Published in final edited form as:

Dev Neurobiol. 2008 June ; 68(7): 981–995. doi:10.1002/dneu.20638.

Sex differences in brain developing in the presence or absence of gonads

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Abstract

Brain sexual differentiation results from the interaction of genetic and hormonal influences. The current study utilized a unique agonadal mouse model to determine relative contributions of genetic and gonadal hormone influences in the differentiation of selected brain regions. SF-1 knockout (SF-1 KO) mice are born without gonads and adrenal glands, and are not exposed to endogenous sex steroids during fetal/neonatal development. Consequently, male and female SF-1 KO mice are born with female external genitalia and if left on their own, die shortly after birth due to adrenal insufficiency. In the present study, SF-1 KO mice were rescued by neonatal adrenal transplantation to examine their brain morphology in adult life. To determine potential brain loci that might mediate functional sex differences, we examined the area and distribution of immunoreactive calbindin and neuronal nitric oxide synthase in the preoptic area and ventromedial nucleus of the hypothalamus, two areas previously reported to be sexually dimorphic in the mammalian brain. A sex difference in the positioning of cells containing immunoreactive calbindin in a group within the preoptic area was clearly gonad-dependent based on the elimination of the sex difference in SF-1 KO mice. Several other differences in the area of ventromedial hypothalamus and in preoptic area were maintained in male and female SF-1 KO mice, suggesting gonad-independent genetic influences on sexually dimorphic brain development.

Keywords

mouse; brain morphology; steroidogenic factor-1; Calbindin D-28k; neural nitric oxide synthase

INTRODUCTION

Sex differences in brain characteristics have been described for a number of brain regions in many species including rat, ferret, gerbil, guinea pig, mouse and human (Tobet and Fox, 1992). Sex differences range in magnitude from gross volumes of a given brain nucleus to the length and number of axons and dendrites, and neuronal ultrastructure. The organizational hypothesis for brain sexual differentiation predicts that gonadal hormones secreted by testes during perinatal development trigger changes in the fetal brain that result in long lasting, if not permanent, consequences for adult brain structure and function (Morris et al., 2004). Several studies have indicated that genes encoded on sex chromosomes may play important role(s) in brain sexual differentiation independently of gonadal hormone action (Arnold, 2004). Two

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studies have shown linear transcripts for Sry in adult (Lahr et al., 1995) and developing (Mayer et al., 2000) mouse brain detected by RT PCR. More recent *in situ hybridization* and immunocytochemical studies showed expression of the Y-chromosome gene *Sry* in the midbrain in adult male mice and rats as well as direct or indirect regulation of tyrosine hydroxylase by Sry (Dewing et al., 2006). Using a genetic approach to create XY and XX male and female mice, sexually dimorphic vasopressin immunoreactivity in the lateral septum was found to depend upon the sex chromosome complement, as XY males and females showed a masculine pattern for immunoreactive vasopressin in the lateral septum, suggesting that other Y linked genes apart from Sry are also involved in brain sexual differentiation (De Vries et al., 2002).

Steroidogenic factor 1 (SF-1, NR5A1), a member of a nuclear receptor superfamily of transcription factors, is expressed in steroidogenic as well as non-steroidogenic tissues. SF-1 knockout mice (SF-1 KO) are born without gonads and adrenal glands, have nonfunctional gonadotrope cells in the pituitary and an altered organization of the ventromedial hypothalamus (Ikeda et al., 1995; Shinoda et al., 1995). In SF-1 KO mice, gonadal ridges form normally on embryonic day 10.5 (E10.5), but undergo apoptosis immediately thereafter and gonadal ridges disappear by day E12.5 (Ikeda et al., 1994). Consequently, SF-1 KO mice develop female internal and external genitalia irrespective of genetic sex. Expression of steroidogenic enzymes in mouse fetal gonads is present around day E13 in testes (fetal ovaries are hormonally inactive; Greco and Payne, 1994) coincident with the start of testosterone production (Gondos, 1980). SF-1 KO mice are not exposed to endogenous gonadal hormones and therefore represent an excellent model to study gonad-dependent versus gonad-independent brain sexual differentiation in the absence of *SF-1* gene. However, absence of gonads does not necessarily preclude exposure to sex steroids from other sources such as placenta, mother or neighboring male WT or +/- siblings during fetal development, but these sources should affect male and female SF-1 KO fetuses randomly.

