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Sex Difference in Calbindin Cell Number in the Mouse Preoptic Area: Effects of Neonatal Estradiol and Bax Gene Deletion

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**SEX DIFFERENCE IN CALBINDIN CELL NUMBER IN THE MOUSE
PREOPTIC AREA: EFFECTS OF NEONATAL ESTRADIOL AND *BAX* GENE
DELETION**

A Thesis Presented
by

RICHARD F. GILMORE III

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University of Massachusetts Amherst in partial fulfillment
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DEDICATION

To my extremely loving family and friends.

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ABSTRACT

SEX DIFFERENCE IN CALBINDIN CELL NUMBER IN THE MOUSE PREOPTIC AREA: EFFECTS OF NEONATAL ESTRADIOL AND *BAX* GENE DELETION

SEPTEMBER 2011

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The sexually dimorphic nucleus of the preoptic area (SDN-POA) was first discovered in rats and is one of the most famous and best studied sex differences in the field of neuroscience. Though well documented in rats (larger in males than females), this sex difference was only recently able to be observed in mice due to the discovery of the protein calbindin-D28k as a marker. Recent studies have shown a larger, more distinct calbindin-immunoreactive (ir) cell cluster in male mice compared to females. However, the exact location of the cluster and whether the sex difference is one of total cell number or cell distribution remains unclear. In this study, we use defined contours to demonstrate that male mice have more calbindin-ir cells than females both in the central cell cluster and areas surrounding the cluster. We also report a full masculinization of these characteristics in females given a single injection of estradiol benzoate (EB) on the day of birth. The potential role of cell death in the development of this sex difference was tested using mice with a deletion of the *bax* gene, which codes for a pro death factor required for the establishment of other sex differences in the mouse brain. We demonstrate that *bax* knockout (KO) mice have more cells in the POA region in general, but eliminating cell death does not affect the development of the sex difference in calbindin-ir cell number, nor does it affect calbindin-ir cell spread. Taken together, this suggests that cell death is not a significant underlying mechanism in the establishment of the sex difference in the calbindin-ir cell cluster in the mouse POA.

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CHAPTER I

INTRODUCTION

Sexual Differentiation

Over 60 years ago, Alfred Jost discovered the importance of secretions from the gonads for the establishment of male-specific differentiation, particularly of the internal and external genitalia (Jost 1947). More recently, it was discovered that sexual differentiation extends to the brain (Gorski, 1985). Sexual dimorphisms in the brain have largely been studied in an attempt to better understand male and female behavior, the role of gonadal hormones in development, and the cellular and molecular mechanisms of gonadal hormones.

Most of the studies on sex differences in the nervous system have focused on rodent models, but neural sex differences have been identified for almost every vertebrate species that has been studied. These sex differences are in most cases dependent on the presence of gonadal hormones prenatally or during postnatal development (Morris et al, 2004). Sex hormones can have an organizational effect on brain morphology and behavior, as Phoenix et al. showed in 1959 by permanently reversing sex behavior in female guinea pigs with only prenatal testosterone propionate exposure (Phoenix et al., 1959), but they also can have direct activational effects on cognition and sex behavior by being present in adulthood (McCarthy et al, 2009).

One mechanism by which developmental hormones establish these sex differences is by modulating neuronal apoptosis, as is shown in the case of the sex difference in neuron number in the principle nucleus of the bed nucleus of the stria

terminalis (BNSTp) (Forger et al., 2004). Other studies have suggested differential cell migration as a mechanism for hormone-dependent sexual dimorphisms in the developing hypothalamus (Tobet et al., 2009), and neurogenesis is also a possible, as yet unconfirmed mechanism. Animal models, particularly rodents, have been extremely important to sexual differentiation research due to brain tissue availability, relatively short life cycle, similarity of known sexually dimorphic regions to humans, established methods of quantifying sexual behavior, possibility of hormonal manipulation, and genetic advantages.

Sexually Dimorphic Nucleus of the Preoptic Area

The sexually dimorphic nucleus of the preoptic area (SDN-POA) is one of the first discovered and best documented sex differences in the field of neuroscience. It was initially identified and described in rats (Gorski et al, 1978), but homologous sexually dimorphic brain regions have been observed in a variety of other species such as ferrets (Tobet et al, 1985), hyenas, (Fenstermaker et al, 1999), and humans (Swaab and Hofman, 1988). This nucleus is located in the preoptic area/ anterior hypothalamus, just lateral to the 3rd ventricle, and ventral to the caudal-most portion of the BNSTp, another well studied sexually dimorphic brain region (Guillamón et al, 1988, Forger et al, 2004). Although the exact function in behavior is not known, the SDN-POA has been associated with promoting male sex behavior in lesion studies in rats (De Jonge et al., 1989), and has been implicated in sexual partner preference in ferrets and humans (Swaab and Hofman, 1995, Paredes and Baum, 1995).

The sex dimorphism in the SDN-POA was first observed in Nissl-stained sections, in which male rats were observed to have a cluster of cells several times larger than that of females (Gorski et al, 1978). Males also have more neurons in this nucleus than in females (Madeira et al, 1999) and these neurons are significantly larger in soma size than in females (Gorski et al, 1980). Gonadectomy on postnatal day 1 of male rats leads to a reduction in volume of the SDN-POA in adulthood (Jacobson et al, 1981), while females treated with testosterone propionate injections from embryonic day 16 to postnatal day 10 show a masculinized SDN-POA volume (Dohler et al, 1984). This indicates that testosterone, secreted from the perinatal testes, is crucial for the development of this sex difference. It has also been shown that gonadectomy after puberty in either sex has no effect on the sex difference in SDN-POA volume (Jacobson et al, 1981), indicating that adult hormone activation is not necessary in maintaining the dimorphism.

Sex differences in neurogenesis do not seem to play a role in the development of sex differences in SDN-POA volume (Jacobson et al, 1985, Orikasa et al, 2010). Apoptosis appears to play a role (Davis et al, 1996) because males and females start out with no sex difference in SDN-POA volume, and greater apoptosis is observed in a subsection of the female SDN-POA between postnatal day 7-10. This increase in cell death is also inhibited by testosterone exposure.

Calbindin-D28k as an SDN-POA Marker

While the SDN-POA can easily be visualized and studied with a Nissl-stain in rats, the cell group is not seen in Nissl-stained mouse brain sections (Young, 1982).

Because of this, it was thought for a long time that mice did not have an SDN-POA. This proved frustrating for researchers since there are advantages to using mice as an animal model for the study of sex differences, including a wide variety of genetic knockout mice that can be used to study the importance of individual genes.

