Neuronal Nitric Oxide Synthase and Calbindin Delineate Sex Differences in the Developing Hypothalamus and Preoptic Area

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Abstract

Throughout the hypothalamus there are several regions known to contain sex differences in specific cellular, neurochemical, or cell grouping characteristics. The current study examined the potential origin of sex differences in calbindin expression in the preoptic area and hypothalamus as related to sources of nitric oxide. Specific cell populations were defined by immunoreactive (ir) calbindin and neuronal nitric oxide synthase (nNOS) in the preoptic area/anterior hypothalamus (POA/AH), anteroventral periventricular nucleus (AVPv), and ventromedial nucleus of the hypothalamus (VMN). The POA/AH of adult mice was characterized by a striking sex difference in the distribution of cells with ir-calbindin. Examination of the POA/AH of androgen receptor deficient Tfm mice suggests that this pattern was in part androgen receptor dependent, since Tfm males had reduced ir-calbindin compared with wild-type males and more similar to wild-type females. At P0 ir-calbindin was more prevalent than in adulthood, with males having significantly more ir-calbindin and nNOS than have females. Cells that contained either ir-calbindin or ir-nNOS in the POA/AH were in adjacent cell groups, suggesting that NO derived from the enzymatic activity of nNOS may influence the development of ir-calbindin cells. In the region of AVPv, at P0, there was a sex difference with males having more ir-nNOS fibers than have females while ir-calbindin was not detected. In the VMN, at P0, ir-nNOS was greater in females than in males, with no significant difference in ir-calbindin. We suggest that NO as an effector molecule and calbindin as a molecular biomarker illuminate key aspects of sexual differentiation in the developing mouse brain.

INTRODUCTION

Throughout the hypothalamus there are several regions known to contain sex differences in specific cellular, neurochemical, or cell grouping characteristics (reviewed Tobet and Fox, 1992). One set of primary factors that contribute to brain sexual differentiation are steroid hormones that are produced after the development of the gonads and act directly in the developing brain (Carrer and Cambiasso, 2002; Tobet, 2002; Morris et al., 2004; Simerly,

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2005). In addition, the actions of genes located on the sex chromosomes are being noted with increasing frequency as potential initiating mechanisms for the differentiation of sex differences in brain (Arnold, 2004). The means by which sex differences develop may be due to differences in virtually all known neurodevelopmental mechanisms, including neurogenesis, migration, cell fate determination, or cell death/survival between males and females (reviewed in Tobet and Hanna, 1997; Tobet, 2002; Simerly, 2002).

There are several ways to categorize the molecular mechanisms that drive brain development with or without sexual differentiation. One class of molecules that control gene expression is transcription factors. For example, the transcription factor steroidogenic factor-1 (SF-1) is essential for development of the ventromedial nucleus of the hypothalamus (VMN; Ikeda et al., 1995; Davis et al., 2004) while the transcription factors SIM1 and ARNT2 are important for the development of the paraventricular nucleus of the hypothalamus (PVN; Michaud et al., 1998, 2000; Hosoya et al., 2001). The expression pattern of other transcription factors suggests roles in the differentiation of regions that are compelling, but less clear in their definition of selective cell groups (e.g., estrogen receptors (ER), Brown et al., 1999; COUP-TF1, Pereira et al., 2000; islet-1, Davis et al., 2004).

Complementary to transcription factors are another class of molecules that could be referred to as effector molecules, which control and contribute to signaling from one cell to another. Of those molecules that might be considered "effectors," the neurotransmitter γ-aminobutyric acid (GABA) might be particularly important in hypothalamic development (McCarthy et al., 2002; McClellan et al., 2006). Another potential molecular effector of differentiation is nitric oxide (NO), which is a product of the enzymatic conversion of L-arginine to citrulline. NO plays many roles in development as well as adulthood. NO helps direct cell migration (Bicker, 2005; Bulotta et al., 2005), cell proliferation (Ciani et al., 2006), and survival (Contestabile and Ciani, 2004), which are all important factors for sexual differentiation. NO is produced by three forms of nitric oxide synthase (NOS): neuronal (nNOS), inducible (iNOS), and endothelial (eNOS) (reviewed Mungrue et al., 2003; Huang, 2004). Since NO is a gaseous molecule that is difficult to isolate *in vivo*, many studies evaluate NOS expression as an indicator of the location of NO production. Sex differences in the adult preoptic area/anterior hypothalamic region (POA/AH) have been found for nNOS mRNA-positive cells in the rat (Ishihara et al., 2002) and immunoreactive (ir) nNOS cells in the mouse (Scordalakes et al., 2002). In the current study we examined the distribution of ir-nNOS in developing mice to determine if they are in position to contribute to the development of sex differences in the hypothalamus.

A third class of molecules, those which might be called "biomarkers" of sexual differentiation, have undetermined functions in development. A potentially important sexually dimorphic biomarker is the calcium binding protein calbindin (a 28 kDa EF hand protein). Calbindin expression is sexually dimorphic in the preoptic area or hypothalamus of the rat (Brager et al., 2000; Sickel and McCarthy, 2000; Scallet et al., 2004; Pei et al., 2006) and rabbit (Bisenius et al., 2006). Although the exact function of calbindin remains unclear, one of the primary suggestions for the function of calbindin has been a potential involvement in cell survival or death (Lephart, 1996; Sickel and McCarthy, 2000). In the current study we examined the distribution of ir-calbindin in developing mice to determine whether this biomarker could be used to follow the development of sex differences in the hypothalamus. To determine the hormone receptor dependence of expression for this key biomarker, we examined the sex difference in the POA/AH distribution of calbindin in androgen receptor disrupted (*Tfm*) mice.

