ neurons were visible as brightly labeled cells with orange cell bodies and dendrites, but a clear nucleus (excitation wavelength range 530–560 nm, emission wavelength range 573–648 nm). Total TH^+ A_{11} neurons and those TH^+ A_{11} neurons also containing fluorogold were counted using the same criteria as described above. The brightness and contrast of photomicrographs selected for publication were not manipulated.

Statistical analysis

To compare among groups, *t*-tests or one-way ANOVAs were performed in SigmaStat software version 2.03 (SysStat Software, Point Richmond, CA). If a significant interaction was detected by ANOVA, Tukey's post hoc test was used for multiple comparisons, and differences with a probability of error of less than 5% were considered statistically significant ($P = 0.05$). For cell-counting studies, effect sizes were computed by determining Cohen's d (difference between the means divided by the pooled standard deviation) and then computing the effect size correlation [$r = d/\sqrt{(d^2 + 4)}$].

RESULTS

Sex difference in spinal cord DA concentration

Genetic male (XY) tfm mice were compared with wild-type (WT) male and female littermates. As shown in Figure 1A, lumbar DA concentrations are significantly higher in WT male mice than in females, but tfm males have concentrations similar to those of females. In contrast, lumbar spinal cord concentrations of norepinephrine (NE) are comparable in samples from the same set of male, female, and tfm mice (Fig. 1B).

No sex difference in DA concentration was observed in the median eminence (Table 1), but females and tfm male mice displayed significantly higher DOPAC/DA ratios than did male mice. Conversely, female mice possess significantly higher DA concentrations in the periventricular nucleus (PeVN) than either WT or tfm male mice. These results are consistent with previous findings and serve as positive controls for these assays (Demarest et al., 1981; Simerly et al., 1985a,b, 1997; Moore and Lookingland, 1995; Lookingland and Moore, 2005). As previously reported, there were no sex differences observed in DA

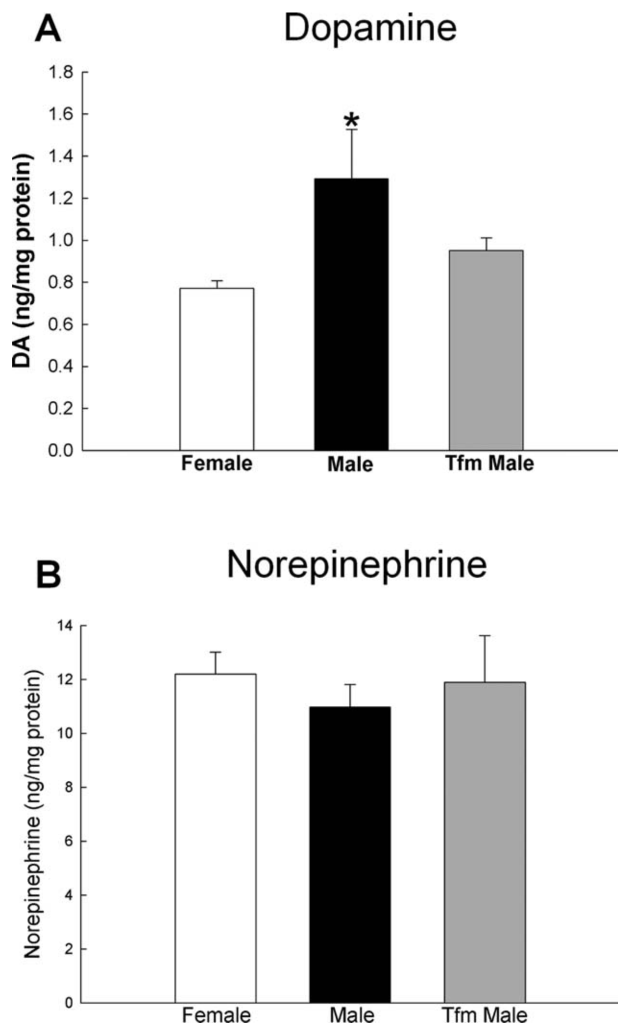

Figure 1. DA (A) and NE (B) concentrations in the lumbar spinal cord of WT female, WT male, and Tfm male mice carrying a dysfunctional allele for androgen receptors. Columns represent the means and error bars ± 1 SEM (n = 7–8 mice per group). *Significantly higher than WT females $P < 0.05$. (one-way ANOVA: $F_{2,21} = 3.72$, $P = 0.041$).

or DOPAC concentrations in the striatum (Pappas et al., 2008).

Hormonal manipulations

Two experiments were performed on adult mice to determine whether the sex difference in lumbar spinal cord DA is due to the presence of adult circulating androgens. As demonstrated in Figure 2A, orchidectomy (Orch) of adult male mice for 1 week had no effect on spinal cord DA concentrations, with or without testosterone (T) replacement. Similarly, no significant changes in lumbar spinal cord DA concentrations were seen with T treatment following ovariectomy (OVX) for 1 week of adult female mice (Fig. 2B), indicating that the sex difference in DA concentrations is independent of adult androgen levels. Neither did we detect changes in DA concentrations in the PeVN, median eminence, and striatum following adult hormone manipulations (data not shown). On the other hand, we found that the adult sex difference in spinal cord DA was affected by neonatal manipulation of androgens. A single injection of T to female mice on day 2 of postnatal life reversed the adult sex difference in DA concentration. Levels of DA in lumbar spinal cord of adult female mice exposed to neonatal T were significantly higher than those of control females, but not significantly different from those of males (Fig. 3).

Sex differences in the number of TH immunoreactive A_{11} neurons

Coronal brain sections through the diencephalon were immunohistochemically stained for TH, and A_{11} DA neurons located between Bregma −1.9 mm and −3.0 mm (Fig. 4) were counted in littermate WT male and female mice or littermate WT male and Tfm male mice. As depicted in Figure 5A, the total number of A_{11} neurons in male mice was significantly higher than in that females.

TABLE 1.

Periventricular nucleus, median eminence, and striatum concentrations of DA and DOPAC and the ratio of DOPAC/DA in male, female, and tfm male mice (mean ± SEM, n = 7–8 mice per group)

	DA (ng/mg protein)	DOPAC (ng/mg protein)	DOPAC/DA
Periventricular nucleus			
Male	4.58 (0.44)	1.67 (0.12)	0.378 (0.034)
Female	11.48 (0.97)[1]	1.97 (0.13)	0.177 (0.013)[1]
tfm Male	5.96 (0.61)	1.95 (0.19)	0.330 (0.027)
Median eminence			
Male	83.59 (11.46)	3.96 (0.87)	0.048 (0.008)
Female	104.68 (11.64)	10.53 (1.47)[1]	0.113 (0.022)[1]
tfm Male	74.48 (18.23)	10.24 (2.93)[1]	0.145 (0.030)[1]
Striatum			
Male	143.31 (7.27)	17.45 (1.28)	0.123 (0.011)
Female	150.00 (12.21)	15.97 (1.78)	0.104 (0.004)
tfm Male	152.36 (9.94)	19.12 (1.85)	0.126 (0.010)

[1]Significantly different from male levels ($P < 0.05$, one-way ANOVA).