Recent studies, however, have found a way to identify the SDN-POA cell group without using Nissl-staining, but instead labeling for the protein calbindin-D28k (Sickel and McCarthy, 2000). Calbindin-D28k is a calcium-binding protein that is responsible for chelating intracellular calcium, which is crucial for many cellular functions such as controlling ion gradients. Calbindin is known to be neuroprotective (Heizmann and Braun, 1992), and Western blot analysis has shown that male rats have significantly higher expression of the protein than females in the medial basal hypothalamic region from embryonic day 17 to postnatal day 1 (Lephart, 1996). Though the mechanism is unknown, one possible explanation of calbindin's anti-apoptotic properties may be its relationship with caspase-3. In osteoblasts, calbindin-D28k has been shown to directly bind and inhibit caspase-3 (Bellido et al, 2000), a protein that is necessary in the pathway of apoptosis in neurons. Calbindin is highly expressed in cells of the amygdala, basal medial hypothalamus (Lephart, 1996), BNST, and the SDN-POA in rats (Sickel and McCarthy, 2000). More recently, calbindin has been used to stain a distinct cluster of cells in the POA of mice.

Calbindin Staining the SDN-POA of Rats

Previous studies of calbindin immunoreactivity in rat brain sections to study the cellular basis of the sex difference of the SDN-POA have provided somewhat conflicting

results. In rats, males have a 2-4 times larger volume of a calbindin-immunoreactive (ir) cell cluster in the SDN-POA than females by postnatal day 12 (Sickel and McCarthy, 2000). Castration of males at birth decreases the volume of the calbindin SDN-POA, and treatment of females with testosterone propionate on the day of birth results in masculinization of the calbindin cell cluster (Sickel and McCarthy, 2000). Estradiol benzoate (EB) treatment of females on the day of birth results in an even larger increase of the calbindin SDN-POA cluster than testosterone, but dihydrotestosterone (DHT) treatment does not affect the cluster volume in females. Since DHT is a form of testosterone that cannot be aromatized to estradiol, this study suggests that estrogen receptors play a role in development of the sex difference. Another study measured a larger calbindin SDN-POA volume in male rats than in females, but also reports that males have significantly more calbindin-ir cells in this cluster (Patisaul et al, 2006). A more recent study has also demonstrated this larger SDN-POA cluster volume in male rats compared to females, but reported no total difference in the *number* of calbindin-ir cells (Orikasa et al, 2010); Sickel and McCarthy (2000) did not report cell counts. These findings together indicate that the nature of the calbindin SDN-POA cell cluster is still unclear in regards to cell number. One possibility by Orikasa et al (2010) is that females have a less defined SDN-POA cell cluster due to a wider spread of calbindin-ir cells. It is possible that researchers only counted cells that were part of the dense calbindin cluster, but previous studies have failed to specifically report where cell counts were performed in this area.

Calbindin-SDN-POA in Mice

While the sex difference in the SDN-POA has been well studied in rats, it was only recently detected in the mouse brain. One study reported that male mice have a nucleus of calbindin-ir cells in the same area as the rat brain, and this cluster is completely absent in females (Büdefeld et al, 2008). In addition, the cluster is eliminated in adult male SR1-knockout mice, which are unable to develop gonads (Büdefeld et al, 2008). This study also counted calbindin-ir cells, however, and reported that the number of calbindin-ir neurons in the region was not sexually dimorphic, a result similar to one of the rat studies (Orikasa et al, 2010). Other recent studies have reported, however, a smaller nucleus in the female mouse SDN-POA with fewer calbindin-ir cells (Bodo and Rissman, 2008; Edelmann et al, 2007). The most recent study of the calbindin SDN-POA in mice reports a larger number of calbindin-ir cells in this cluster in males than females (Orikasa and Sakuma, 2010). Two studies have examined hormone dependence on this sex difference. Bodo and Rissman (2008) used DHT treatment at birth to partially masculinize the calbindin SDN-POA of female mice, suggesting that the sex difference in the calbindin SDN-POA is partially dependent on androgen receptor activation in mice. However, Orikasa and Sakuma (2010) report that while the female cluster calbindin-ir cell number is masculinized via a single injection of testosterone on the day of birth or daily injections of estradiol benzoate from postnatal day 1 through 5, it is not affected by neonatal DHT treatment. This study also shows that the mouse calbindin-ir cluster is not affected by adult gonadectomy in either sex, indicating that activational hormones do not contribute to the sex difference.

Research on the calbindin-ir SDN-POA in mice has not provided clear results on the characteristics and mechanism of development of this sexual dimorphism. The study that reported no visible nucleus of calbindin-ir cells in the female mouse SDN-POA (Büdefeld et al, 2008; Fig. 1.1A) also reports no sex difference in calbindin-ir cell number, but it is not clear how or where cell counts were performed in the absence of the cluster in females.

Other studies report a larger number of calbindin-ir SDN-POA cells in male mice than females. Edelman et al, (2007) comment that males have more calbindin-ir SDN-POA cells than females, but they do not actually provide the data nor indicate the methods or location of cell counts (Fig. 1.1B). Bodo and Rissman (2008) report that male mice have over twice as many calbindin-ir cells as control females in the SDN-POA, however, the photomicrographs from this study also show two calbindin-ir cell clusters in this area and it is not entirely clear which cells were counted (Fig. 1.1C).

Photomicrographs presented in the papers published to date appear to show two different calbindin-ir cell clusters (Fig. 1.1A-C). The more dorsal cluster is identified as the SDN-POA in the Büdefeld study, but according to the location relative to the third ventricle it appears that the ventral cluster (as indicated by Edelman et al, [2007, Fig. 1.1B]) would be more similar to the SDN-POA observed in rats. The cells of the cluster analyzed by Büdefeld et al, (2008; Fig. 1.1A) may actually belong to the tail end of the BNST.