In the current study we focused on three regions characterized in previous studies for sex differences and neuroendocrine functions. Rostrally we examined the region of the

anteroventral periventricular nucleus (AVPv) of the preoptic area (Simerly et al., 1985; Sumida et al., 1993; Lund et al., 2000) that we have previously characterized for a unique apposition of cells containing estrogen receptor alpha (ERα) versus islet-1 (Davis et al., 2004). More caudally, we examined the boundary region at the caudal POA/AH that we have previously characterized for developmental sex differences in mice for ERβ and GABA$_B$R1 (Wolfe et al., 2005). Finally, in the medial basal hypothalamus, we analyzed the VMN where ongoing studies are characterizing factors that control its development (McClellan et al., 2006). The results show selective sex differences in the expression of either calbindin or nNOS in the POA/AH, AVPv region, and the VMN.

METHODS

Animals

Thy1-YFP mice generated by Feng et al. (2000) were purchased from Jackson Laboratories (Bar Harbor, ME; referred to as B6.Cg-Tg(Thy1-YFP)16Jrs/J). Mice were housed in plastic cages with bedding (autoclaved sanichips, Harlan Teklad, Madison, WI) in a 14:10 light–dark cycle (lights on 07:00). Mice were provided standard rodent chow (#8640, Harlan Teklad, Madison, WI) and water *ad libitum*, at Colorado State University's laboratory animal facility. Pups taken within 24 h of birth, postnatal day 0 (P0), were anesthetized using cryoanesthesia. Sex was established by direct visual inspection of the gonads. Animals were transcardially perfused with 5 mL of 4% formaldehyde (Polysciences Inc., Warrington, PA) and 16% methanol-free formaldehyde stock in 0.1M phosphate buffer (PB, pH 7.4) using a 27-gauge needle hand-held syringe under constant observation with a dissecting scope. The heads were post-fixed overnight in 4% formaldehyde, and stored in 0.1M PB prior to sectioning in the coronal plane.

Tfm mice were generated at the University of Virginia by mating females carrying the Tfm mutation, originally purchased from Jackson Laboratory (C57BL/6J-A^{w-J}-Ta +/+ Ar^{Tfm}), with WT males, all mice were in a C57BL/6J background. To distinguish Tfm, wild type, and carrier females, the offspring were screened by PCR amplification of tail DNA as previously described (Scordalakes and Rissman, 2004). The mice were maintained on a 12:12 h light–dark cycle (lights off at 1200 h EDT). Food (Harlan Teklad Mouse/Rat Sterilizable Diet #7012) and water were provided *ad libitum*. Tfm animals between the ages of 18–21 days were taken from their mother at time of weaning. Animals were anesthetized using an overdose of sodium pentobarbital. Animals were perfused with heparinized-saline solution (~10 mL) followed by 4% paraformaldehyde (40 mL). After perfusions, brains were removed and post-fixed in 4% paraformaldehyde for 1 h, then placed in 30% sucrose overnight. Cryoprotected brains were frozen in 2-methylbutane and stored at −70°C until sectioning. Tissue was cut in a coronal plane at 30 μm on a cryostat. Alternating sections were collected into vials and stored at −20°C in antifreeze until processing. All animal procedures were approved by the appropriate Institutional Animal Care and Use Committee.

Immunocytochemistry

P0 brains were dissected out of the skull, embedded in 5% agarose, and sectioned at 50 μm using a vibrating microtome (Leica VT-1000S). ir-nNOS was determined using a rabbit polyclonal C-terminal directed antiserum acquired from ImmunoStar Inc. (Hudson, WI; diluted1/10,000). ir-Calbindin was determined using a mouse monoclonal D-28K directed antiserum from Sigma-Aldrich (Saint Louis, MO; diluted 1/40,000). Controls for nNOS and calbindin immunocytochemistry included exclusion of primary antibody, in which case no reaction products were detected. Other controls included comparing antibodies from different sources. ir-Calbindin using the mouse monoclonal D-28K from Sigma-Aldrich was compared with a rabbit polyclonal affinity purified D-28K calbindin antiserum from

Chemicon International (Temecula, CA; diluted 1/2500). nNOS C-terminal antiserum from Immunostar Inc. was compared with a rabbit polyclonal N-terminal nNOS antiserum from Zymed Laboratories, Inc. (San Francisco, CA; diluted 1/1000). In both cases, immunoreactivity was similar among antisera directed against the same protein. As an additional control for immunoreactive nNOS, we used brain sections from mice in which Gyurko et al. (2002) deleted exon 6 of the nNOS gene, in these mice ir-nNOS was not detected.

Immunocytochemical procedures were conducted on free-floating sections in a similar manner to those previously reported (Davis et al., 2004; Wolfe et al., 2005). In brief, the tissue sections were pretreated at 4°C with $0.1 M$ glycine in $0.05 M$ phosphate-buffered-saline (PBS; pH 7.5) for 30 min and 0.5% sodium borohydride in PBS for 15 min. For tissue sections assigned for ir-calbindin, an antigen retrieval procedure was used to enhance the immunoreactive signal (Dellovade et al., 2001). To perform antigen retrieval, sections were washed in $0.05 M$ sodium citrate (pH 8.6) for 1 h at room temperature (RT) and then washed in preheated $0.05 M$ sodium citrate for 30 min at 80°C. Sections were returned to RT and washed thrice in PBS for about 5 min. All tissue sections were subsequently washed in 5% normal goat serum (NGS) with 0.3% TritonX-100 (Tx) and 1% H202 in PBS for a minimum of 30 min. Sections were then incubated with primary antibodies diluted with 1% BSA and 0.3% Tx in PBS over 2–3 nights at 4°C.