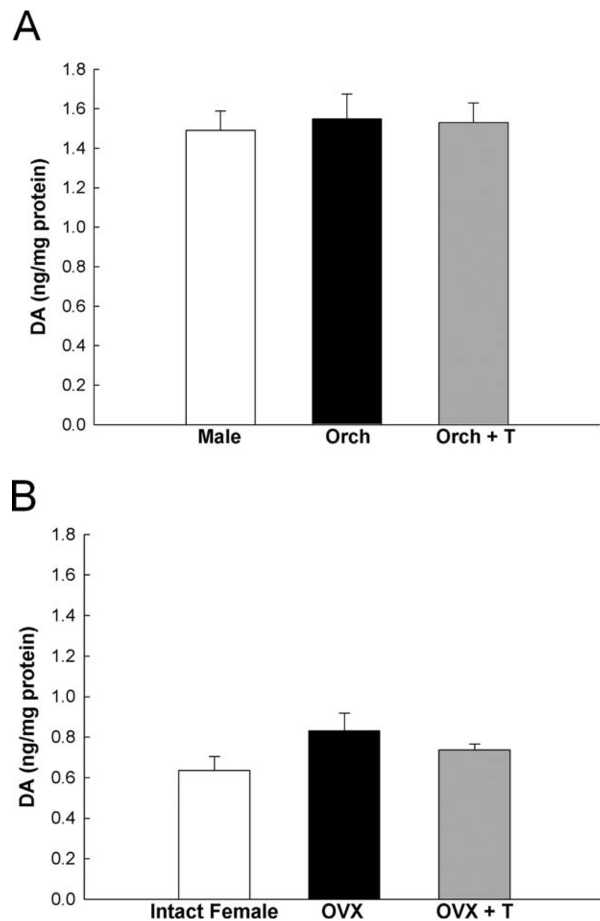

Figure 2. DA concentrations in the lumbar spinal cord of gonadally intact males or males orchidectomized (Orch) as adults (**A**) and gonadally intact females or females ovariectomied (OVX) as adults (**B**) with or without testosterone (T) administration. Columns represent the means and error bars ± 1 SEM (n = 6–8 mice per group).

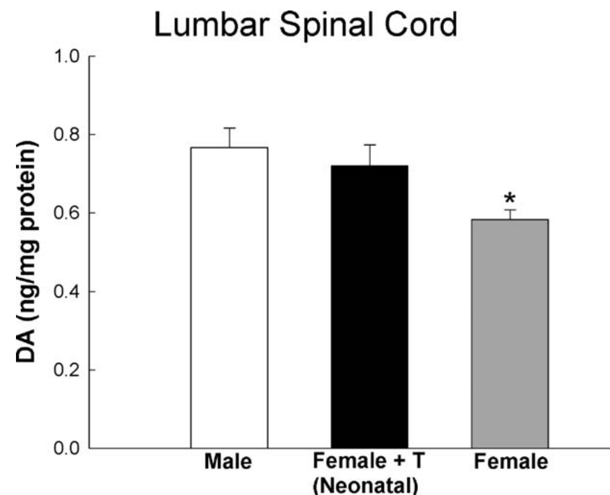

Figure 3. DA concentrations in the lumbar spinal cord of male and female mice that received testosterone (T) or oil vehicle on postnatal day 2. Columns represent the means and error bars ± 1 SEM (n = 9–10 mice per group). *DA concentrations significantly lower than male controls $P < .05$ (one-way ANOVA: $F_{2,27} = 4.06$, $P = 0.02$).

TH-immunoreactive neurons in A_{11} were subdivided into a "rostral A_{11}" group, beginning at Bregma –2.4 and extending rostrally, and a "caudal A_{11}" group, which included TH^+ neurons distributed caudally from Bregma –2.4 (Fig. 4). We found a significant sex difference in the number of TH^+ neurons in the caudal but not the rostral portion of A_{11}. Similarly, WT male mice contained significantly more A_{11} neurons compared with Tfm male littermates in this same caudal region (Fig. 5B). Cell soma area was measured in 45–50 caudal-dorsal neurons per animal, and there was no significant difference in area between males (218.2 ± 10.55 μm^2) and females (210.5 ± 13.8 μm^2), so no attempt was made to count cells stereologically.

Ten days following injection of fluorogold into the lumbar enlargement of the spinal cord (Fig. 6A,B), diencephalic coronal brain sections were stained for TH. As positive control, fluorogold-labeled (but TH negative) neurons were present in the primary motor cortex (Fig. 6C). Neurons in this region were seen bilaterally, confirming that the fluorogold injection was present in both sides of the lumbar spinal cord gray. TH^+ A_{11} neurons (Fig. 6D) were colocalized with fluorogold (Fig. 6E,F), and the total numbers of TH neurons and those TH^+ neurons containing fluorogold were counted. No other DA system of the brain exhibited fluorogold colocalization with TH^+ cell bodies, demonstrating that the A_{11} system is the sole supraspinal source of lumbar spinal cord DA (Fig. 6G–I). Male mice contained significantly more fluorogold-labeled TH^+ A_{11} neurons than females (Fig. 7A), demonstrating a sex difference in lumbar-projecting A_{11} neurons. The sex difference in total TH^+ A_{11} cell number was replicated (Fig. 7B), and approximately 50% of the total diencephalic A_{11} TH^+ neurons in each sex contained fluorogold (Fig. 7C).

DISCUSSION

Lumbar spinal cord DA concentration

The present results demonstrate a sex difference in lumbar spinal cord DA in mice, with males possessing higher DA concentrations than females or tfm males. Because the majority of DA measured in axon terminal regions is stored in synaptic vesicles (Carlsson, 1975), DA concentrations in regions containing axon terminals are generally accepted to reflect the actual density of axon terminals. For example, in the sexually dimorphic anteroventral PeVN, female mice have three to four times more TH-immunoreactive cell bodies and two to three times greater density of fibers than do males (Simerly