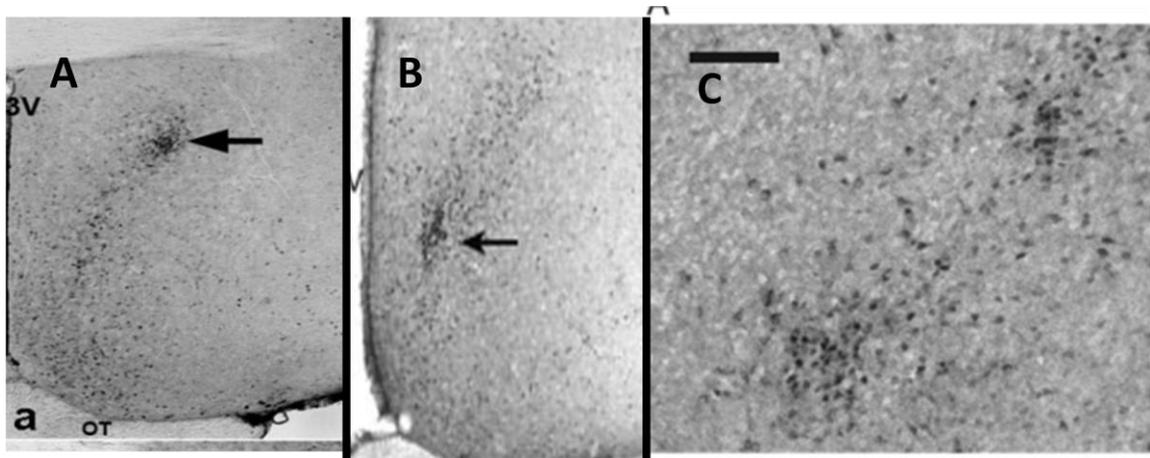


Figure 1.1A-C – Photomicrographs of calbindin-stained sections from previous calbindin SDN-POA mouse studies. (A) Photomicrograph from Büdefeld et al (2008) paper with arrow indicating the calbindin-ir cluster used in analysis. (B) Photomicrograph from Edelmann et al (2007) paper with arrow indicating the calbindin-ir cluster used in analysis. (C) Photomicrograph from Bodo and Rissman (2008) paper showing two apparent calbindin-ir cell clusters.

The Orikasa and Sakuma (2010) paper, which reported significantly more calbindin-ir cells in male mice, is in many ways the most thorough and systematic study of the calbindin SDN-POA of mice, and it is clear that only the ventral cluster was included in the cell counts. Parameters for counting, however, are again unclear (e.g. how cell counts were obtained for females since they do not have a cluster).

If the sexual dimorphism in the mouse SDN-POA is to be fully understood, there must be a more consistent characterization of the calbindin-ir cell cluster. The sex difference in calbindin-ir cell number, if one does exist, should be explicitly and clearly documented. This study aims to address the question of a sex difference in calbindin-ir cell number in the SDN-POA of mice, but to design a more consistent and fully described method of cell counting. A second goal is to evaluate the possibility of differential calbindin-ir cell spread in males and females, which may have led others to conclude that

there is no sex difference in calbindin-ir cell number (Büdefeld et al, 2008, Orikasa et al, 2010). The third goal is to begin to understand the cellular mechanism of this sex difference by testing the role of cell death.

CHAPTER II

A SEX DIFFERENCE IN CALBINDIN-IR CELL NUMBER IN THE SDN-POA AND THE EFFECT OF NEONATAL TREATMENT WITH ESTRADIOL

Introduction

As mentioned above, several past studies in mice report a significantly higher number of calbindin-ir cells in the SDN-POA of male mice than of females (Edelmann et al, 2007; Rissman and Bodo, 2008; Orikasa and Sakuma, 2010). However, the figures from those papers suggest substantial inconsistency in defining the relevant cell group. Moreover, Büdefeld et al, (2008) report that the sex difference is not one of cell number, but of position: whereas only male mice have the distinctive cluster of calbindin-ir cells in the POA, there is no sex difference in total calbindin cell number within the region in general. This study also does not specify the rostrocaudal extent of the cluster examined.

Some of this confusion may be due to the fact that the BNSTp also possesses calbindin-ir cells, and is located in close proximity to the SDN-POA. The SDN-POA in the rat is found lateral to the third ventricle, about midway between the dorsal and ventral extremes of the ventricle, at the rostrocaudal level of the crossing of the anterior commissure. This is also the location of a prominent calbindin-ir cluster in male mice. Based on the angle that brain sections are cut, it is possible that this SDN-POA cluster will be found in the same coronal section as the caudal BNSTp. This could make indentifying the SDN cells separate from the BNSTp cells difficult, and may be the reason for the inconsistencies in the previous published papers. The goals of this study

were therefore threefold: first, to count calbindin-ir cells in the SDN-POA of male and female mice in a defined and consistent manner. To achieve this, I designed a contour based on the cluster shape with fixed location relative to the third ventricle and to the optic chiasm, and counted within this contour in all animals. This way, even if there was no distinct cluster of calbindin-ir cells in the section, it was still possible to place the SDN-POA contour and perform cell counts. Secondly, differential spread of calbindin-ir cells was investigated via use of a grid contour. Using this type of contour, the number of calbindin-ir cells in the POA region surrounding the defined cluster could be measured and analyzed. Finally, this study was designed to determine whether any sex difference in calbindin-ir cell number was due to neonatal exposure to estradiol. Work from the Forger lab previously showed that a single injection of EB in mice on the day of birth masculinized BNSTp cell number in females (Hisasue et al, 2010). I examined whether or not this same treatment eliminates the calbindin SDN-POA sex difference.

Materials and Methods

Animals and EB Treatment

Wildtype adult C57Bl/6J mice were used from our breeding colony. Animals were housed under 14:10 light/ dark conditions at 22 C. All procedures were approved by the Institutional Animal Care and Use Committee at the University of Massachusetts, Amherst.

Animals were injected on the day of birth with either 20 μ g of EB in 25 μ L peanut oil with 10% DMSO or just 25 μ L peanut oil with 10% DMSO as a control (N = 6 per group). Mice were sacrificed and their brains collected at day 55-60. Brains were

immersion fixed in 5% acrolein in .01M phosphate buffer for four hours, and then submerged in 30% sucrose. Coronal sections were cut into three series at 30 μ m on a freezing microtome. The steps up to this point were conducted by Megan Varnum in the Forger lab. I used one of the three series for the analysis described below.

Immunocytochemistry for Calbindin-D28k

Pilot work on the brains of untreated adult C57Bl/6J mice was performed to choose the antibody for this study and to determine optimal primary anti-calbindin-D28k concentration. Two different antibodies (monoclonal calbindin-D28k antiserum [C9848; clone CB-955; Sigma-Aldrich, St. Louis, MO] and polyclonal calbindin-D28k antiserum [C2724; Sigma-Aldrich, St. Louis, MO]) were tested with concentrations ranging between 1:2000 and 1:10000. The monoclonal antibody at a concentration of 1:5000 was determined to produce the best staining and was used throughout this thesis.