To determine potential brain loci that might mediate functional sex differences, we examined the area and distribution of immunoreactive calbindin D28k (calbindin; (Brager et al., 2000) and neuronal nitric oxide synthase (nNOS; (Scordalakes et al., 2002) in the preoptic area (POA) and ventromedial nucleus of the hypothalamus (VMH) in adult WT and SF-1 KO mice. As SF-1 KO mice normally die shortly after birth due to adrenal insufficiency, all SF-1 KO mice were rescued by corticosteroid treatment and adrenal transplantation and maintained into adulthood as described previously (Majdic et al., 2002). Since sex differences in gene expression are often more prominent under the influence of testosterone (Tobet and Fox, 1992), all mice in the current study were treated with testosterone propionate for one week prior to sacrifice. To the extent that SF-1 KO mice are not exposed to endogenous gonadal hormones, sex differences in gene expression between SF-1 KO mice of both sexes are likely due to effects of sex chromosomes.

METHODS

Animals and tissue recovery

C57BL/6J SF-1 heterozygous mice (SF-1^{+/-} backcrossed to C57BL/6J for more than 10 generations and inbred for more than 10 generations) were bred to generate SF-1 KO (SF-1^{-/-}) and littermate wild type (WT) control mice and maintained at 11:13 dark-light cycle with regular chow (Altromin, Lage, Germany) and water *ad libitum*. Normally, SF-1 KO mice die within 24h of birth due to adrenal insufficiency. Therefore, all newborn pups received daily subcutaneous (s.c.) injections of 50µl of corticosteroids in corn oil (400µg/ml hydrocortisone (Sigma, Steinheim, Germany), 40ng/ml dexamethasone (Sigma) and 25ng/ml fludrocortisone acetate (Sigma)) until animals were genotyped on days 6 - 7 postnatally using a PCR assay. Adrenal glands, transplanted into SF-1 KO pups of both sexes, were always from the WT (+/

+) female donors to insure that the adrenals were fully functional (+/- adrenals may be compromised; Bland et al. 2004). Adrenal transplantations were done on postnatal days 7 or 8 as described previously (Majdic et al., 2002). After adrenal transplantation, SF-1 KO animals received four more s.c. injections of corticosteroids until weaning at 21 days. Control WT male and female mice (+/+ genotype) were derived from the same litters as SF-1 KO mice or in rare cases (when no littermates were available) from other age-matched litters, received corticosteroid injections postnatally, and were gonadectomized after weaning before the onset of puberty between P21 and P25. For gonadectomies, WT mice were anesthetized with a mixture of ketamine (Vetoquinol Biowet, Gorzowie, Poland; 100 µg/g BW), xylazine (Chanelle Pharmaceuticals Ltd., Loughrea, Ireland; 10 µg/g BW) and acepromazine (Fort Dodge Animal Health, Fort Dodge, IA, USA; 2 µg/g BW). All mice were weighed once a week until 12 weeks of age. Before sacrifice at 6 months of age, all mice received daily s.c. injections of 50 µl of testosterone-propionate (TP, 2mg/ml; Fluka, Steinheim, Germany) in corn oil vehicle for seven days. On the seventh day, mice were anesthetized with a mixture of ketamine, xylazine and acepromazine and perfused with 4% paraformaldehyde (Sigma) in 0.01M phosphate buffered saline (PBS – pH = 7.4; Sigma). After removal, brains were postfixed in the same fixative overnight at 4°C and then stored until immunocytochemical processing in 0.01M PBS at 4°C. Several mice were sacrificed without TP treatment, but otherwise processed following exactly the same procedure. All animal experiments were done according to ethical principles and in accordance with EU directive (86/609/EEC). Animal experiments were approved by the Veterinary commission of Slovenia and the Animal Care and Use Committee at Colorado State University.

SF-1 genotyping and sex determination

Tissue from mice 6 – 7 days of age were used to determine SF-1 genotype and chromosomal sex. DNA samples were obtained by tail clipping and digested in a thermostatic shaker in 200 µl of PCR DNA buffer (Promega, Madison, WI, USA) containing 0.15 mg of Proteinase K (Sigma) at 55°C overnight. 3 µl of lysate was used for PCR reaction as described before (Luo et al., 1994).