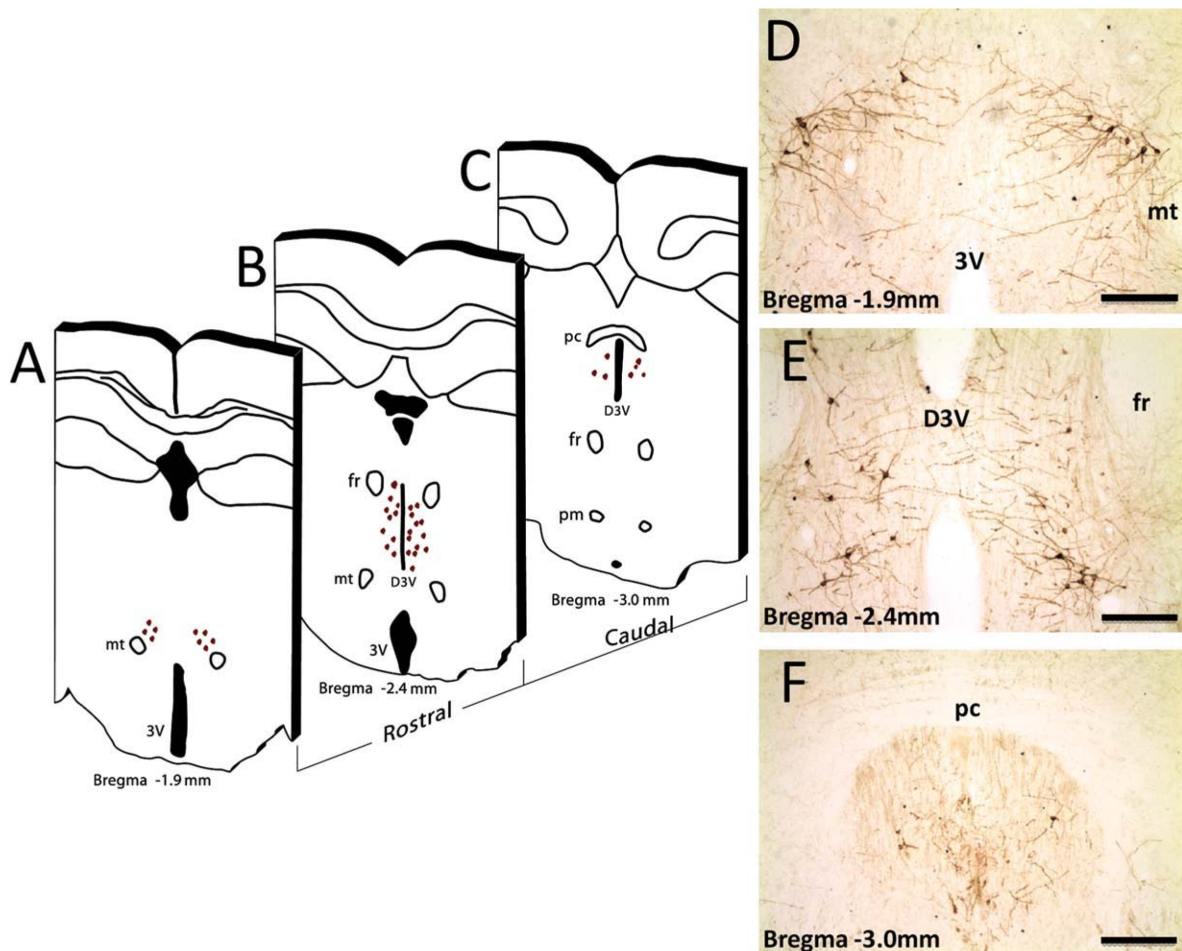

Figure 4. A_{11} coordinates and representative corresponding TH-stained sections from a single male mouse. The A_{11} region begins approximately 1.9 mm posterior to Bregma. In this rostral portion, cells cluster bilaterally near the midline, dorsal to the periventricular DA nuclei, near the third ventricle (3V), and medial to the mammillothalamic tracts (mt). At the A_{11} midregion, neurons begin to appear in more dorsal areas, close to the midline, surrounding the dorsal third ventricle (D3V) and appearing medial to the fasciculus retroflexus (fr). At the caudal A_{11} border, cells cluster ventral to the posterior commissure (pc). At this point, size discrimination is important while counting, because TH-positive neurons of the ventral tegmental area begin to appear. A_{11} neurons taper off once the substantia nigra appears in the ventral midbrain. Scale bars = 200 μm. [Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com.]

et al., 1985a,b). This difference in fiber density approximates the two- to threefold increase in PeVN DA concentrations seen in the current study. Accordingly, it is reasonable to surmise that differences in spinal cord DA concentrations are due to differences in DA neuronal innervation to the spinal cord.

Alternatively, it is possible that DA could be present as a precursor in descending noradrenergic fibers and axon terminals in the spinal cord, but several findings suggest that this is not the case. There is 10 times more NE than DA in the spinal cord (Skagerberg et al., 1982; Skagerberg and Lindvall, 1985), but the sex difference and the effect of the tfm allele on DA concentrations were not reflected in spinal cord NE concentration. NE and DA concentrations decline at different rates after transection or destruction of the spinal cord (Magnusson, 1973; Hedeman et al., 1974), and selective depletion of spinal cord NE with 6-hydroxydopamine, the NE-specific neurotoxin DSP-4 (Mouchet et al., 1982; Skagerberg et al., 1982; Holstege et al., 1996), or destruction of the locus coeruleus (Commissiong et al., 1978) leaves spinal cord DA terminals largely intact. Taken together, these results are consistent with the conclusion that DA concentrations measured in the spinal cord reflect the density of axon terminals of A_{11} DA neurons rather than precursor DA in noradrenergic fibers.

Diencephalic DA neuron development

In rodents, the distribution pattern and morphological features of diencephalic DA neurons are similar to those in adult animals by postnatal day 9 (Dahlstrom and Fuxe,

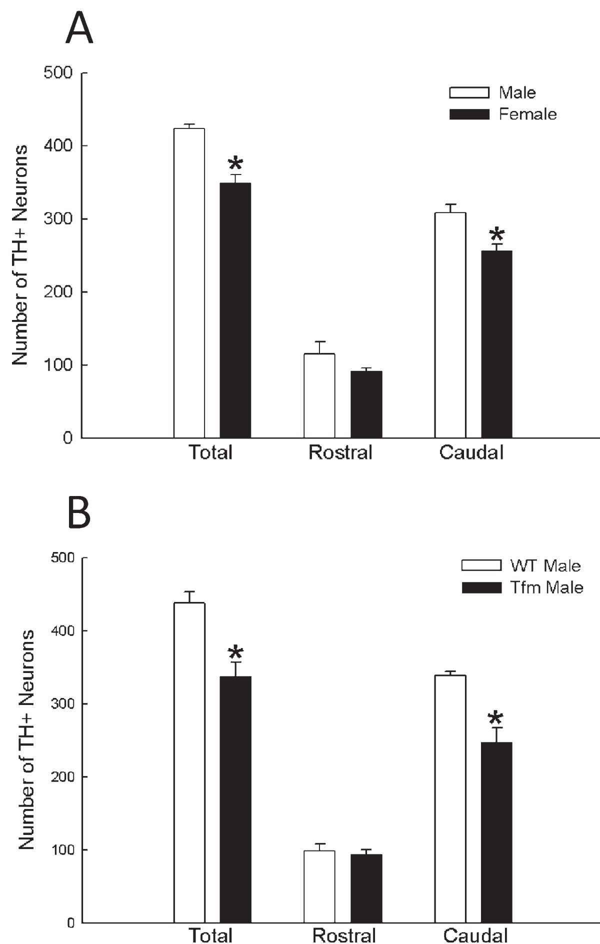

Figure 5. Counts of total, rostral, and caudal TH-immunoreactive A_{11} neurons in the diencephalon of littermate male vs. female mice (**A**) or WT male vs. tfm male mice (**B**). Coronal sections between Bregma –1.9 and Bregma –3.0 mm were immunostained for TH, and A_{11} neurons were counted in ImageJ. A_{11} neurons rostral to the midpoint at Bregma –2.4 were classified as "rostral," and neurons caudal to the midpoint were classified as "caudal." Columns represent means and error bars ± SEM (A: n = 3 male, n = 4 female; B: n = 3 WT male, n = 4 tfm male). *Female or tfm male values significantly lower than WT males ($P < 0.05$, *t*-test). Effect size correlation, r = 0.904 (A), r = 0.835 (B) for total counts and r = 0.822 (A), r = 0.856 (B) for caudal counts.

1964; van den Pol et al., 1984; Borisova et al., 1991). Androgen treatment of females before postnatal day 5 impairs adult reproductive behavior (Nwagwu et al., 2005) and fertility (Hutter and Gibson, 1988) and affects development of monoamine systems in the diencephalon (Siddiqui and Gilmore, 1988; Siddiqui and Shah, 1997). This period of sensitivity to the developmental effects of androgens on these functions may overlap with the establishment of the sexual dimorphism in spinal cord DA, insofar as administration of T to female mice on postnatal day 2 abolishes the sex difference in lumbar spinal cord DA. The presence of AR mRNA in the brain during early embryogenesis (Young and Chang, 1998) and the expression of AR and estrogen receptors in the diencephalon 7 days before birth (Vito and Fox, 1981) support this conclusion.