Immunocytochemistry for calbindin-D28k was carried out in mesh-bottomed cups and all incubations and rinses were done at room temperature. Free floating sections were incubated in the following solutions: 3 \times 5 min in Tris-buffered saline (TBS; 0.05 M Tris, 0.9% NaCl, pH 7.6); 0.05 M sodium citrate in TBS for one hour; 3 \times 5 min in TBS; 0.1 M glycine in TBS for 30 min; 3 \times 5 min in TBS. Sections were then incubated in blocking solution (20% normal goat serum (NGS), 0.3% Triton-X (Labchem, Inc., Pittsburgh, PA) and 1% H₂O₂ in TBS) for 30 min before being transferred to 2 ml Eppendorf tubes containing primary antibody (2% NGS, 1% H₂O₂, 0.3% Triton-X and 1:5000 mouse monoclonal anti-calbindin-D28k antiserum [C9848; clone CB-955; Sigma-Aldrich, St. Louis, MO]). After incubating overnight, sections were returned to wells and rinsed with

TBS containing 1% NGS and 0.02% Triton-X for 3×10 min. Next, sections were incubated in secondary antiserum (1:250 biotinylated goat anti-mouse IgG, [Vector Laboratories, Burlingame, CA, USA] in TBS with 2% NGS and 0.32% Triton-X) for 1 h, followed by 3×10 min rinses in TBS containing 0.2% Triton-X. Sections were then incubated in avidin–biotin complex in TBS (ABC Elite Kit, Vector Laboratories), followed by 3×15 min rinses in TBS. Lastly, sections were incubated in diaminobenzidine (DAB Kit, Vector Laboratories, Burlingame, CA, USA) for ~3 min, followed by 4×5 min rinses in TBS. Sections were mounted onto slides and coverslipped with Permount (Fisher Scientific).

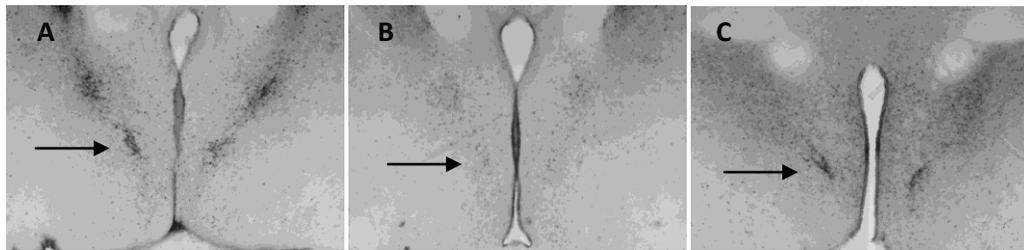


Figure 2.1A-C- Photomicrographs of calbindin-stained sections of a (A) control male, (B) control female, and (C) EB-treated female mouse. Arrow indicates the position of the calbindin-ir cell cluster that was used in analysis for this study.

Calbindin-ir Cluster Cell Counts using a Defined Contour

All measurements were made on slides coded to conceal the sex and treatment of the animals. A distinctive cluster of calbindin-ir cells was found in the POA of all males. This cluster was ellipsoidal in shape and centered at about 670 μ m dorsal to the optic chiasm and 275 μ m lateral to the third ventricle; the cluster angled away from the third ventricle dorsally (i.e., dorsal cells are slightly more lateral than ventral cells). The cluster was usually present in one or two of the sections in males. Since every third 30 μ m section was collected, this implies that the cluster extends approximately 100-200 μ m

rostrocaudally. Females also had calbindin-ir cells in this region, but they did not form a distinct cluster.

In a first, non-quantitative analysis of calbindin immunoreactivity, two independent observers, blind to group, scored the presence or absence of a distinct cluster in all animals. Six males, no females (0/6), and four of the six EB-treated females were scored as having this cell cluster (Fig. 2.1).

To quantify this apparent sex difference, counts of calbindin-ir cells were made in both sexes using StereoInvestigator software (MicroBrightfield, Williston, VT). An ellipsoid contour was defined ($\sim 65,000\mu\text{m}^2$; major axis $365\mu\text{m}$ and minor axis $220\mu\text{m}$) and aligned with respect to the optic chiasm ($\sim 675\mu\text{m}$ dorsal) and ventricle ($\sim 275\mu\text{m}$ lateral) to capture the calbindin cluster as described above in males (Fig. 2.2A). The size of the ellipse was somewhat larger than the cluster in males to ensure that all calbindin-ir cells of the cluster were counted in each animal. Sections with the largest SDN-POA calbindin-ir cell cluster positioned as described above were first identified by eye. Two counts were then performed for each animal. In the majority of cases (13/18), these were left and right SDN-POA of the same section. In a few cases, presumably due to the angle of the cut, the two largest clusters were in the adjacent sections. In cases where the sections with the largest cell cluster were not immediately obvious, three or four counts were made and the two with the greatest amount of calbindin-ir cells were combined and used for analysis.

Calbindin-ir Grid Analysis

We also performed grid counts to address the possibility that the sex difference in calbindin-ir cell number is due to distribution of cells rather than absolute number. To count calbindin-ir cells in the area around the SDN-POA, a grid contour (525 μ m by 525 μ m) was constructed consisting of nine identical blocks (each 175 μ m by 175 μ m) and calbindin-ir cells were counted within each block. The nine blocks were summed for total counts. The grid was aligned with the edge of the third ventricle for all animals, with the ventral edge of the grid approximately 350 μ m from the optic chiasm (Fig. 2.2B). In this position, most of the calbindin-ir cluster as defined above fell within the central block of the grid (block #5).