Immunocytochemistry on floating sections

Brains were embedded in 5% agarose (Sigma) and sectioned at 50 µm in cold 0.01M PBS using a vibrating microtome (Integralslice 7550 MM, Campden Instruments, UK). Sections were incubated in 0.1M glycine (Sigma) in 0.01M PBS for 30 min followed by incubation in 0.5% sodium borohydride (Sigma) for 15 min at 4°C. Glycine and sodium borohydride were washed out with 15 min and 20 min washes in 0.01M PBS. Sections were blocked in 5% normal goat serum (Chemicon, Temecula, CA, USA) containing 0.5% Triton X-100 (Promega) and 1% H₂O₂ (Merck, Darmstadt, Germany) for 30 min at 4°C. Mouse primary antibodies against Calbindin D-28k (Sigma) and rabbit primary antiserum against nNOS (Immunostar, Hudson, WI, USA) were diluted 1:20000 and 1:10000, respectively, in 0.01M PBS containing 1% bovine serum albumin (Sigma) and 0.5% Triton X-100. Sections were incubated with primary antibodies over 2-3 nights at 4°C with shaking. Sections were then washed in 0.01M PBS containing 1% normal goat serum and 0.02% Triton X-100 four times 15 minutes at room temperature. Biotinylated secondary antibodies (Jackson ImmunoResearch, West Grove, PA, USA) against primary mouse (1:2500) or rabbit (1:500) antibodies were diluted in 0.01M PBS containing 1% normal goat serum and 0.5% Triton X-100. Sections were incubated with secondary antibodies for two hours, followed by 4 washes (15 minutes each) in 0.01M PBS buffer containing 0.02% Triton X-100. Streptavidin – HRP complex (Jackson ImmunoResearch) was diluted 1:2000 in 0.01M PBS solution containing 0.5% TritonX-100. Sections were incubated with Streptavidin – HRP for 1 hour at room temperature and then washed in Tris-buffered saline (0.05M Tris-HCl/0.9% NaCl; pH 7.5; Sigma) for 1 hour at room temperature. Antigen-antibody complexes were visualized as a black reaction product by

incubating sections in 0.025% DAB/Ni substrate (Sigma) in Tris-buffered saline (pH 7.5) containing 0.02% H₂O₂ for 5 min at room temperature. After mounting, sections were dried and coverslipped using hydrophobic medium (Pertex, Burgdorf, Germany).

Data collection and analyses

Digital images of brain regions of interest were obtained using a Nikon microphot FXA microscope with Sony 3CCD camera. Images were enhanced for contrast using Photoshop software package (Adobe Version 8.0). For nNOS analyses, coronal sections of the region containing the rostral part of the anteroventral periventricular preoptic area (AVPV; all brain anatomical terms and corresponding abbreviations are taken from Paxinos and Franklin, 2001) were taken (0.26 mm rostral from Bregma according to stereotaxic coordinates, (Paxinos and Franklin, 2001)) and calbindin and nNOS immunoreactivity was analyzed in coronal sections containing caudal aspects of the POA at the rostral boundary with the anterior hypothalamus (POA/AH; 0.1 mm caudal from Bregma) under 40× magnification; the third ventricle and base of the brain were considered as reference boundaries. The medial divisions (BSTMPM – posteromedial and BSTMPI – posterointermedial) of the bed nucleus of the stria terminalis (BST) were studied in sections corresponding to coronal section 0.22 mm caudally from Bregma. Digital images were obtained under 60× magnification with the stria medullaris of thalamus and stria terminalis being reference boundaries. The amygdala and the ventromedial nucleus with its surrounding area in gonadectomized WT mice and corresponding ventromedial hypothalamic region in SF-1 KO mice were analyzed in sections corresponding to areas 1.7 mm caudally from Bregma and taken under 60× magnification. Reference boundaries were the third ventricle and base of brain for VMH sections and reference points for amygdala were the edge of the optic tract and external capsule (longer side of an examined area was positioned 45° aside from a perpendicular dorsal-ventral axis). Due to the possibility of asymmetry in antigen detection between the left and right sides of the brain, we always chose the side with more cells detected. As the sections were run free-floating and the brains were not notched ahead of time, the actual left or right side of the brain was unknown.

The number of immunoreactive cells in each region was evaluated using Image J software (NIH, Bethesda, MD, USA). All analyzed regions were divided into grid squares as described in previous analyses (Davis et al., 2004; Wolfe et al., 2005) to provide a mechanism to discern objective changes in the positions of cells. For the VMH and BST regions we used a grid of 9×10 squares, measuring 85×85 µm each when taken under 60× magnification. The number of immunoreactive cells was counted in each box and all boxes in the grid with the exception of calbindin immunopositive cells in BST, where only the grid delimiting the BSTMPM region (7 × 5 squares) was analyzed. In the caudal POA/AH region, only the region under the anterior commissure extending 768 µm laterally from the third ventricle was analyzed (6×10 squares measuring 128×128 µm under 40× magnification). To examine a rostral part of the POA we examined a region including the rostral AVPV and extending 512 µm laterally from the third ventricle and 768 µm dorsally from the base of each brain that was analyzed. nNOS immunoreactivity in cell bodies and fibers was quantified using custom software (Surfkvad; made by Dr. Marko Kreft, Institute of pathophysiology, Faculty of Medicine, Ljubljana); that divides an image into 6×8 squares and calculates a percentage of dark area for each square. To standardize the collection immunoreactive area data, all images were taken under the same illumination and converted to grayscale. Grayscale images were subjected to threshold conversion to selectively identify immunoreactive elements using Photoshop software package Adobe Version 8.0 with threshold limit always set at 50%. Black and white images were then analyzed with Surfkvad software.