After primary antibody incubation, tissue sections were washed at RT four times in PBS containing 1% NGS and 0.02% Tx for 15 min each and incubated in the proper secondary antibody diluted with 1% NGS and 0.32% Tx in PBS for 2 h. Donkey anti-rabbit IgG biotinylated secondary antibody from Jackson ImmunoResearch (West Grove, Pennsylvania; diluted 1/500) was used for ir-nNOS and donkey anti-mouse IgG biotinylated secondary antibody (Jackson ImmunoResearch; diluted 1/2500) was used for ir-calbindin. Sections were subsequently rinsed and incubated in peroxidase-conjugated streptavidin (Jackson ImmunoResearch; diluted 1/2500) in PBS with 0.32% Tx for 1 h. After 1 h of washes using $0.05 M$ Tris-buffered saline (TBS, pH 7.5), sections were placed in a diaminobenzidine (DAB) solution (0.025% 3,3′-DAB, 0.2% nickel ammonium sulfate, and 0.02% hydrogen peroxide in TBS) for 5 min to produce a black reaction product. Following immunocytochemistry, tissue sections were mounted on gelatin-subbed slides, dehydrated, and coverslipped with Permount.

Analysis

Only one coronal section per animal was analyzed for each region. Tfm mice were compared with wild-type litter-mates, and analyzed only in the caudal POA/AH. The immunoreactive markers used defined unique cell subpopulations within the regions of interest. Digital mages (10×) were obtained using a Spot Insight QE camera (Diagnostic Instruments, Sterling Heights, MI) with 1600×1200 pixel resolution, mounted on an Olympus BH-2 photomicroscope. Adobe Photoshop (version CS) was used to optimize contrast in digital images. To ensure that digital image processing did not influence the results, all digital images were contrast adjusted following a standard procedure (levels adjustment followed by "unsharp mask") and investigators were blind to treatment group. Since the distribution of immunoreactive cells varies relative to the angle at which the brain is cut, tissue sections were grouped by angle and referred to as angle A, B, or C (see Fig. 1). Comparisons were only made between brains at matched angles. Data are presented for the angle that most clearly demonstrated significant sex differences.

The POA is located at the rostral tip of the hypothalamus at the base of the diencephalon and stretches dorsally to the anterior commissure (AC). Sections were analyzed only in the caudal POA at the boundary region between the POA and anterior hypothalamus, ventral to

the midline crossing of the AC. Only the side with the most immunoreactivity in each POA/AH section, at angle C (see Fig. 1) was analyzed. Following contrast enhancement, images were cropped along the third ventricle and at the base of the AC. IPLab software (Scanalytics, Fairfax, VA) was used to quantify the clearly defined immunoreactive cells and fibers in the region of interest. The distributions, as well as the total area, of immunoreactive markers were analyzed using a grid system that used a reference point to create columns and rows 100 μm across (Davis et al., 2004; Wolfe et al., 2005; Bisenius et al., 2006). For POA/AH images, the reference point was the point at which the third ventricle meets the AC. The angle of section is a key element in evaluating sex differences in cell grouping in the POA/AH (Tobet and Fox, 1992). For the current study, an angle that contains the caudal aspects of the AC and rostral aspects of the optic chiasm contained the region of the POA/AH that revealed the greatest sex difference. Statistical analyses for each age and marker were based on the number of rows (dorsal–ventral) and columns (medial–lateral) needed to cover the critical region of interest. The size of the grid reflects the area of immunoreactivity analyzed. At P0, calbindin was analyzed in a 6 row × 6 column grid and nNOS was analyzed in a 7 row × 6 column grid. In adulthood, C57BL/6J animals were analyzed in a 7 row × 7 column grid, while Tfm animals and littermate controls were analyzed in a 9 row × 7 column grid. The data as immunoreactive areas were then analyzed using a sex × column (in females using a "group" × column) two-way ANOVA with column (i.e., location) analyzed as a repeated measure (JMP 5.1.2, SAS Institute). Tfm males were compared with wild-type males.

The region of AVPv lies just lateral to the opening of the third ventricle and is in the periventricular zone of the POA. After capturing the image and optimizing the contrast at angle C (see Fig. 1), each of the AVPv image was cropped along the third ventricle and the base of the brain. IPLab software was then used to circle and determine the area of the region of dense fibers, if present, in each image. The average area of the region of dense fibers was used to approximate the same sized area for all sections even when a region of dense fibers was not clearly discernible. The area of the immunoreactive fibers and cells within the circled region was obtained. The data was analyzed using a one-way ANOVA for sex effects (SPSS 11.0). ir-Calbindin was not seen in the AVPv at P0.

The VMN is positioned in the medial hypothalamus, close to the base of the diencephalon and above the arcuate nucleus and median eminence. The VMN, based on shape and position within the forebrain, has been categorized into four major planes moving from rostral to caudal (McClellan et al., 2006). Only the central most region of the VMN at angle B (see Fig. 1) was analyzed. After contrast enhancement, VMN images were adjusted so that the edge of the image was aligned along the third ventricle and at the base of the brain. In contrast to the POA/AH analysis, IPLab software and the grid system were used to define the region of interest to measure the total area of the clearly defined immunoreactive cells and fibers. The reference points for the VMN were the third ventricle and base of the brain. To be considered the first row of the grid, which is along the base of the brain, at least half of the boxes in that row had to contain tissue. In the VMN at P0 calbindin occupied a 5 row × 6 column grid and nNOS occupied a 7 row × 7 column grid. The sections were then separated by sex and analyzed in a one-way ANOVA for total area of immunoreactivity (JMP 5.1.2, SAS Institute).

RESULTS

POA/AH

A striking sex difference in ir-calbindin was seen in the POA/AH of adult mice. In adult C57BL/6J mice, males had a notable cell grouping in the medial region of the POA/AH that was not discernible in females [arrows in Fig. 2(A,B)]. Using a two-way ANOVA with

location as a repeated measure there was a main effect of sex [Fig. 2(C); $F(1,24) = 1.6$, $p < 0.05$; $n = 5$ males, 5 females] with no significant interaction. Nonetheless, the difference was primarily due to the greater than twofold difference occurring 100–300 μm from the third ventricle. In a replication and extension using Tfm mice, there was a significant main effect of group ($F(2,36) = 12.6$, $p < 0.001$; $n = 3$ wild-type males, 3 wild-type females, 3 Tfm males). The majority of the immunoreactive calbindin was located within 400 μm of the third ventricle. Male mice had 3–4 times the amount of ir-calbindin than had females, and had 2–3-fold more ir-calbindin than had Tfm male mice with a disrupted androgen receptor [Fig. 2(D)].