Orchidectomy in male rats reduces plasma testosterone within several hours of surgery, and testosterone replacement with Silastic capsules returns testosterone levels to those of intact males within 30 minutes (Krey and McGinnis, 1990). Because orchidectomy of adult male mice and T administration to ovariectomized adult female mice did not affect spinal cord DA concentration 1 week following manipulations, a longer time course may be required for an additional activational function of androgens in adults. AR activation changes morphology and connectivity of the SNB in adult animals, without affecting cell numbers (Breedlove and Arnold, 1981), and these effects on measures such as cell soma area require up to 1 month following castration (Hamson et al., 2009) and mimic the time course for castration-induced changes in cell soma area in the sexually dimorphic nucleus of the preoptic area in rats (Dugger et al., 2008). On the other hand, activity of the hypothalamic tuberoinfundibular DA neurons increases within 1 week following castration, an effect that is reversed within 1 day of T replacement (Toney et al., 1991). Because castration-induced changes in nearby hypothalamic DA nuclei occur within 1 week, it is likely that the length of T treatment used in the present study was sufficient to see any potential activational effects in the A_{11} neurons.

The numbers of TH-immunoreactive neurons in the caudal diencephalon in male mice (424 ± 6) in the present study are similar to those described for male rats (448 ± 58; Ondo et al., 2000) and were significantly higher than those found in female mice (349 ± 11). In a companion study, WT male A_{11} cell counts (438 ± 15) were also significantly higher than those of Tfm males (338 ± 19). There was no sex difference in the size of TH^+ neuronal somata, suggesting that AR activation does not affect this morphological property. Moreover, this evidence indicates that cell size did not bias our counts of TH^+ neurons. This result, coupled with the sparse, uneven distribution of A_{11} neurons in both sexes, led us to count consecutive sections rather than use a stereological approach to count cells. To correct for potential double counting of cell profiles, the Abercrombie formula was used as described above. These data suggest that the androgen-dependent sex difference in TH-immunoreactive diencephalic neurons correlates with the observed sex difference in lumbar spinal cord DA concentrations.

If the difference in cell number, like the sex difference in spinal cord DA, is dependent on organizational effects of neonatal androgens as our data suggest, studies in other androgen-dependent sexual differentiated systems

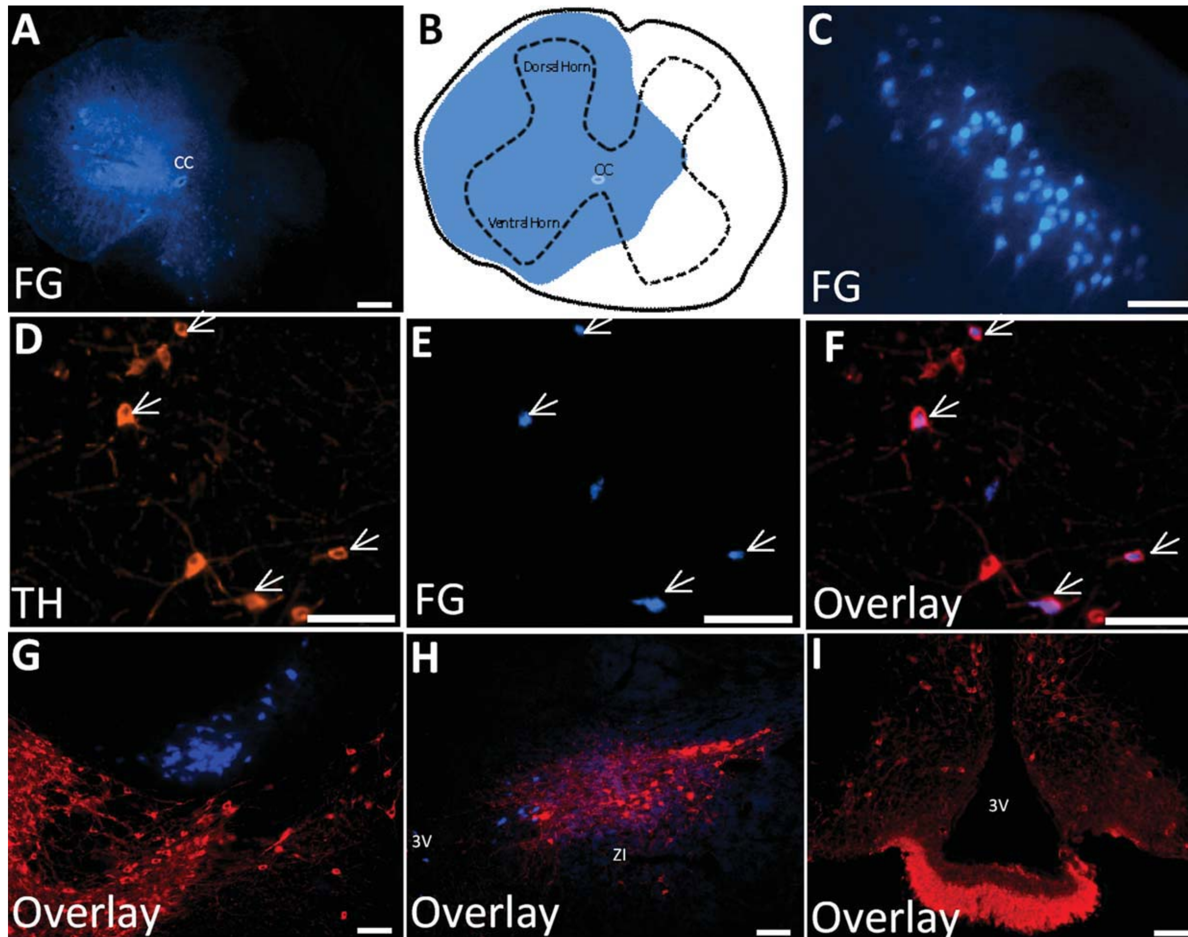

Figure 6. Fluorescence immunohistochemistry following injection of fluorogold (FG) into the lumbar enlargement of the spinal cord. Mice received a unilateral laminectomy in vertebra L1, and 0.5 μl of 4% FG fluorogold was injected into the gray matter of the spinal cord on one side (**A**,**B**). The primary motor cortex (positive control region) includes many large FG-labeled cortical neurons that do not express TH (**C**). TH, FG, and overlay images (**D–F**) in the caudal A_{11} region demonstrate singly and dually labeled neurons that we counted. Other DA systems of the brain such as the A8/9 substantia nigra and A10 ventral tegmental area (G), A13 medial zona incerta (H), and A12 arcuate nucleus (I) contain no apparent double-labeled TH^{+}/FG^{+} cell bodies. CC, central canal; D3V, dorsal third ventricle; ZI, zona incerta; ME, median eminence. Scale bars = 100 μm.

suggest specific hypotheses. For example, if target neurons innervated by the A_{11} DA neurons express AR, then androgen may act upon those target cells to provide trophic support during development to enhance the survival of A_{11} neurons, akin to the effect of androgen acting on target muscles to spare SNB motor neurons from develomental cell death (Nordeen et al., 1985; Freeman et al., 1996; Morris et al., 2004). As SNB motoneurons are putative targets of A_{11} DA neurons in males, AR activation in postsynaptic muscle fibers preserving afferent SNB neurons could extend to the A_{11} DA neurons in the diencephalon. Males with dysfunctional AR are like females in having a reduced amount of DA in their lumbar spinal cords, indicating that masculinization of this DA system is mediated by AR, rather than through the aromatization of T to estrogens and action of estrogen receptors. Because the SNB system is also masculinized via activation of AR rather than estrogen receptors, masculine development of spinal cord DA and the A_{11} DA diencephalospinal neurons may be directly linked to the SNB system. AR acting in the muscles prevents SNB motoneurons from dying, which might in turn prevent A_{11} DA neurons from dying.