POA/AH and VMH brain regions from three randomly chosen animals from each group were inspected for size of calbindin and nNOS immunoreactive cells. For each region 12

immunoreactive cells were chosen in each of three fields under 200× magnification. Cell size (area in square microns) was measured on Nikon microphot FXA microscope using Lucia G software.

Data were analyzed either by using two-factor analysis of variance (ANOVA; sex × genotype) to detect sex differences in total number of cells or immunoreactivity in a given area (for BST and caudal POA), or using a three-factor ANOVA with the inclusion of location as a repeated measure in addition to the independent variables of sex and genotype (for VMH and AVPV). In some cases Fisher LSD posthoc analyses were used to determine statistical differences between sexes within each genotype. All statistical analyses were performed with SPSS 14.0 software package for PC (SPSS Inc., Chicago, IL, USA) with statistical significance considered at $p < 0.05$. All data are presented as Mean ± SEM.

RESULTS

Cells containing immunoreactive nNOS and calbindin were found throughout regions stretching from the opening of the third ventricle, dorsally to the BST, lateral to the amygdala, and caudally to the region of the ventromedial nucleus. A pilot experiment showed greater levels of immunoreactivity in brains from castrated WT mice that were treated with TP compared to oil vehicle. To insure that all comparisons were made in animals under the same adult hormone conditions (Tobet and Fox, 1992), data were only collected from mice that received the same dose of testosterone for 1 week prior to sacrifice. The data are presented by region, but each result should be considered in one of three categories related to sexual differentiation: 1) gonad-dependent sexual differentiation for sex differences in WT that are not found in KO's; 2) gonad-independent sexual differentiation for sex differences in WT that are still found in KO's; and 3) although none were identified in the current study, gonad-independent sexual differentiation that would be 'compensatory' (DeVries, 2005; Palaszynski et al., 2005) and would be identified by sex differences not found in WT, but discernible in KO's or reversed between WT and KO.

Preoptic area/anterior hypothalamus (POA/AH)

In the caudal POA/AH of the mouse brain there is a cluster of calbindin immunopositive cells in the coronal plane 0.1 mm from Bregma; this is potentially homologous to the sexually dimorphic calbindin cell cluster previously described in rats (Sickel and McCarthy, 2000) and seen previously in mice (Hall et al., 2000; Edelmann et al., 2007). The calbindin immunopositive cell cluster in this region was present only in WT males, and absent in all other groups of mice (Fig. 1). Interestingly, quantitative analyses revealed similar numbers of calbindin immunopositive cells in the region in all four groups of mice (312 ± 43 WT M ($n = 6$); 424 ± 84 WT F ($n = 5$); 236 ± 46 KO M ($n = 7$); 333 ± 29 KO F ($n = 8$)), suggesting that neurons expressing calbindin were present in the area but did not form a distinct nucleus in mice not exposed to gonadal hormones during perinatal life. This provides strong evidence for gonad-dependent sexual differentiation. Cell size was similar between all four groups ($82.44 \pm 5.19 \mu\text{m}^2$ in WT males, $80.96 \pm 7.39 \mu\text{m}^2$ in WT females, $66.54 \pm 4.48 \mu\text{m}^2$ in KO males and $75.17 \pm 5.59 \mu\text{m}^2$ in KO females).

Immunoreactivity for nNOS in the caudal POA/AH did not reveal any distinct cell groups. However, the quantitative analysis of total immunoreactivity in fibers and cells showed a significant effect of sex ($F(1,23) = 10.53$, $p < 0.01$) with males of both genotypes (WT and SF-1 KO) having greater immunoreactive area than female mice. In the absence of a statistically significant sex × genotype interaction ($p > 0.10$), this difference is considered evidence for gonad-independent sexual differentiation (Fig. 2).

Rostral AVPV area

The rostral ventral area including the AVPV is an area in which sex differences in cell number and phenotype have been described previously (Simerly, 2002). While few calbindin immunopositive cells were found in this region, nNOS immunoreactivity was found in both cell bodies and fibers (Fig. 3). As AVPV differs in size between males and females, numbers of cells were analyzed in horizontal bins from dorsal to ventral locations (repeated measures using dorsal/ventral location as a third variable). Statistical analyses revealed a significant main effect of sex for the number of cells in the AVPV and surrounding area ($F(1,21) = 4.89$, $p < 0.05$), with males of both genotypes having more cells than females throughout the region (Fig. 4). In the absence of a statistically significant sex $\times$ genotype interaction ($p > 0.10$), this difference is considered evidence for gonad-independent sexual differentiation.