Mice at P0 were used to determine if sex differences arose during development. In the POA/AH, males had significantly more ir-nNOS and ir-calbindin than females (see Fig. 3). There was a significant main effect of sex for ir-nNOS with males having a significantly greater area of immunoreactivity than have females [Fig. 3(C); $F(1, 78) = 4.36$, $p < 0.05$; $n = 9$ males, 6 females]. There was not a significant interaction (sex × location as a repeated measure; $p > 0.40$). Nonetheless, the sex difference in ir-nNOS was most apparent in the medial portion of the POA/AH (~200–400 μm lateral to the third ventricle). The main variations in expression occurred dorsally, just ventral to the AC [arrowheads in Fig. 3(A,B)], and in a ventral stripe of cells [see arrows in Fig. 3(A,B)]. There was a main effect of sex for ir-calbindin with significantly more ir-calbindin in males than in females [Fig. 3(F); $F(1, 48) = 7.84$, $p < 0.01$; $n = 6$ males, 5 females]. Again, there was not a significant interaction (sex × location as a repeated measure; $p > 0.90$). Nonetheless, the cells and fibers with ir-calbindin appeared more densely localized in males between 200 and 400 μm lateral to the third ventricle [Fig. 3(D)], whereas the ir-calbindin was more spread in females [Fig. 3(E)].

There was a striking relationship between the distribution of ir-nNOS and ir-calbindin, shown in both the pseudocolor overlay of adjacent tissue sections immunoreacted for either nNOS (red) or calbindin (green) [Fig. 4(A)], or in double-label immunofluorescence in the same section (similarly nNOS red, calbindin green) [Fig. 4(B)]. These two cell populations distinctly border each other rather than overlap. Dense nNOS-ir (red) was located adjacent to the third ventricle in the dorsal POA/AH. ir-Calbindin (green) was found just lateral and ventral to ir-nNOS. The apposition of these two cell groups formed a pattern in which ir-nNOS surrounded the more interior ir-calbindin.

AVPv

In the rostral POA, in the region of the AVPv, there was notably more ir-nNOS in males than in females (see Fig. 5), although the immunoreactivity itself was light compared to darker cells in the surrounding region. Nonetheless, the sex difference was so striking that the sex of each brain could be determined by looking for the region of dense fibers [arrows in Fig. 5(A,B)]. When quantifying differences, males had a threefold larger immunoreactive area than had females in the same relative region [Fig. 5(C); $F(1, 9) = 11.47$, $p < 0.01$; $n = 6$ males, 5 females]. No ir-calbindin was observed in this region at P0.

VMN

In the more caudal hypothalamus, in the region of the VMN, there was significantly more ir-nNOS cells and fibers in females [Fig. 6(B)] when compared with males [Fig. 6(A); $F(1, 15) = 6.61$, $p < 0.05$; $n = 10$ males, 7 females]. Unlike the POA/AH, the sex difference in the VMN could not be localized to a particular subregion (see Fig. 6). There was not a significant difference in the cells and fibers containing ir-calbindin in the VMN [Fig. 6(F); $p > 0.50$; $n = 4$ males, 5 females].

DISCUSSION

Sex differences in morphology have been described in regions of the hypothalamus and preoptic area of a number of species (Tobet and Fox, 1992). Some of these sex differences are relatable to particular cell phenotypes (e.g., galanin in ferrets; Park et al., 1997 or vasopressin in a number of species; De Vries and Panzica, 2006). We have recently shown that the positions of identified cells at E17 may be one aspect of sex-dependent characteristics during development with potential consequences for longer-term sex differences (particularly ERβ expression; Wolfe et al., 2005). In the current study we examined two additional markers for sex differences in development. Calbindin, serves as a potential biomarker for sex differences in brain structure across several species (Sickel and McCarthy, 2000; Bisenius et al., 2006). The other, nNOS, is an enzyme responsible for the synthesis of NO, which is likely bioactive in the developing brain. Based on the sex-dependent distribution of nNOS and calbindin, NO may be an effector molecule in the determination of selected sex differences in development. Using calbindin as a biomarker for sexual differentiation in the POA/AH, the data in the current study suggests significant androgen actions in the mouse brain based on decreases in Tfm mice. This contrasts with a previous examination of immunoreactive calbindin in the POA/AH of ERαKO mice that did not show differences from wild-type (Hall et al., 2000).