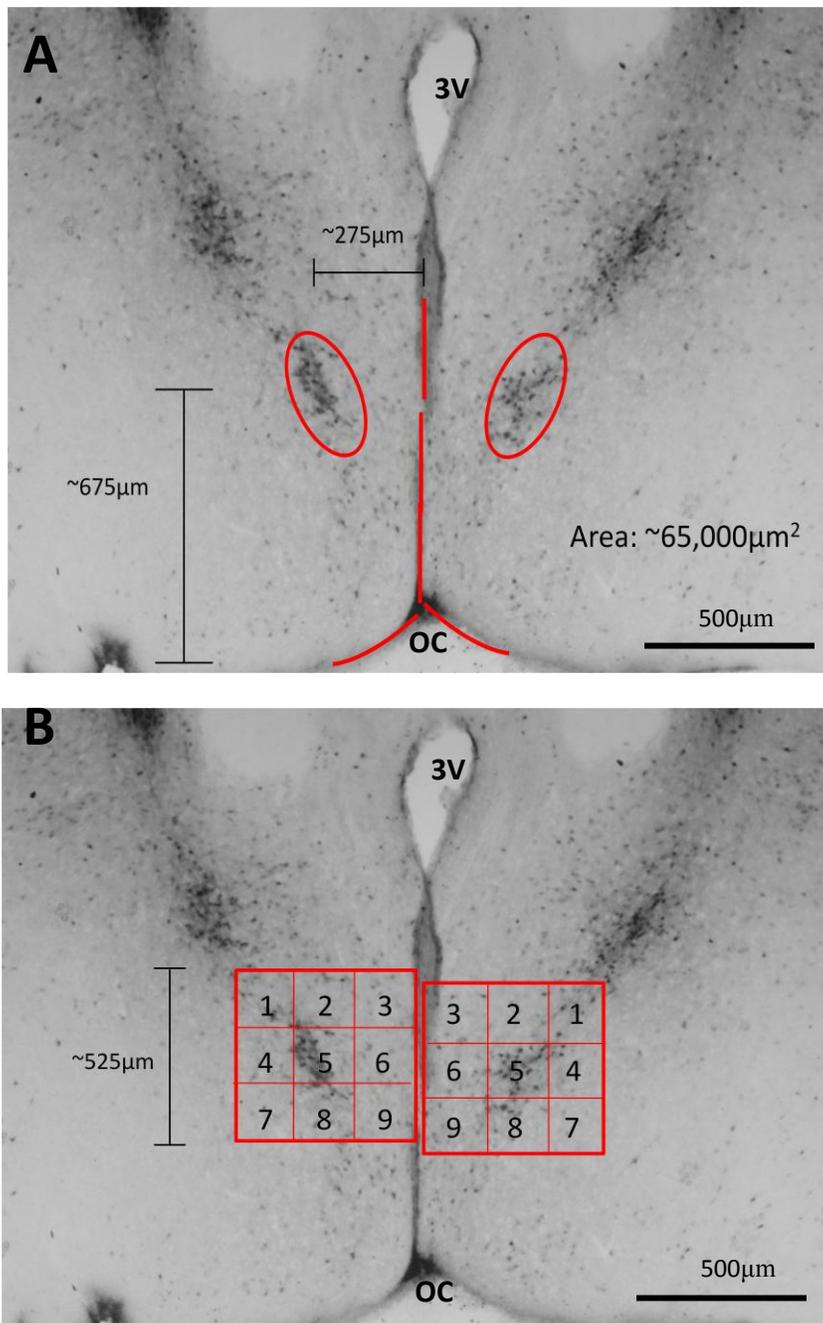


Figure 2.2- Representative photomicrograph taken at 4x magnification of the calbindin SDN-POA cluster of a male mouse aligned with (A) the ellipse contour and (B) the grid contour. 3V = third ventricle, OC = optic chiasm. Scale bar = 500µm.

Statistics

One-way analysis of variance (ANOVA) was used to compare mean calbindin-ir cell number within the ellipsoid contour and the grid count (individual grid blocks and total) among the groups. In cases of a significant main effect ($p < .05$) Fisher's least significant difference (LSD) post hoc tests were performed.

Results

SDN-POA Cluster Cell Counts

ANOVA revealed a main effect of group on counts of calbindin-ir cells that fell within the ellipsoid contour (Fig. 2.3; $F_{2, 15} = 29.97$; $p < 0.0005$). Calbindin-ir cell number was significantly greater in control males than in females ($p < 0.0005$). Females treated with a single injection of EB on the day of birth also had significantly more calbindin-ir cells than control females ($p < 0.0005$). The EB-treated females were not different from males, indicating a full masculinization of the calbindin SDN-POA cell cluster in females after neonatal treatment with EB.

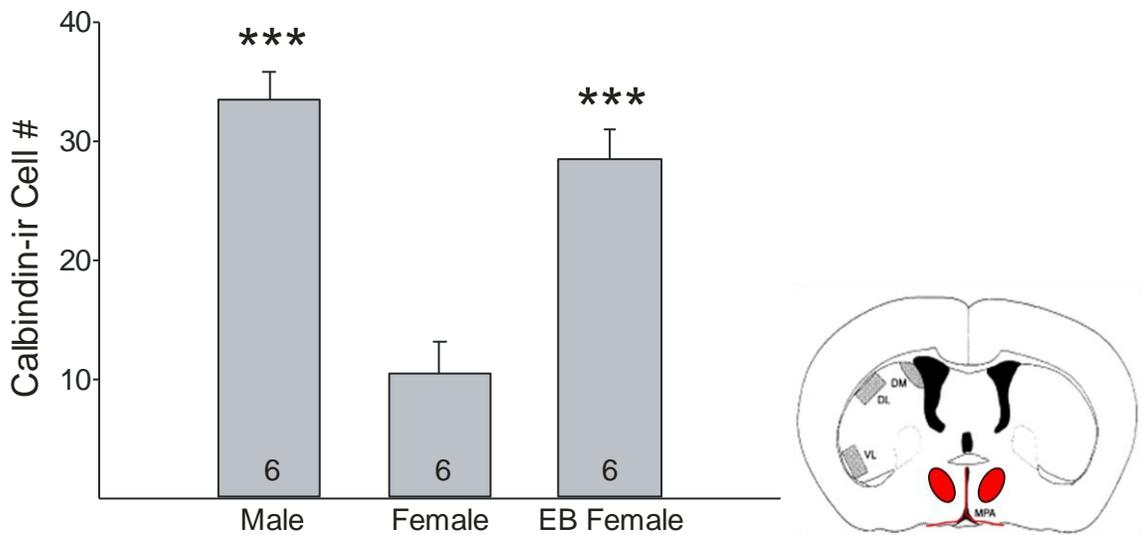


Figure 2.3 – Mean (\pm SEM) number of calbindin-ir cells in the ellipsoidal contour for male, female, and EB-treated female mice ($N = 6$ /group). (Asterisks indicate significantly different from female group: ***, $p < 0.0005$)

Grid Contour Cell Count Analysis

Cell counts for the entire grid (total counts) and for each of the nine blocks of the grid were compared among groups. For total calbindin-ir cell counts, there was a significant effect of group ($F_{2, 15}$; $p = 0.006$). Males and EB-treated females had significantly more cells than control females (Fig. 2.4; $p = 0.002$ and $p = 0.016$, respectively), and were not significantly different from each other. These results are consistent with those seen in the previous analysis, but also show that expanding the area in which calbindin-ir cells are counted does not eliminate the sex difference.