Bed nucleus of the stria terminalis – medial division (BSTMPM and BSTMPI)

Components of the BST have been noted for sex differences in several species (De Vries and Panzica, 2006). We examined calbindin expression in the medial portion of the BST in coronal brain sections positioned 0.22 mm caudally from Bregma. The area analyzed extended between the stria terminalis and the stria medularis of the thalamus and contained BSTMPM and BSTMPI subdivisions. More than 95% of the calbindin immunopositive cells were positioned in the BSTMPM and all differences refer to the BSTMPM subdivision. Calbindin positive cells were counted in a single coronal section in a grid 7 by 5 squares containing the BSTMPM (Fig. 5). The number of calbindin immunopositive cells was greater in WT males than in WT females, and lower in SF-1 KO males in comparison to WT males and females and SF-1 KO females. Fundamentally, female levels of expression were similar in both genotypes, while male levels were lower in the SF-1 KO. This led to a significant sex $\times$ genotype interaction ($F(1,21) = 4.99$, $p < 0.05$; Fig. 6). There was a trend for KO females to have more cells than KO males, appearing opposite to the WT, but posthoc test indicated that this was not statistically significant. Were this difference significant, this would be an example of a sex difference that would be masked by the presence of gonads (Palaszynski et al., 2005). As the sex difference in WT was not seen in the SF-1 KO, this provides evidence for gonad-dependent sexual differentiation.

Similar to calbindin, nNOS was also expressed in the BST area, although the immunopositive cells were not limited to the BSTMPM area (Fig. 7). Therefore, the analysis was done in two ways; first, the whole coronal region containing the BSTMPM and the surrounding area and, second, using the same area as for calbindin (7×5 squares delineating BSTMPM). The total number of immunopositive cells was similar between all four groups and there was no statistically significant interaction either for sex $\times$ genotype both in the whole region or when the number of cells in the same grid (7 by 5 squares) as for calbindin was counted. However, there was a strong statistical trend ($p = 0.061$) for males of both genotypes to have more nNOS immunopositive cells in the whole area than either female WT or SF-1 KO mice (data not shown).

Amygdala

Another location that is significantly connected to BST and hypothalamus is the amygdala, and particularly the medial amygdaloid nuclei. Immunoreactive calbindin and nNOS were detected in both lateral and medial amygdaloid nuclei, but there was no indication of influences of sex or genotype (data not shown).

Ventromedial hypothalamus

As the cells of the VMH are rearranged in SF-1 KO mice (Dellovade et al., 2000; Davis et al., 2004), we focused on the coronal plane through the center of the nucleus found in wild type

control mice (coronal sections 1.7 mm caudal from Bregma). The positions of calbindin and nNOS immunopositive cells were altered in SF-1 KO mice, as predicted based on the previous studies showing altered positions of cells in this region of SF-1 KO mice at birth. Immunoreactive calbindin and nNOS neurons were found ventrolaterally in WT mice, but were positioned closer to the third ventricle in SF-1 KO mice (Fig. 8 and 9). The similar numbers of neurons in both genotypes is consistent with the notion that these may be the same neurons that did not migrate properly, yet retained some phenotypical characteristics.

For calbindin, quantitatively (Fig. 10), the altered distribution was reflected in a significant interaction of genotype $\times$ sex $\times$ location (distance from third ventricle; $F(9,270) = 6.53$, $p < 0.001$). The interaction was due to the translocation of a sex difference present in both genotypes (females greater than males in both WT and SF-1 KO mice) in the number of calbindin cells from lateral positions in WT mice to more medial positions in SF-1 KO mice. Whether or not the calbindin positive cells were the same cells in WT and SF-1 KO mice, the results nevertheless provide evidence for gonad-independent sexual differentiation in the SF-1 KO mice.

Average cell size of calbindin positive cells in the VMH region was significantly greater in WT males in comparison to other three groups ($129 \pm 4.12 \mu\text{m}^2$ in WT males, $100.17 \pm 7.01 \mu\text{m}^2$ in WT females, $92.89 \pm 3.33 \mu\text{m}^2$ in KO males and $94.11 \pm 3.95 \mu\text{m}^2$ in KO females; $p < 0.01$), suggesting gonad-dependent sexual differentiation. While the presence of a group difference in cell size renders interpretation of cell counts more complicated, the significant difference in this case was in cell position – not number.