The dysfunctional tfm allele did not affect the sex difference in PeVN DA. This result was expected, in that estrogen binding at the estrogen receptor occurs at normal levels in tfm male mice (Attardi et al., 1976), and the PeVN DA sex difference is a result of the aromatization of androgens to estrogens and subsequent binding to estrogen receptors alpha and beta (Simerly et al., 1997; Bodo et al., 2006).

Site of androgen action

AR is widely expressed throughout the brain, including in regions containing DA cell bodies such as the midbrain

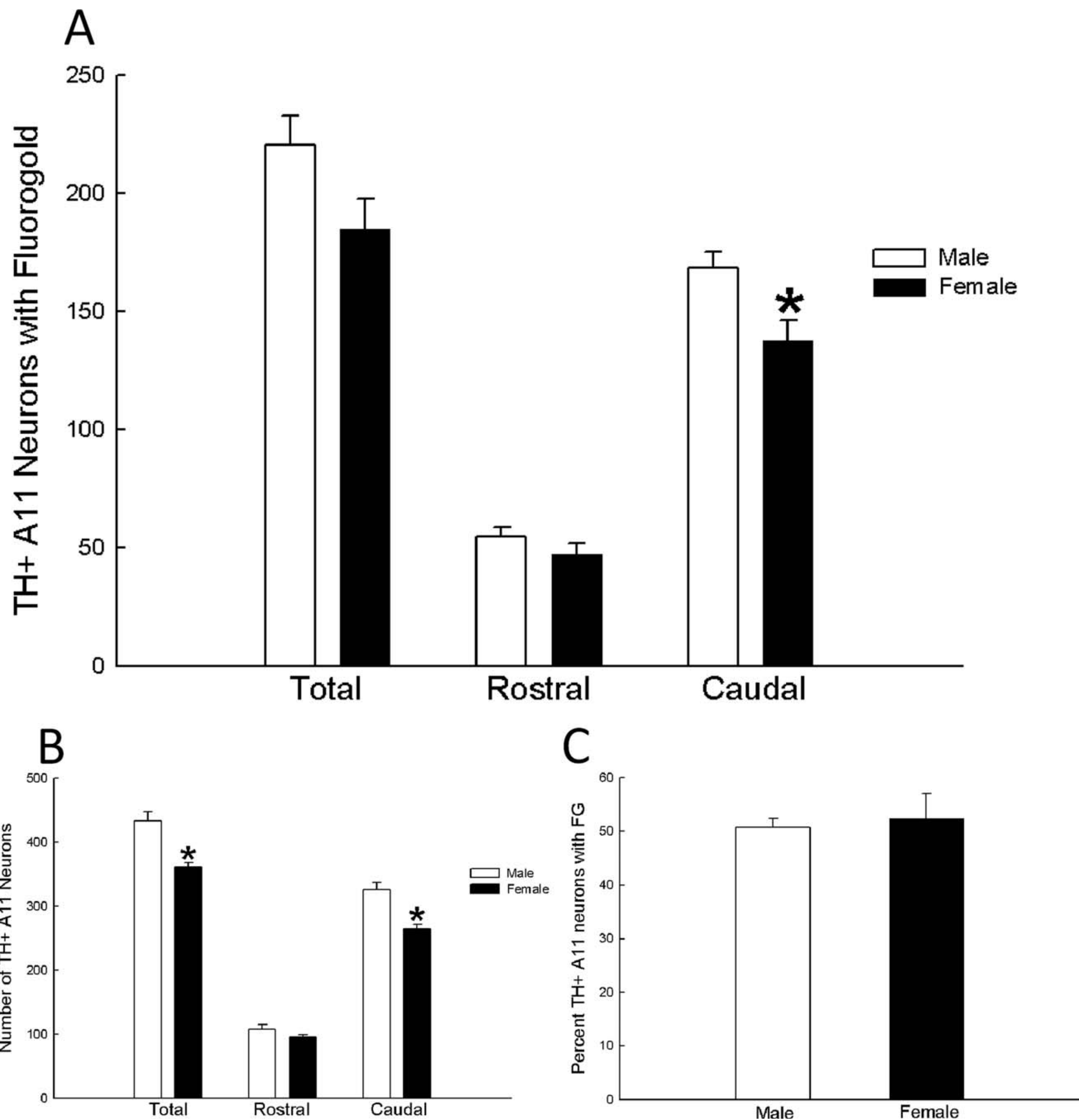

Figure 7. Counts of the number of TH-immunoreactive A_{11} neurons (total, rostral, and caudal) retrogradely identified with fluorogld in the diencephalon of male and female mice (**A**), total number of TH^{+} A_{11} cell counts (**B**) and the percentage of TH^{+} A_{11} neurons retrogradely labeled with fluorogold (**C**). Ten days following lumbar enlargement injection of fluorogold, coronal sections between Bregma –1.9 and -3.0 mm were immunostained for TH. Total TH^{+} A_{11} neurons and those A_{11} neurons containing fluorogold were counted in ImageJ. Values are means $\pm$ SEM based on n = 3 mice/ group). *Female values significantly lower than WT male values ($P < 0.05$, *t*-test). Effect size correlation, r = 0.754 for TH^{+}/FG^{+} caudal counts (A), r = 0.882 for TH^{+} total counts (B), r = 0.876 for TH^{+} caudal counts (B).

substantia nigra and ventral tegmental area (Simerly et al., 1990; Kritzer, bib45; Ravizza et al., 2002; Kritzer and Creutz, 2008) and the hypothalamic arcuate and periventricular nuclei (Heritage et al., 1980; Lorenz et al., 2005). ARs are expressed during both development and adulthood in these regions, and AR immunoreactivity is present in mature astrocytes in the mediobasal hypothalamus in adult rats (Lorenz et al., 2005), but autoradiographic and fluorescence histochemistry studies suggest that there are no AR or estrogen receptors expressed in A_{11} DA neurons (Heritage et al., 1980). Insofar as the sex differences measured in the present study are dependent on the actions of androgens, it is likely that androgens are exerting an effect on neurons afferent or efferent to

the A_{11} region, rather than a direct action on the A_{11} DA neurons. Because AR can transactivate transcriptional activity of the TH gene in a ligand-dependent manner and a putative androgen response element is located upstream of the TH promoter (Jeong et al., 2006), there may also be a direct link between androgen action and TH expression in A_{11} DA neurons.