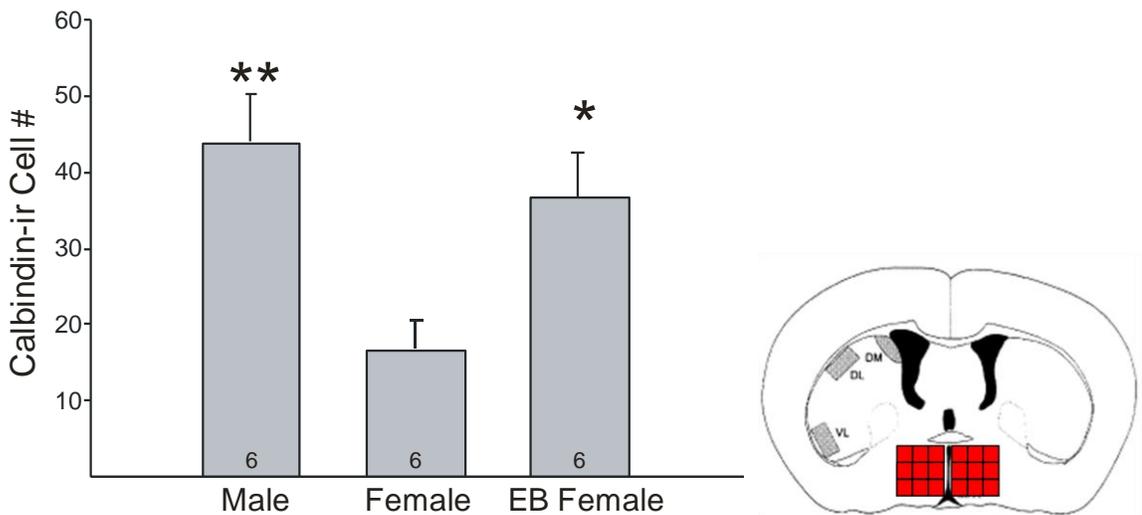


Figure 2.4 – Mean (\pm SEM) number of calbindin-ir cells in the total grid contour (blocks #1-9) for male, female, and EB-treated female mice ($N = 6$ /group). (Asterisks indicate significantly different from female group: *, $p < .05$; **, $p < .01$)

We performed separate ANOVAs for each of the nine individual blocks in the grid. Block #5 (which captured the calbindin-ir cluster as defined above) contained about half of the total calbindin-ir cells in each group and was the only block of the grid that differed significantly among the test groups (Fig. 2.5; $F_{2, 15} = 7.553$; $p = 0.005$). As expected, males and EB-treated females both had significantly more calbindin-ir cells

than control females ($p = 0.002$ and $p = 0.029$, respectively) but did not differ from each other in block #5 cell counts.

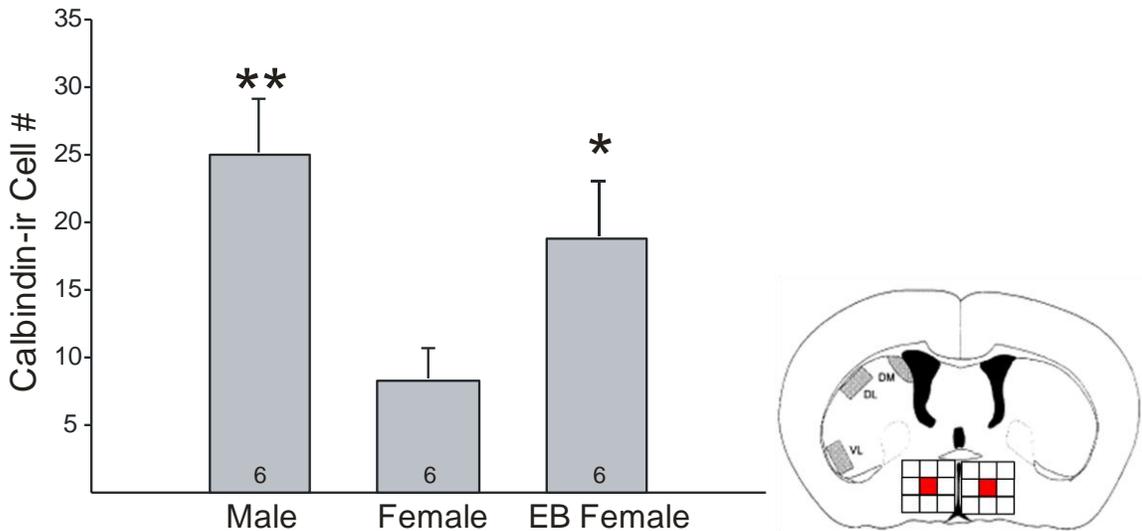


Figure 2.5 – Mean (\pm SEM) number of calbindin-ir cells in block #5 of the grid contour for male, female, and EB-treated female mice ($N = 6$ /group). (Asterisks indicate significantly different from female group: *, $p < .05$; **, $p < .01$)

Although no other single block showed a significant difference in calbindin-ir cell counts by group, we noted that males tended to have more calbindin-ir cells than females in all blocks. Indeed, if an analysis is performed of all blocks except block #5 (i.e., the sum of blocks #1-4 and 6-9), a significant main effect is observed (Fig. 2.6; $F_{2, 15}=6.115$; $p=0.011$). Once again, males and EB-treated females had significantly more calbindin-ir cells in these blocks than control females ($p=0.006$ and $p=0.013$, respectively) but did not differ from each other. Thus, we find no evidence for the suggestion that females have as many calbindin-ir cells as males, but that they are more broadly distributed. If anything, in the region surrounding the main calbindin cluster, males also have more calbindin-ir cells than females.

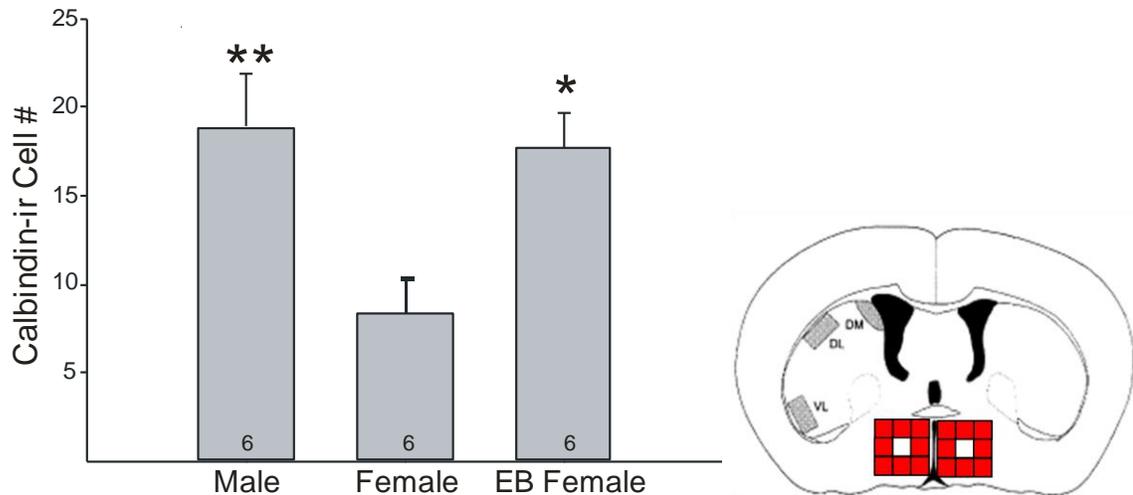


Figure 2.6 – Mean (\pm SEM) number of calbindin-ir cells in all blocks of the grid contour except for block #5 for male, female, and EB-treated female mice (N = 6/group). (Asterisks indicate significantly different from female group: *, $p < .05$; **, $p < .01$)