POA/AH

Sex differences in the brain morphology of adult animals often arise as sex differences in development. The sex difference in ir-calbindin in the POA/AH in the current study fits this pattern. However, sex differences in development can sometimes carry forward with a different appearance in adulthood. In both the current study and in a companion study of older mice (Majdic et al., 2006), a much tighter distribution of ir-calbindin cells was a striking characteristic found in adult males relative to adult females. From the perspective of developmental relationship of the cell groups containing ir-calbindin and ir-nNOS, it is important to consider that nNOS generates NO that may function to help create sex differences in other characteristics such as cells containing ir-calbindin (current study) or ERβ (Wolfe et al., 2005). Steroid hormones, acting through ER-α and androgen receptors, play crucial roles in regulating nNOS in adults (Scordalakes et al., 2002). Gonadal steroid hormones may influence sex differences in ir-calbindin by either altering nNOS or by acting directly on cells containing ir-calbindin. We found that androgen receptor defective Tfm male mice had significantly reduced ir-calbindin expression compared to wild-type mice suggesting that androgen receptors may be necessary for the normal male pattern of ir-calbindin in the POA/AH, in agreement with some earlier work in rats (Watson et al., 1998). Since there is more ir-calbindin in the Tfm male than in the wild-type female, other factors must also influence ir-calbindin. Other previous work in rats had suggested that calbindin expression in the rat sexually dimorphic nucleus (SDN) of the POA was fully estrogen dependent (Sickel and McCarthy, 2000). The current result in mice suggests that there may be synergies between androgens and estrogens in the murine POA/AH. An alternative in addition to hormones, may be sex chromosome complement that might also affect sex differences in neural differentiation. For example there is direct evidence for effects of sex chromosome complement on ir-AVP in the laternal septum (Gatewood et al., 2006; De Vries et al., 2002) and for nNOS in the POA (Budefeld et al., Sex differences in brain morphology in agonadal adult steroidogenic factor-1 knockout mice, submitted for publication). Given that the Tfm mice are XY, yet lack functional androgen receptors their ir-calbindin, which is intermediate between WT males and females may be influenced by both hormone-dependent and -independent factors.

The POA/AH is important for regulating homeostatic, neuroendocrine, and behavioral functions. It is a region where hormones dramatically influence development and where

hormone-secreting cells regulate physiology and behavior. This region of the mammalian forebrain is where sex differences are most common (reviewed Tobet and Fox, 1992). Sex differences in peptides, receptors, and protein content have been repeatedly shown in the peripubertal and adult POA/AH (Scouten et al., 1985; Simerly et al., 1985; Micevych et al., 1987; Bloch et al., 1992; 1993; Dubois-Dauphin et al., 1996; Grattan and Selmanoff, 1997; Segarra et al., 1998). Nonetheless, sex differences in the mouse POA/AH have been less well characterized, possibly because some sex differences in mice may be strain dependent (Brown et al., 1999). Unlike the rat, the mouse has not been shown to have a specific sexually dimorphic nuclear group in the POA (Brown et al., 1999). However, the current study demonstrates that ir-calbindin in C57BL/6J mice occupies a similar location in the POA/AH to the rat SDN. The current study shows significantly more of both immunoreactive nNOS and calbindin in male mice compared to females in the POA/AH for animals on a C57BL/6J background.

AVPv

The hypothalamus is comprised of a highly interconnected and integrated series of nuclear groups and scattered neuroendocrine effector cells. Sex differences in one component are often mirrored by sex differences in another (Simerly, 1998; Segovia et al., 1999). For example, the AVPv projects to several regions including the preoptic area and the medial hypothalamus. The POA receives projections from the amygdala and the VMN and projects extensively including to regions thought to aid in neuroendocrine responses. The VMN also has extensive projections to and from the amygdala. Sex differences in various aspects of morphology have been reported in all of these regions. In the region of AVPv, the results of the current study indicated that males had an immunoreactive region of dense fibers three times larger than females. The AVPv has been extensively characterized as sexually dimorphic (Simerly et al., 1985; Sumida et al., 1993; Lund et al., 2000; Lephart et al., 2001; Orikasa et al., 2002; Rubin et al., 2006). While some data suggests late development for aspects of AVPv sexual dimorphism (Davis et al., 1996; Hutton et al., 1998), other evidence, now including the current study, indicates that sex differences are also detectable during perinatal development (Arai et al., 1994; Sumida et al., 1993).

VMN

The VMN plays an important role in regulating female sexual behavior, energy balance, and feeding behavior (reviewed McClellan et al., 2006). The VMN has been described as sexually dimorphic for specific cell types in a number of studies (Ikeda et al., 2003; Sá and Madeira, 2005). The current study found that females have significantly more ir-nNOS when compared with males while there were no apparent differences in the amount of immunoreactive calbindin in the VMN. Interestingly, our study in older mice found sex differences in wild-type mice in the VMN for calbindin with females having more immunoreactive cells than have males (Majdic et al., 2006). Thus, NO may contribute to the development of sex differences in VMN characteristics that only become apparent later in life.

In summary, evidence from a number of sources suggests that the expression of nNOS and calbindin is regulated by steroid hormones. The current study suggests that androgen receptor action is one factor that helps determine calbindin expression in the POA/AH. In the POA/AH, there is an intriguing spatial pattern between cells that contain immunoreactive nNOS and calbindin. As a developmental effector, NO derived from the enzymatic activity of nNOS may thereby influence the development of cells containing ir-calbindin. In the AVPv region, where males have three times the amount of ir-nNOS in fibers compared to females, late developing sex differences in tyrosine hydroxylase (Tobet and Hanna, 1997) or incoming fibers (Hutton et al., 1998) may be influenced. In the VMN

where females have significantly more ir-nNOS when compared to males, VMN characteristics at later ages may be influenced by developmental sex differences in the action of NO.