Similar to calbindin, there was a significantly altered distribution of immunoreactive nNOS elements that also depended upon sex and genotype. There was a significant interaction of genotype $\times$ sex $\times$ location (distance from third ventricle; $F(9, 270) = 2.97$, $p < 0.01$). The influence of sex was notably more subtle than for calbindin and appears to be based in a lateral sex difference in immunoreactivity in WT, that was absent in the SF-1 KO mice as the immunoreactivity became concentrated more medially (Fig. 11). The absence of a sex difference in the SF-1 KO is consistent with the subtle sex difference in nNOS expression to be gonadal-dependent. Cell size was similar between all four groups ($193 \pm 8.00 \mu\text{m}^2$ in WT males, $198.19 \pm 5.95 \mu\text{m}^2$ in WT females, $193.34 \pm 13.70 \mu\text{m}^2$ in KO males and $206.84 \pm 3.10 \mu\text{m}^2$ in KO females).

Body weights

Body weight monitoring revealed significant sex differences, regardless of genotype. At 12 weeks of age, WT female mice had greater body weights (25.1 ± 0.5 g, $n=19$) than WT males (24.4 ± 0.4 g, $n=24$). In SF-1 KO mice the sex difference was more notable with female SF-1 KO mice were more than 7% heavier (27.1 ± 1.1 g, $n=16$) than males (25.2 ± 0.5 g, $n=30$). The statistical analysis yielded statistically significant main effects for both sex ($F(1,85) = 4.29$, $p < 0.05$) and genotype ($F(1,85) = 5.09$, $p < 0.03$). The lack of statistical interaction ($p > 0.30$) suggests that females of both genotypes were heavier.

DISCUSSION

Gonadal hormones influence the differentiation of mammalian brain structures during development. Full development of brain structures and neurochemical phenotypes result from hormone influences acting at multiple periods during the lifespan, including fetal development, during puberty, and following activational hormone exposure in adulthood (Simerly, 2002; Tobet, 2002; Arnold, 2004; Sisk and Zehr, 2005). In mice lacking transcription factor SF-1, gonads regress completely before E12.5 (Luo et al., 1994), before the onset of gonadal steroid production (Greco and Payne, 1994). Therefore, these mice are not exposed to endogenous

gonadal hormones during fetal development and they provide a unique model to study gonad independent sexual differentiation of the mammalian brain. In the present study such mice were raised to adulthood to examine sex differences in the expression and distribution of two markers of hypothalamic cell phenotypes; calbindin and nNOS. The data showed regionally selective and significant sex differences in the distribution of hypothalamic markers in WT mice that were either eliminated in SF-1 KO mice (presumably gonad-dependent) or maintained in SF-1 KO mice (presumably gonad-independent or genetic based). Specifically, the data suggest the sexual differentiation of selected aspects of calbindin expression in the POA/AH and BST and nNOS expression in the VMH are likely gonad-dependent, while sex differences in the expression of immunoreactive nNOS in the POA/AH, AVPV, BST and calbindin in the VMH are more likely to take place in the absence of the *SF-1* gene and consequently gonads. It is still possible, however, that aspects of the observed differences, particularly in the VMH area, might also arise as a consequence of the disrupted SF-1 gene.

The POA/AH is one of the most well characterized regions for sexual dimorphisms across vertebrate species (Tobet and Fox, 1992). The POA/AH is a region rich in neurons expressing estrogen receptors alpha and beta as well as androgen receptors (Simerly et al., 1990; Mitra et al., 2003; Sato et al., 2005). The development (Henderson et al., 1999; Knoll et al., 2007) and function (Hori et al., 1988; Segovia and Guillaumon, 1993; Hull et al., 1999) of cells in the POA/AH are strongly affected by actions of gonadal steroids in various species. In the current study, cells containing immunoreactive calbindin were found in a nuclear configuration in adult WT males, in agreement with previous findings (Hall et al., 2000; Edelmann et al., 2007). This cell cluster is similar in location to the sexually dimorphic nucleus of the rat POA that is also calbindin immunopositive (Sickel and McCarthy, 2000; Scallet et al., 2004; Pei et al., 2006; Patisaul et al., 2007). In the current study, the distinct cell cluster was absent in ovariectomized WT control mice, as well as in SF-1 KO mice of both sexes, suggesting that prenatal or neonatal exposure to gonadal steroids is required for the development of this sex difference as suggested in rats (Sickel and McCarthy, 2000). As the total number of calbindin positive cells in this area did not differ between all 4 experimental groups it provides further evidence that one role of hormonal signals for brain sexual differentiation is the positioning of cellular elements (Tobet and Fox, 1989; Orikasa et al., 2002; Wolfe et al., 2005; Knoll et al., 2007).

In a study of immunoreactive nNOS in the POA/AH of mice at birth, a sex difference was observed that was complementary to the pattern of calbindin immunopositive cells (Edelmann et al., 2007). In the current study, while the distribution of nNOS immunopositive cells in the dorsal POA/AH was not dependent upon genotype (data not shown), the area of immunoreactivity was greater in males than females similar to the pattern in development. This was independent of genotype suggesting a potential developmental gonad-independence of this characteristic. Similarly in the more rostral region of AVPV, males of both genotypes also had significantly higher number of nNOS immunopositive cells and larger nNOS immunoreactive area (data not shown) than females in agreement with a study in rats (Ishihara et al., 2002). The larger difference between WT males and females in comparison to SF-1 KO males and females in the AVPV region might suggest that the sex difference initially produced by the action of genes on sex chromosomes might increase upon developmental exposure to sex steroids.