Finally, we noted that in some cases the calbindin-ir cell cluster extended into block #1, and the dorsolateral corner of block #1 also contain what appeared to be calbindin-ir cells of the ventral BNST in some cases. To evaluate whether this affected our results, we analyzed calbindin counts in all blocks except #1 and #5. This did not change the pattern of results: males and EB females still outpace control females in calbindin-ir cell number ($p = 0.026$; $p = 0.029$, respectively; data not shown).

Discussion

My findings are consistent with the previous papers reporting that male mice have a distinct cluster of calbindin-ir cells in the SDN-POA (Rissman and Bodo, 2008; Edelman et al, 2007; Orikasa and Sakuma, 2010). Using a pre-defined, consistent contour, I find that males have about three times more cells in this cluster than females. My results are also consistent with a previous study (Orikasa and Sakuma, 2010), demonstrating that daily injections of $2\mu\text{g}$ of EB through postnatal day 1-5 in female

mice is sufficient to masculinize the female calbindin-ir cell number in this cluster; here, we produced a full masculinization of the calbindin-ir SDN-POA with a single injection of EB on the day of birth. This confirms that, as is the case for rats, developmental estrogens play a crucial role in the development of the male SDN-POA. As mentioned above, activational effects of gonadal hormones do not seem to play a role, since previous studies have reported that gonadectomy of either sex after puberty has no effect on the sex difference in volume of the SDN-POA in rats (Jacobson et al, 1981), and gonadectomy of mice on postnatal day 42 did not affect the calbindin-ir cell cluster of males or females when examined on day 65 (Orikasa and Sakuma, 2010.)

One possible explanation for the sex difference in the calbindin-ir cluster could be that the calbindin-ir cells in the female mouse SDN-POA do not aggregate as in males, and instead spread out into neighboring brain regions. Büdefeld et al, (2007) noted the lack of a cluster in female mice, but cell counts did not reveal a sex difference in calbindin cell number in the POA area of mice. This study appears to have performed these cell counts on calbindin-ir cells closer to the tail end of the BNST, but it still suggests that the sex difference might result from differential migration of calbindin-ir cells in males and females. Similarly, a recent study in rats (Orikasa et al, 2010) suggested a differential spread of calbindin-ir SDN-POA cells between males and females. To test for the possibility that females have as many calbindin-ir cells as males, but less tightly clustered, we used a grid contour to count calbindin-ir cells from the wall of the third ventricle to the area lateral of the cell cluster. The grid also covered an area extending about 175 μ m dorsal and ventral to the male-specific cluster and encompassed the majority of the POA in these sections.

We found that males and EB-treated females had a higher number of calbindin-ir cells than did control females within the entire grid; these groups also had higher calbindin-ir cell counts in block #5 (the block corresponding to the cluster) and, importantly, in the eight blocks surrounding the male-specific cluster. Since even the areas surrounding the presumed SDN-POA calbindin cell cluster display this sex difference in cell number, it indicates that a more diffuse spread or looser clustering of calbindin-ir cells in female mice is not likely the cause of the sexual dimorphism.

Thus, differential cellular migration does not appear to be a factor in the establishment of the SDN-POA sex difference in mice, although we cannot rule out the possibility that in females calbindin-ir cells are localized to distant areas, beyond even the grid parameters, or spreading rostrocaudally since this study only measured this cell cluster two-dimensionally. The sex difference in Nissl-stained SDN-POA in rats is thought to be due to developmental cell death (Davis et al, 1996). Thus, in the next study, we looked towards neuronal apoptosis as the next logical mechanism to investigate.

CHAPTER III
THE EFFECT OF *BAX* GENE KNOCKOUT ON CALBINDIN-IR CELL
NUMBER AND LOCATION IN THE SDN-POA

Introduction

The previous experiment demonstrated that the sex difference in the number of calbindin-ir cells in the SDN-POA is dependent on neonatal EB exposure, and differential cell localization does not seem to play a role in the development of the SDN-POA sex difference in mice. Neurogenesis is also unlikely to play a role because cells of the SDN-POA are born prenatally (i.e. before the neonatal hormone surge), at least in rats (Jacobson, 1981, Orikasa et al, 2010). In this part of the study, I looked at the potential effect of cell death in the development of this sexual dimorphism. It is possible that females have fewer calbindin-ir neurons in this brain region due to neuronal apoptosis in development.

Neuronal cell death during development is controlled by the Bcl-2 protein family (White et al, 1998). Some of these proteins, such as Bcl-2, are known to prevent apoptosis, but others, such as Bax, promote cell death. Bax deletion has been shown to increase cell number in many areas of the brain in mice. It has also been previously reported that targeted deletion of the cell death gene *bax* in mice eliminates the sex difference in cell number seen in the BNSTp (Holmes et al, 2009), AVPV (Forger et al, 2004), and SNB (Jacob et al, 2005). Although the exact molecular mechanisms of this process are not yet fully understood, it is known that Bax promotes apoptosis by

oligomerizing at the mitochondrial membrane. These Bax oligomers activate downstream caspases (Fig. 3.1), which then cause cell death.