The sex difference in TH-immunoreactive cell number in the caudal-dorsal diencephalon likely contributes to at least part of the sex difference in spinal cord DA concentrations, inasmuch as lumbar injection of fluorogold leads to significantly more labeled A_{11} neurons in males than in females. Activation of AR may also cause more extensive branching of A_{11} DA axon terminals within the lumbar spinal cord of males, particularly because androgen expands the dendritic arbor of SNB motoneurons (Goldstein et al., 1990), a putative target for such DA-containing nerve terminals. In addition, androgens are known to up-regulate the expression of genes associated with neuronal development and growth (Shugrue and Dorsa, 1993; Lustig, 1994) and enhance the rate of axon and neurite outgrowth in brainstem motor neurons (Yu and Srinivasan, 1981; Yu and Yu, 1983) as well as neurite outgrowth in catecholamine neurons (Reisert et al., 1987).

Distribution of A_{11} TH-immunoreative neurons in the diencephalon

In the present study, the rostral and caudal borders of the diencephalon containing A_{11} DA neurons were 1.9 mm and 3.0 mm posterior to Bregma, respectively. Although a sex difference in the total number of TH-positive neurons was established, a difference in distribution was also observed. A "midregion landmark" was determined to be Bregma –2.4 mm, which is the point where A_{11} DA neurons appear more dorsally. Accordingly, A_{11} DA neurons rostral to this landmark were considered rostral A_{11}, while neurons caudal to the midpoint were classified as caudal A_{11}. This is the first report delineating such an organization of A_{11} DA neurons. No sex difference in numbers of TH-immunoreactive neurons was detected within the rostral A_{11} region, but males had significantly more neurons than females or tfm males in caudal region. Sex differences in DA concentrations are most prominent in the lumbar region of the spinal cord (Pappas et al., 2008), so these results suggest a possible rostrocaudal topographic organization of A_{11} DA neurons in the diencephalon that contributes to the sex difference in DA concentration in the lumbar spinal cord. Injection of the retrograde tract tracer fluorogold into the lumbar enlargement of the spinal cord supports this suggestion, insofar as fluorogold-labeled TH^{+} A_{11} neurons were distributed throughout the entire A_{11} region, but the sex difference in double-labeled neurons was again evident only in the caudal most portion of the A_{11}. Accordingly, there may be a specific subset of neurons in the caudal A_{11} region in male mice not present in females.

Sexually dimorphic function of spinal cord DA

Immunocytochemical and in situ hybridization studies directed toward D2 receptors show strong labeling in the sexually dimorphic SNB and the nearby dorsolateral nucleus (Van Dijken et al., 1996), suggesting the presence of presynaptic A_{11} DA axon terminals. As these motor nuclei innervate sexually dimorphic striated muscles controlling the penis, A_{11} diencephalospinal DA innervation may play a role in male sexual function. Indeed, systemic administration of the DA agonist apomorphine promotes erection (Melis et al., 1987), and intrathecal apomorphine delivered to the lumbosacral spinal cord facilitates erection in spinal cord-injured rats (Giulianu et al., 2002). A_{11} diencephalospinal DA innervation to sexually dimorphic motor nuclei in the lumbosacral spinal cord likely underlies the sex difference in DA concentrations in the spinal cord observed in the present study, but a specific functional role for such a projection has not been determined. Sex differences in DA innervation to the spinal cord may also underlie sex differences in pain thresholds, response to analgesia, and tolerance for noxious stimuli that have been observed in humans (Unruh, 1996; Berkley, 1997; Hurley and Adams, 2008) and rodents (Archer, 1975; Kim et al., 1999; Mogil et al., 2000). The sex bias in response to pain has been attributed to differences in both central and spinal nociceptive modulation by opioids (Kepler et al., 1989, 1991; Cicero et al., 1996; Kest et al., 2000; Claiborne et al., 2006) and spinal activation of α2-adrenoreceptors (Thompson et al., 2008). Activation of DA receptors in the spinal cord and stimulation of A_{11} DA neurons have inhibitory, antinociceptive effects (Jensen and Yaksh, 1984; Fleetwood-Walker et al., 1988; Wei et al., 2009), so lower spinal cord DA innervation in females may also contribute to the lower pain tolerance in females. Further investigation of this sexually dimorphic DA neuronal system may elucidate its specific modulatory functions and allow for manipulations of the systems that it regulates.

ACKNOWLEDGMENT

The authors thank Ms. Kathy Lookingland for assistance in creating Figure 4.

LITERATURE CITED

Archer J. 1975. Rodent sex differences in emotional and related behavior. Behav Biol 14:451–479.

Attardi B, Geller LN, Ohno S. 1976. Androgen and estrogen receptors in brain cytosol from male, female, and testicular feminized (tfm/y hermaphrodite) mice. Endocrinology 98: 864–874.

Barasi S, Duggal N. 1985. The effect of local and systemic application of dopaminergic agents on tail flick latency in the rat. Eur J Pharmacol 117:287–294.

Barbeau H, Rossignol S. 1991. Initiation and modulation of the locomotor pattern in the adult chronic spinal cat by noradrenergic, serotonergic, and dopaminergic drugs. Brain Res 546:250–260.

Barriere G, Mellen N, Cazalets JR. 2004. Neuromodulation of the locomotor network by dopamine in the isolated spinal cord of newborn rat. Eur J Neurosci 19:1325–1335.

Berkley KJ. 1997. Sex differences in pain. Behav Brain Sci 20: 371–380.

Bodo C, Kudwa AE, Rissman EF. 2006. Both estrogen receptor-alpha and -beta are required for sexual differentiation of the anteroventral periventricular area in mice. Endocrinology 147:415–420.

Borisova NA, Sapronova AY, Proshlyakova EV, Ugrumov MV. 1991. Ontogenesis of the hypothalamic catecholaminergic system in rats: synthesis, uptake and release of catecholamines. Neuroscience 43:223–229.

Breedlove SM, Arnold AP. 1980. Hormone accumulation in a sexually dimorphic motor nucleus in the rat spinal cord. Science 210:564–566.

Breedlove SM, Arnold AP. 1981. Sexually dimorphic motor nucleus in the rat lumbar spinal cord: response to adult hormone manipulation, absence in androgen insensitive rats. Brain Res 225:297–307.

Carlsson A. 1975. Monoamine-depleting drugs. Pharmacol Ther B1:393–400.

Cicero TJ, Nock B, Meyer ER. 1996. Gender-related differences in the antinocicptive properties of morphine. J Pharmacol Exp Ther 279:767–773.

Claiborn J, Nag S, Mokha SS. 2006. Activation of opioid receptor like-1 receptor in the spinal cord produces sex-specific antinociception in the rat: estrogen attenuates antinociception in the female, whereas testosterone is required for the expression of antinociception in the male. J Neurosci 26:13048–13053.

Clemens S, Rye D, Hochman S. 2006. Restless legs syndrome revisiting the dopamine hypothesis from the spinal cord perpective. Neurology 67:125–130.

Commissiong JW, Galli CL, Neff NH. 1978. Differentiation of dopaminergic and noradrenergic neurons in rat spinal cord. J Neurochem 30:1095–1099.

Daan S, Damassa D, Pittendrigh CD, Smith ER. 1975. An effect of castration and testosterone replacement on a circadian pacemaker in mice (*Mus musculus*). Proc Natl Acad Sci U S A 72:3744–3747.

Dahlstrom A, Fuxe K. 1964. Evidence for the existence of monoamine neurons in the central nervous system. I. Demonstration of monoamines in the cell bodies of brain stem neurons. Acta Physiol Scand 62(Suppl 232):1–55.