It is interesting to note that AVPV is a region normally considered to develop as larger in several characteristics (i.e., cell number) in females than males (Murakami and Arai, 1989; Simerly, 1989; Davis et al., 1996). A previous study of genetic factors versus gonadal steroid contributions to the expression of tyrosine hydroxylase in AVPV using XX and XY males and females (De Vries et al., 2002) suggested that hormonal factors are solely responsible for sexual differentiation. In that study, XX and XY females had more tyrosine hydroxylase immunopositive cells than XX or XY males. Similarly, apoptosis in the male AVPV are thought

to be due to gonadal steroid actions (Murakami and Arai, 1989; Forger et al., 2004). By contrast, in the current study nNOS immunoreactivity in this region was greater in males than females, suggesting a potential for genetic contribution to the development of a male-biased measure in this region that is usually considered female biased. Whether there is a relationship between the direction of influence (e.g., male > female) and the extent of genetic versus gonadal factors remains to be determined.

Sex differences have been described in the encapsulated region of the BST in rats with males having 97% greater volume than females (Hines et al., 1992). Cells in the BST have been noted with a number of sexually dimorphic characteristics and they are connected to other sexually dimorphic sites (Krettek and Price, 1978; Swanson and Cowan, 1979; Hutton et al., 1998), and several studies have suggested that the BST is involved in the regulation of different sex specific behaviors such as male mounting behavior (Valcourt and Sachs, 1979). In the current study, we examined calbindin expression in the BST and found a significant interaction between sex and genotype in the BSTMPM area. The difference was due to a decrease in SF-1 KO male mice with little to no change in SF-1 KO female mice in respect to their WT counterparts. A sex difference in WT mice that is not found in SF-1 KO mice is consistent with a gonadal contribution to sexual differentiation (Forger et al., 2004; De Vries, 2005).

The VMH is a brain region that had long been thought to be important for body weight regulation (Brobeck, 1946; Gold, 1970). Newer studies showed that some effects attributed to the VMH may have been due to circuitry involving arcuate and paraventricular hypothalamic nuclei (Schwartz et al., 2000). Nonetheless, molecular studies are once again providing evidence of a direct involvement of the VMH in body weight regulation (Butler et al., 2000; Dhillon et al., 2006; King, 2006). SF-1 KO mice become obese in adult life (Majdic et al., 2002) and they have selective disruption of cellular topography in the VMH area (Ikeda et al., 1995; Shinoda et al., 1995; Dellovade et al., 2000; Tran et al., 2003; Davis et al., 2004). Interestingly, body weights between SF-1 KO male and female mice differed even though mice of both sexes were morphologically female from the perspective of secondary sexual characteristics. Although in the current study the difference at 12 weeks of age was only 7%, previous data from SF-1 KO mice raised to adulthood showed larger differences at 12 weeks of age with SF-1 KO females (31.3 ± 1.1 g, n=11; (Majdic et al., 2002) approximately 23% heavier than SF-1 KO males (25.8 ± 1.1 g, n=7; $p = 0.017$; (Majdic and Parker, unpublished observation) from the same cohort of mice as (Majdic et al., 2002). Sex differences in feeding behavior and body weight have long been attributed to the developmental actions of sex steroids (Bell and Zucker, 1971; Nance and Gorski, 1975) that may be attributable to actions in the VMH (Beatty et al., 1975).

The VMH is a site with a long history of findings related to sex differences in morphology and molecular phenotypes that make the findings of sex differences in the VMH in the current study particularly relevant to a body weight phenotype (Matsumoto and Arai, 1983, 1986; Romano et al., 1990; Coirini et al., 1992; Madeira et al., 2001; Sa and Madeira, 2005). We found that the distribution of immunoreactive nNOS and calbindin cells was altered in the VMH of SF-1 KO mice with immunoreactive cells positioned in more dorsomedial positions in comparison to brains from WT control mice. A significant sex difference in the number of immunoreactive calbindin cells in the VMH area of WT mice was maintained in SF-1 KO mice along with reorganization of the region with SF-1 KO males having fewer cells than SF-1 KO females. In the WT mice, the calbindin immunopositive cells are located normally in the ventrolateral VMH where they normally would be more likely associated with sexual receptivity than energy balance. It should be noted, however, that a recent study (Musatov et al., 2007) suggests that cells containing estrogen receptor alpha in the ventrolateral portion of the VMH are also involved in metabolic homeostasis. In the SF-1 KO where lateral cells become located more medially (Dellovade et al., 2000; Davis et al., 2004), such as the calbindin immunopositive

cells in the present study, a change in connectivity or phenotype may be likely (Tran et al., 2003) and this might be connected to the body weight phenotype in SF-1 KO mice. As previous studies have shown sex differences in the expression and regulation of proteins important for neuronal connectivity (Lustig et al., 1991), it will be important to determine how aspects of connectivity are altered in SF-1 KO mice, that might be connected to sex differences found in this area.