Acknowledgments

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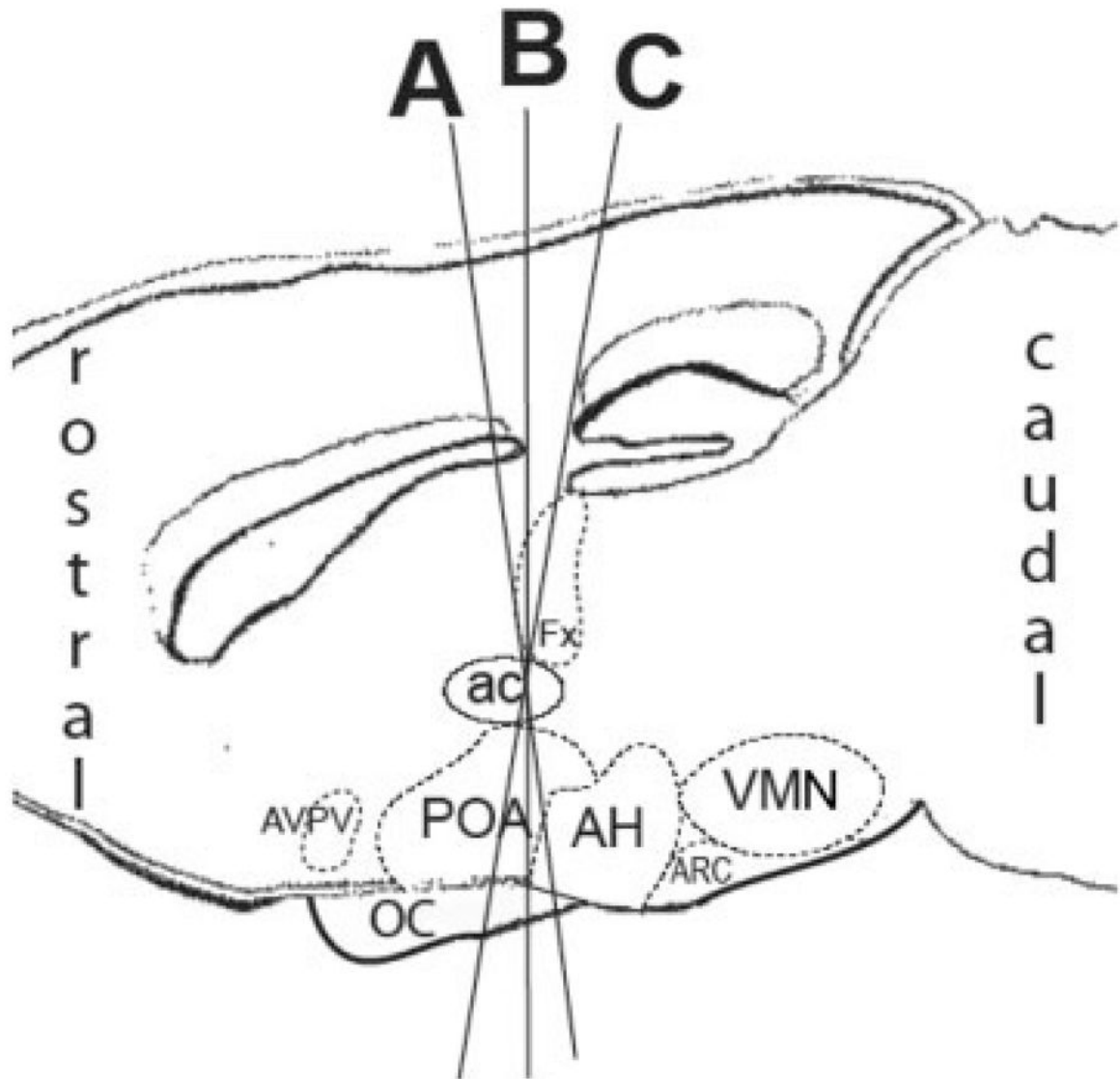
Figure 1.
Sagittal section of the mouse brain illustrates different angles of sectioning (A, B, or C), which create alternate ventral/dorsal alignments of brain regions. In coronal plane A, the preoptic area overlays different regions of the anterior hypothalamus than in coronal plane B or C. AVPV, anteroventral periventricular nucleus of the preoptic area; POA, preoptic area; ac, anterior commisure, OC, optic chiasm; Fx, fornix; AH, anterior hypothalamus; ARC, arcuate; VMN, ventromedial nucleus of the hypothalamus.
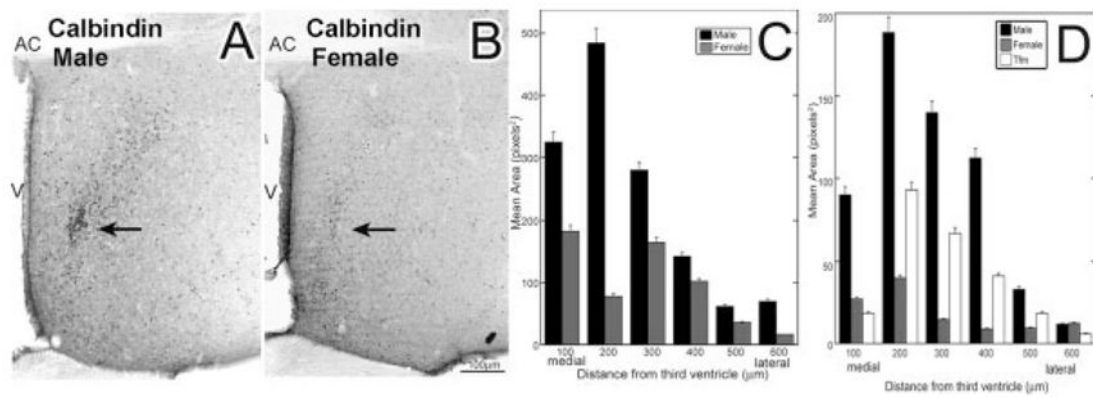
Figure 2.
In the POA/AH, adult C57BL/6J mice, males and females differed in the total number of cells that contained immunoreactive calbindin. Males (A) had more ir-calbindin cells and fibers than females (B), $p < 0.01$ (data are mean ± SEM). Positional analysis shows that the sex difference was primarily due to fourfold differences in the amount of ir-calbindin at 100–200 μm away from the third ventricle (C). Similarly, in the POA/AH of weanling males, females, and Tfm mice, the areas of ir-calbindin differed significantly (D). Wild-type