Davis EC, Popper P, Gorski RA. 1996. The role of apoptosis in sexual differentiation of the rat sexually dimorphic nucleus of the preoptic area. Brain Res 734:10–18.

Demarest KW, McKay DW, Riegle GD, Moore KE. 1981. Sexual differences in tuberoinfundibular dopamine nerve activity induced by neonatal androgen exposure. Neuroendocrinology 81:222–227.

Dugger BN, Morris JA, Jordan CL, Breedlove SM. 2008. Gonadal steroids regulate neural plasticity in the sexually dimorphic nucleus of the preoptic area of adult male and female rats. Neuroendocrinology 88:17–24.

Fishman RB, Chism L, Firestone GL, Breedlove SM. 1990. Evidence fo androgen receptors in sexually dimorphic perineal muscles of neonatal male rats. Absence of androgen accumulation by the perineal motoneurons. J Neurobiol 21: 694–704.

Fleetwood-Walker SM, Hope PJ, Mitchell R. 1988. Antinociceptive actions of descending dopaminergic tracts on cat and rat dorsal horn somatosensory neurons. J Physiol 399: 335–348.

Forger NG, Breedlove SM. 1986. Sexual dimorphism in human and canine spinal cord: role of early androgen. Proc Natl Acad Sci U S A 83:7527–7531.

Franklin KBJ, Paxinos G. 1997. The mouse brain in stereotaxic coordinates. San Diego, CA: Academic Press.

Freeman LM, Watson NV, Breedlove SM. 1996. Androgen spares androgen-insensitive motoneurons from apoptosis in the spinal nucleus of the bulbocavernosus in rats. Horm Behav 30:424–433.

Gao X, Zhang Y, Wu G. 2001. Effects of dopaminergic agents on carrageenan hyperalgesia after antrathecal administration to rats. Eur J Pharmacol 418:73–77.

Giulianu F, Allard J, Rampin O, Droupy S, Benoit G, Alecandre L, Bernabe J. 2002. Pro-erectile effect of systemic apomorphine: existence of a spinal site of action. J Urol 167: 402–406.

Goldstein LA, Kurz EM, Sengelaub DR. 1990. Androgen regulation of dendritic growth and retraction in the development of a sexually dimorphic spinal nucleus. J Neurosci 10: 935–946.

Guillery RW. 2002. On counting and counting errors. J Comp Neurol 447:1–7.

Hamson DK, Morris JA, Breedlove SM, Jordan CL. 2009. Timecourse of adult castration-induced changes in soma size of motoneurons in the rat spinal nucleus of the bulbocavernosus. Neurosci Lett 454:148–151.

Hedeman LS, Shellenberger MK, Gordon JH. 1974. Studies in experimental spinal cord trauma. Part I: alterations in catecholamine levels. J Neurosurg 40:37–43.

Heritage AS, Stumpf WE, Sar M, Grant LD. 1980. Brainstem catecholamine neurons are target sites for sex steroid hormones. Science 207:1377–1379.

Holstege JC, Van Dijken H, Buijs RM, Goedknegt H, Gosens T, Bongers CMH. 1996. Distribution of dopamine immunoreactivity in the rat, cat, and monkey spinal cord. J Comp Neurol 376:631–652.

Hurley RW, Adams MC. 2008. Sex, gender, and pain: an overview of a complex field. Anesth Analg 107:309–317.

Hutter HS, Gibson MJ. 1988. Effect of neonatal androgenization on positive feedback in female mice. Biol Reprod 38: 636–638.

Jensen TS, Yaksh TL. 1984. Effects of an intrathecal dopamine agonist, apomorphine on thermal and chemical evoked noxious responses in rats. Brain Res 296:285–293.

Jeong H, Kim M, Kwon J, Kim K, Seol W. 2006. Regulation of the transcriptional activity of the tyrosine hydroxylase gene by androgen receptor. Neurosci Lett 396:57–61.

Kepler KL, Kest B, Kiefel JM, Cooper ML, Bodnar RJ. 1989. Roles of gender, gonadectomy and estrous phase in the analgesic effects of intracerebroventricular morphine in rats. Pharmacol Biochem Behav 34:119–127.

Kepler KL, Standifer KM, Paul D, Kest B, Pasternak GW, Bodnar RJ. 1991. Gender effects and central opioid analgesia. Pain 45:87–94.

Kest B, Sarton E, Dahan A. 2000. Gender differences in opioid-mediated analgesia: animal and human studies. Anesthesiology 93:539–547.

Kim SJ, Calejesana AA, Lia P, Weia F, Zhuoa M. 1999. Sex differences in late behavioral response to subcutaneous formalin injection in mice. Brain Res 829:185–189.

Krey LC, McGinnis MY. 1990. Time-courses of the appearance/disappearance of nuclear androgen + receptor complexes in the brain and adenohypophysis following testosterone administration/withdreawal to castrated male rats: relationships with gonadotropin secretion. J Steroid Biochem 35:403–408.

Kritzer MF. 1997. Selective colocalization of immunoreactivity for intracellular gonadal hormone receptors and tyrosine hydroxylase in the ventral tegmental area, substantia nigra, and retrorubral fields in the rat. J Comp Neurol 379: 247–260.

Kritzer MF, Creutz LM. 2008. Region and sex differences in constituent dopamine neurons and immunoreactivity for intracellular estrogen and androgen receptors in mesocortical projections in rats. J Neurosci 28:9525–9535.

Levant B, McCarson KE. 2001. D3 dopamine receptors in rat spinal cord: implications of sensory and motor function. Neurosci Lett 303:9–12.

Lindley SE, Gunnet JW, Lookingland KJ, Moore KE. 1990. 3,4-Dihydroxyphenylacetic acid concentrations in the intermediate lobe and neural lobe of the posterior pituitary gland as an index of tuberohypophysial dopaminergic neuronal activity. Brain Res 506:133–138.

Lookingland KJ, Moore KE. 2005. In: Dunnett SB, editor. Functional anatomy of hypothalamic dopaminergic neuroendocrine systems. Handbook of Chemical Neuroanatomy, vol20. Amsterdam: Elsevier. p433–521.

Lorenz B, Garcia-Segura LM, Doncarlos LL. 2005. Cellular phenotype of androgen receptor-immunoreactive nuclei in the developing and adult rat brain. J Comp Neurol 492: 456–468.

Lowry OH, Rosebrough NJ, Farr AL, Randall RJ. 1951. Protein measurement with the Folin phenol reagent. J Biol Chem 193:265–275.

Lustig RH. 1994. Sex hormone modulation of neural development in vitro. Horm Behav 28:383–395.

Magnusson T. 1973. Effect of chronic transection on dopamine, noradrenaline, and 5-hydroxytryptamine in the rat spinal cord. Naunyn-Schmiedeberg Arch Pharmacol 278: 13–22.

Melis MR, Argiolas A, Gessa GL. 1987. Apomorphine-induced pernile erection and yawning: site of action in the brain. Brain Res 415:98–104.

Mogil JS, Chesler EJ, Wilson SG, Juraska JM, Sternberg WF. 2000. Sex differences in thermal nociception and morphine antinociception in rodents depend on genotype. Neurosci Biobehav Rev 24:375–389.

Monks DA, O'Bryant EL, Jordan CL. 2004. Androgen receptor immunoreactivity in skeletal muscle: enrichment at the neuromuscular junction. J Comp Neurol 473:59–72.