In summary, our results show that some sex differences in brain are present in agonalal SF-1 KO mice that likely arise due to different complements of sex chromosomes or disruption of a functional *SF-1* gene and not due to differential exposure to sex steroids during prenatal/early postnatal development. The results of the current study with the SF-1 KO mice demonstrate significant differentiating roles for gonadal hormones, but also that specific sex differences persist in the absence of exposure to gonadal hormones during prenatal and neonatal life, and therefore arise as a likely consequence of differences in sex chromosomes that may or may not also depend on the presence of functional SF-1 in the VMH.

Acknowledgements

We are thankful to Dr. Marko Kreft for providing Surfkvad software. We thank Dr. Keith Parker and members of his laboratory for continuing discussions of the role of SF-1 and the consequences of its disruption. This study was supported by grants from NIH (MH61376 (SAT), TW005922 (SAT, GM)), ARRS (Slovenian research agency) P4-0053 and ICGEB (GM). Tomaz Büdefeld was supported by fellowship from foundation Stein and Neza Grgurevic is supported by Ph.D. fellowship from ARRS.

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Fig. 1. Digital images show calbindin immunopositive cells formed a distinct nucleus in caudal POA/AH in the brains from WT males only (a, arrow) while this nucleus was absent in all three other groups of mice (b- WT female, c – SF-1 knockout male, d – SF-1 knockout female; 3V – third ventricle, OT – optic tract).

Fig. 2.

Quantification of nNOS immunoreactivity in the caudal POA/AH analyzed as total immunoreactive area in coronal sections (mean $\pm$ S.E.M.) revealed statistically significant effect of sex in both WT and SF-1 knockout mice ($p < 0.01$) suggesting a gonadal hormone independent sex difference.

Fig. 3. Digital images in the region of AVPV show both cell bodies and fibers were immunopositive for nNOS (a – WT male, b – WT female, c – SF-1 knockout male, d – SF-1 knockout female; 3V – third ventricle, OT – optic tract).

Fig. 4. Quantification of nNOS immunopositive cells in the rostral AVPV area analyzed by rows (mean $\pm$ S.E.M.) revealed statistical significant interaction between location and sex ($p < 0.05$) with males of both genotypes having more cells than females throughout the region.

Fig. 5. Digital images show calbindin immunopositive cells in the BSTM area in WT (a – male, b – female) and SF-1 knockout (c – male, d – female) mice. Bold black lines mark the BSTMPM area that was analyzed for cell numbers.

Fig. 6.

Quantification of calbindin immunopositive cells in the BSTMPM area analyzed as a total number of cells in the nucleus (mean $\pm$ S.E.M.) revealed statistical significant interaction of sex and genotype between WT and SF-1 knockout mice of both sexes ($p < 0.05$).

Fig. 7.
Digital images show nNOS immunopositive cells in BSTM area in WT (a – male, b – female) and SF-1 knockout (c – male, d – female) mice.

Fig. 8. Digital images of the region of the VMH show calbindin immunopositive cells were concentrated in ventrolateral positions in WT males (a) and females (b), but were translocated to more dorsomedial positions in male (c) and female (d) SF-1 knockout mice. The third ventricle provides the left margin of the tissue in each image; ARC – arcuate nucleus, VMH – ventromedial nucleus.

Fig. 9.

Digital images of the region of the VMH show nNOS immunopositive cells were concentrated in ventrolateral positions in WT males (a) and females (b), but were translocated to more dorsomedial positions in male (c) and female (d) SF-1 knockout mice. The third ventricle provides the left margin of the tissue in each image; ARC – arcuate nucleus, VMH – ventromedial nucleus.

Fig. 10.

Quantification of calbindin immunopositive cells in the VMH area analyzed by columns relative to the 3rd ventricle (mean $\pm$ S.E.M.) revealed significant differences in the location of immunoreactive cells between WT and SF-1 knockout mice ($p < 0.001$).

Fig. 11.

Quantification of nNOS immunopositive cells in the VMH area analyzed by columns relative from 3rd ventricle (mean $\pm$ S.E.M.) also revealed significant interaction between sex, genotype and location ($p < 0.01$).