males had three to four times the amount of ir-calbindin than had wild-type females and had 2–3-fold more ir-calbindin than had Tfm mice, whether measured in dorsal/ventral distances away from the AC or in medial/lateral distances from the third ventricle (D), $p < 0.01$ (data are mean ± SEM). AC, anterior commissure; V, third ventricle.
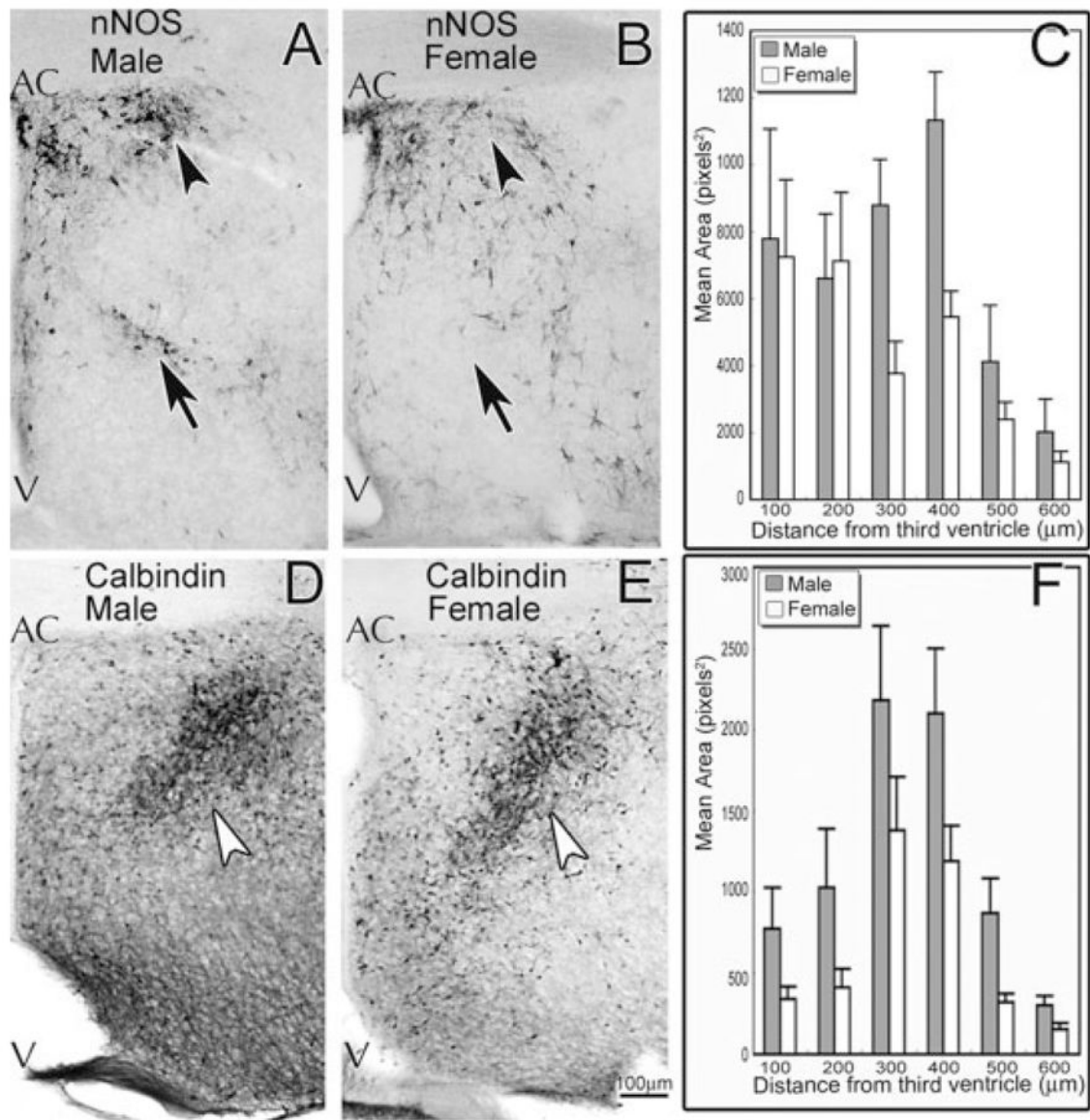
Figure 3.
In the POA/AH at birth (P0) male mice had more immunoreactive nNOS and calbindin cells and fibers than had females. Sex differences were found approximately 200–400 μm lateral to the third ventricle (C) with more ir-nNOS in males (A) than in females (B). Differences in nNOS expression occurred dorsally, ventral to the AC (black arrowheads), and in a ventral stripe of cells that was oriented medial/lateral (black arrows). ir-Calbindin was more densely localized in males (D) compared to more spread in females (E, white arrowheads). Males and females differed in the total number of cells that contained ir-calbindin (F), $p < 0.01$ (data are mean ± SEM). AC, anterior commissure; V, third ventricle.
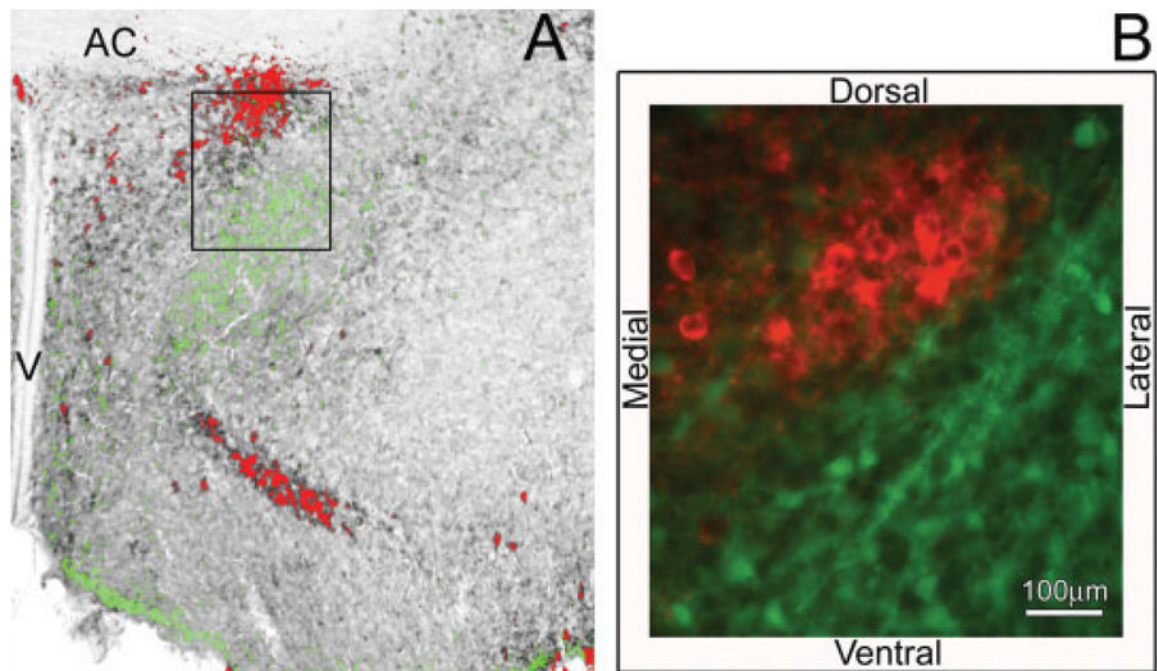
Figure 4.