Moore KE, Lookingland KJ. 1995. Dopaminergic systems in the hypothalamus. In: Meltzer H, editors. Psychopharmacology: the fourth generation of progress. New York: Raven Press. p245–256.

Morris JA, Jordan CL, Breedlove SM. 2004. Sexual differentiation of the vertebrate nervous system. Nat Neurosci 7: 1034–1039.

Mouchet P, Guerin B, Feuerstein C. 1982. Dissociate destruction of noradrenaline and dopamine descending projections in the thoracic spinal cord of the rat. Life Sci 30: 373–381.

Niel L, Shah AH, Lewis GA, Mo K, Chatterjee D, Fernando SM, Hong MH, Chang WY, Vollmayr P, Rosen J, Miner JN, Monks DA. 2009. Sexual differentiation of the spinal nucleus of the bulbocavernosus is not mediated solely by androgen receptors in muscle fibers. Endocrinology 150: 3207–3213.

Nordeen EJ, Nordeen KW, Sengelaub DR, Arnold AP. 1985. Androgens prevent normally occurring cell death in a sexually dimorphic spinal nucleus. Science 229:671–673.

Nwagwu MO, Baines H, Kerr JB, Ebling FJP. 2005. Neonatal androgenization of hypogonadal (hpg) male mice does not abolish estradiol-induced FSH production and spermatogenesis. Reprod Biol Endocrinol 3:48.

Ondo WG, He Y, Rajasekaran S, Le WD. 2000. Clinical correlates of 6-hydroxdopamine injections into A_{11} dopaminergic neurons in rats: a possible model for restless legs syndrome. Mov Disord 15:154–158.

Palkovits M. 1973. Isolated removal of hypothalamic or other brain nuclei of the rat. Brain Res 59:449–450.

Pappas SS, Behrouz B, Janis KL, Goudreau JL, Lookingland KJ. 2008. Lack of D2 receptor mediated regulation of dopamine synthesis in A_{11} diencephalospinal neurons in male and female mice. Brain Res 1214:1–10.

Ravizza T, Galanopoulou AS, Veliskova J, Moshe SL. 2002. Sex differences in androgen and estrogen receptor expression in rat substantia nigra during development: an immunohistochemical study. Neuroscience 115:685–696.

Reisert I, Han V, Lieth E, Toran-Allerand D, Pilgrim C, Lauder J. 1987. Sex steroids promote neurite growth in mesencephalic tyrosine hydroxylase immunoreactive neurons in vitro. Int J Dev Neurosci 5:91–98.

Ridet JL, Sandillon F, Rajaofetra N, Geffard M, Privat A. 1992. Spinal dopaminergic system of the rat: light and electron microscopic study using an antiserum against dopamine, with particular emphasis on synaptic incidence. Brain Res 598:233–241.

Shughrue PJ, Dorsa DM. 1993. Gonadal steroids modulate the growth-associated protein GAP-43 (neuromodulin) mRNAin postnatal rat brain. Brain Res Dev Brain Res 73:123–132.

Siddiqui A, Gilmore DP. 1988. Regional differences in the catecholamine content of the rat brain: effects of neonatal castration and androgenization. Acta Endocrinol 118: 483–494.

Siddiqui A, Shah BF. 1997. Neonatal androgen manipulation differentially affects the development of monoamine systems in rat cerebral cortex, amygdala, and hypothalamus. Brain Res Dev Brain Res 98:247–252.

Simerly RB, Swanson LW, Gorski RA. 1985a. The distribution of monoaminergic cells and fibers in a periventricular preoptic nucleus involved in the control of gonadotropin release: immunohistochemical evidence for a dopaminergic sexual dimorphism. Brain Res 330:55–64.

Simerly RB, Swanson LW, Handa RJ, Gorski RA. 1985b. Influence of perinatal androgen on the sexually dimorphic distribution of tyrosine hydroxylase-immunoreactive cells and fibers in the anteroventral periventricular nucleus of the rat. Neuroendocrinology 40:501–510.

Simerly RB, Chang C, Muramatsu M, Swanson LW. 1990. Distribution of androgen and estrogen receptor mRNA-containing cells in the rat brain: an in situ hybridization study. J Comp Neurol 294:76–95.

Simerly RB, Zee MC, Pendleton JW, Lubahn DB, Korach KS. 1997. Estrogen receptor-dependent sexual differentiation of dopaminergic neurons in the preoptic region of the mouse. Proc Natl Acad Sci U S A 94:14077–14082.

Skagerberg G, Lindvall O. 1985. Organization of diencephalic dopamine neurons projecting to the spinal cord in the rat. Brain Res 342:340–351.

Skagerberg G, Bjorklund A, Lindvall O, Schmidt RH. 1982. Origin and termination of the diencephalo-spinal dopamine system in the rat. Brain Res Bull 9:237–244.

Thompson AD, Angelotti T, Nag S, Mokha SS. 2008. Sex-specific modulation of spinal nociception by α2-adrenoceptors: differential regulation by estrogen and testosterone. Neuroscience 153:1268–1277.

Toney TW, Lookingland KJ, Moore KE. 1991. Role of testosterone in the regulation of tuberoinfundibular dopaminergic neurons in the male rat. Neuroendocrinology 54:23–29.

Unruh AM. 1996. Gender variations in clinical pain experience. Pain 65:123–167.

Van den Pol AN, Herbst RS, Powell JF. 1984. Tyrosine hydroxylase-immunoreactive neurons of the hypothalamus: a light and electron microscopic study. Neuroscience 13: 1117–1156.

Van Dijken H, Dijk J, Voom P, Holstege JC. 1996. Localization of dopamine D2 receptor in rat spinal cord identified with immunocytochemistry and in situ hybridization. Eur J Neurosci 8:621–628.

Vito CC, Fox TO. 1981. Androgen and estrogen receptors in embryonic and neonatal rat brain. Brain Res 254:97–110.

Wei H, Viisanena H, Pertovaara A. 2009. Descending modulation of neuropathic hypersensitivity by dopamine D2 receptors in or adjacent to the hypothalamic A_{11} cell group. Pharmacol Res 59:355–363.

Yokoyama C, Okamura H, Nakajima T, Taguchi J, Ibata Y. 1994. Autoradiographic distribution of [^{3}H]YM-09151-2, a high-affinity and selective antagonists ligand for the dopamine D2 receptor group, in the rat brain and spinal cord. J Comp Neurol 344:121–136.

Young WJ, Chang C. 1998. Ontogeny and autoregulation of androgen receptor mRNA expression in the nervous system. Endocrine 9:79–88.

Yu WH, Srinivasan R. 1981. Effect of testosterone and 5alpha-dihydrotestosterone on regeneration of the hypoglossal nerve in rats. Exp Neurol 71:431–435.

Yu WH, Yu MC. 1983. Acceleration of the regeneration of the crushed hypoglossal nerve by testosterone. Exp Neurol 80: 349–360.

Zhu H, Clemens S, Sawchuk M, Hochman S. 2007. Expression and distribution of all dopamine receptor subtypes (D1–D5) in the mouse lumbar spinal cord: a real-time polymerase chain reaction and non-autoradiographic in situ hybridization study. Neuroscience 149:885–897.

Zuloaga DG, Morris JA, Jordan CL, Breedlove SM. 2008. Mice with the testicular feminization mutation demonstrate a role for androgen receptors in the regulation of anxiety-related behaviors and the hypothalamic-pituitary-adrenal axis. Horm Behav 54:758–766.