In the POA/AH, a striking relationship was seen in the apposition of cells containing immunoreactive nNOS and calbindin at P0. A pseudocolor overlay of adjacent sections containing nNOS-ir (red) or calbindin-ir (green) illustrates this apposition (A). The box in the overlay image of panel A depicts the approximate location of the boundary in the fluorescent double-label experiment (B). Dense ir-nNOS (red) was located adjacent to the third ventricle and ir-calbindin (green) was found just lateral and ventral to ir-nNOS.
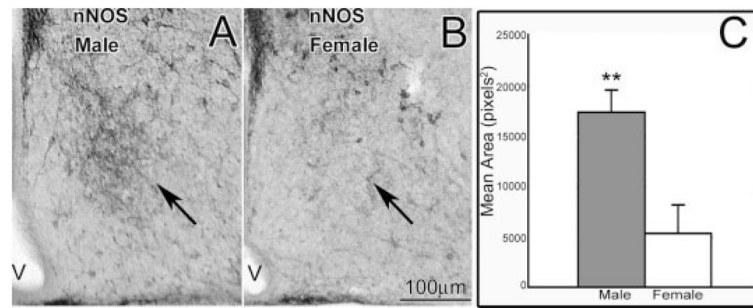
Figure 5.
In the region of AVPv at birth (P0), males (A) and females (B) differed in the amount of fibers containing immunoreactive nNOS. Males had 300% more ir-nNOS than females (C, **$p < 0.01$; data are mean ± SEM). V, third ventricle.
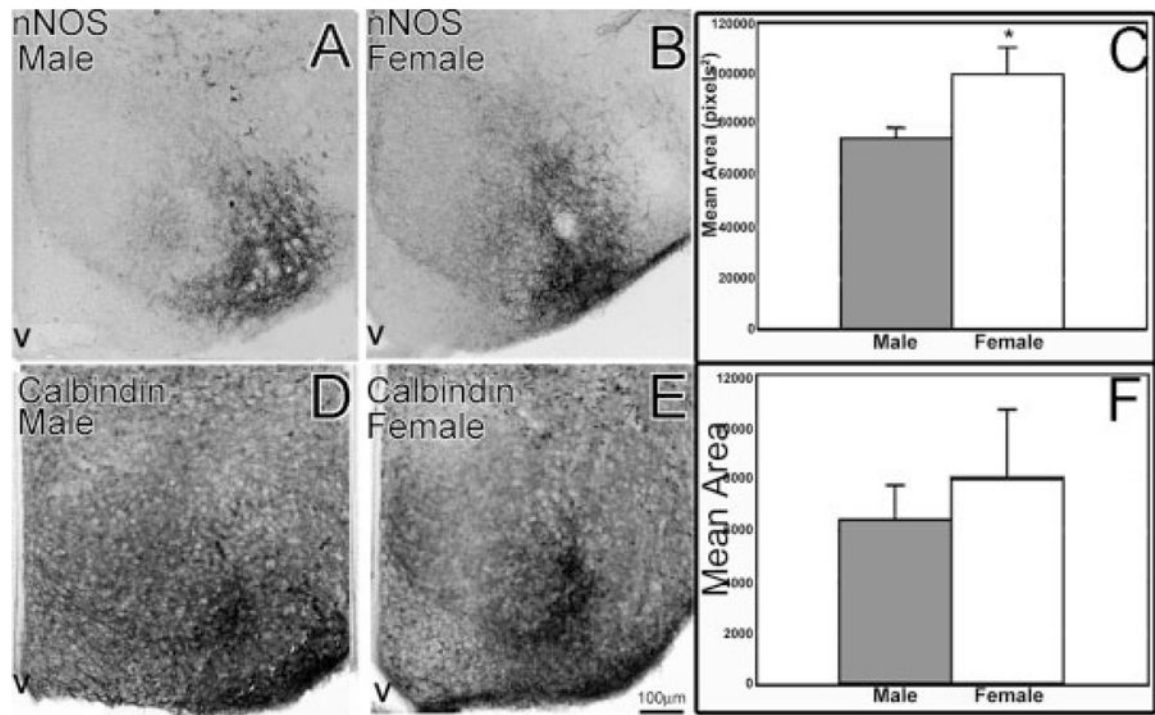
Figure 6.
In the VMN at birth (P0) males and females differed in the amount of cells and fibers containing ir-nNOS, but not ir-calbindin. Females (B) had more ir-nNOS than had males (A) as illustrated in the graph of mean immunoreactive areas (C, $*p < 0.05$; data are mean $\pm$ SEM). There was no significant difference in the amount of ir-calbindin between males (D) and females (E) as depicted by the graph (F). V, third ventricle.