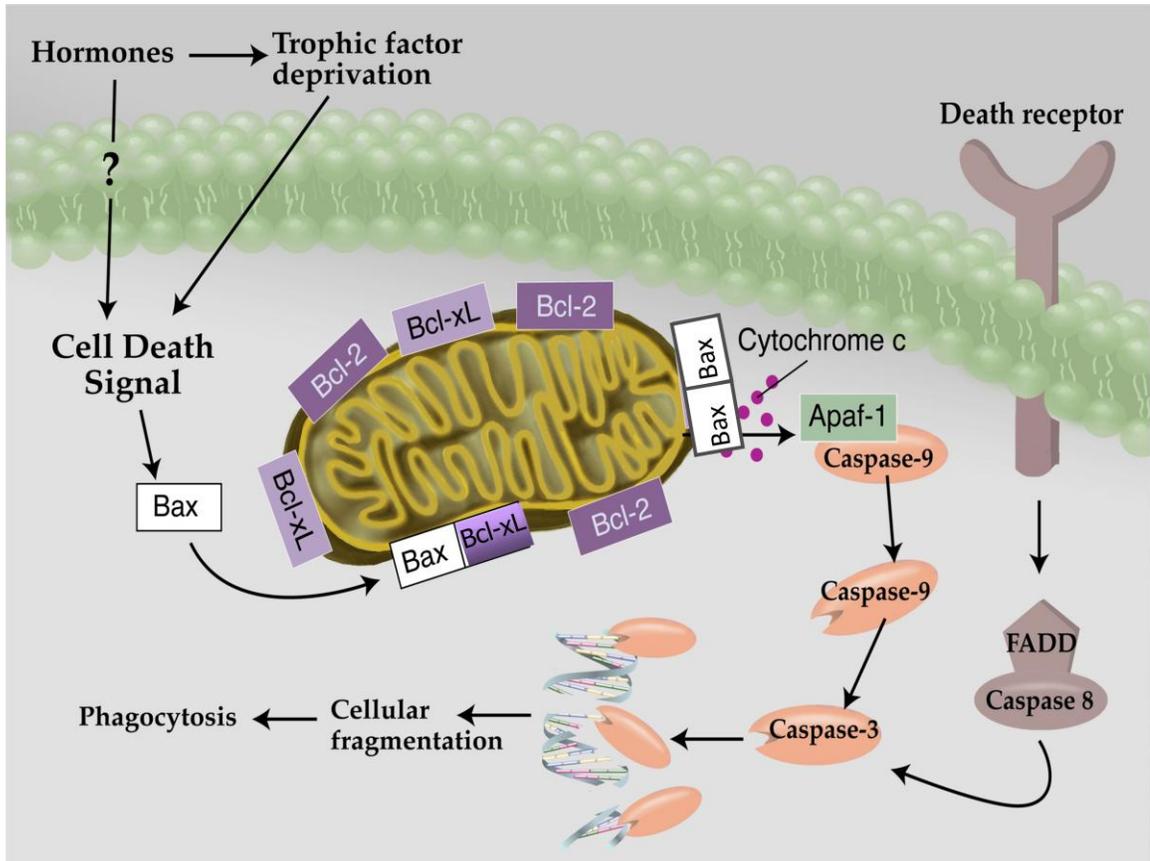


Figure 3.1—Illustration showing the role of Bax in apoptosis. When a cell receives a death signal, Bax protein is targeted to the mitochondrial membrane, triggering release of cytochrome-c. This causes activation of a series of caspase proteins, resulting in cell death. (Adapted from Forger, 2009; artwork by Jay Alexander).

In this study, *bax* knockout (KO) mice were compared to wildtype (WT) mice in order to observe any effect of *bax* deletion on calbindin-ir cell number in the SDN-POA. I predicted that if the sex difference is due to Bax-dependent cell death, then deletion of the *bax* gene would masculinize the calbindin-ir cell number in the SDN-POA cell cluster in female mice.

Materials and Methods

Animals

WT (*bax* +/+) and *bax* KO (*bax* -/-) mice were generated by pairing mice heterozygous for the *bax* gene deletion. As before, animals were housed under 14:10 light dark conditions at 22° C. All procedures were approved by the Institutional Animal Care and Use Committee at the University of Massachusetts, Amherst. Tail tissue samples from the animals were genotyped for *bax* gene status via PCR using previously established primers (White et al, 1998). Animals were killed in adulthood (60-90 days), and their brains were fixed as previously described (N = 6 per group). Sections were cut into two series at 30µm.

Immunocytochemistry for Calbindin-D28k and Cell Counts

Immunocytochemistry for calbindin-D28k was performed for this study exactly as described in Chapter 2. Cell counts were also performed on StereoInvestigator using the same SDN-POA cluster contour and nine-block grid contour as in the previous experiment. To perform a count of total neuron number, coverslips were soaked off after the calbindin-ir cell counts were made, and sections were counter-stained with thionin (Fig 3.2). Total cell counts were performed within the ellipsoidal contour in the same sections as the calbindin-ir cell counts were performed.

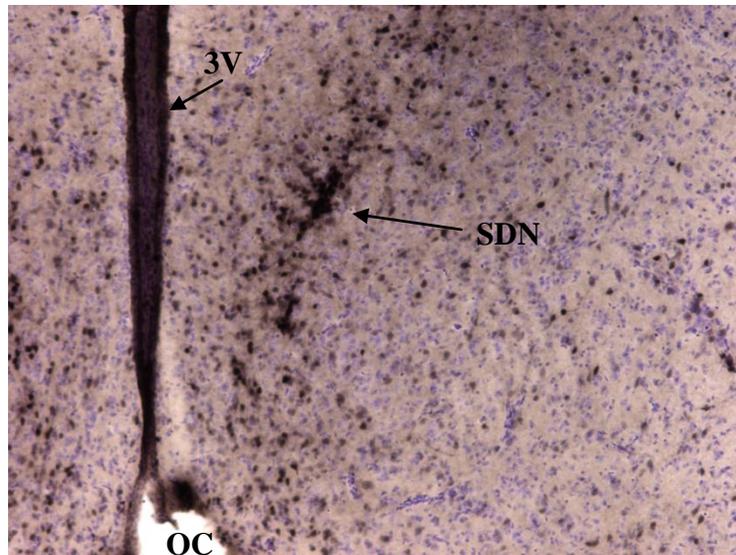


Figure 3.2 – Photomicrograph (10X magnification) of a male counterstained brain section. The darkly labeled cells are from the calbindin ICC. The blue cells seen throughout the section are stained with thionin. (3V = third ventricle; OC = optic chiasm)

Statistics

Calbindin-ir cell count and total (thionin plus calbindin) cell count data were analyzed via two-way ANOVAs to test for significant differences between sex or genotype, and sex by genotype interactions.

Results

SDN-POA Cluster Ellipse Cell Counts

Calbindin-ir cells were counted in the pre-defined ellipsoidal SDN-POA contour. As observed in the previous experiment, there was a main effect of sex, with males having significantly more calbindin-ir cells than females (Fig. 3.3; $F_{3, 20} = 33.144$; $p < 0.0005$). There was no significant effect of *bax* genotype nor any sex by genotype

interaction, indicating that *bax* gene deletion does not interfere with development of the sex difference in calbindin-ir number in the male-specific cluster.

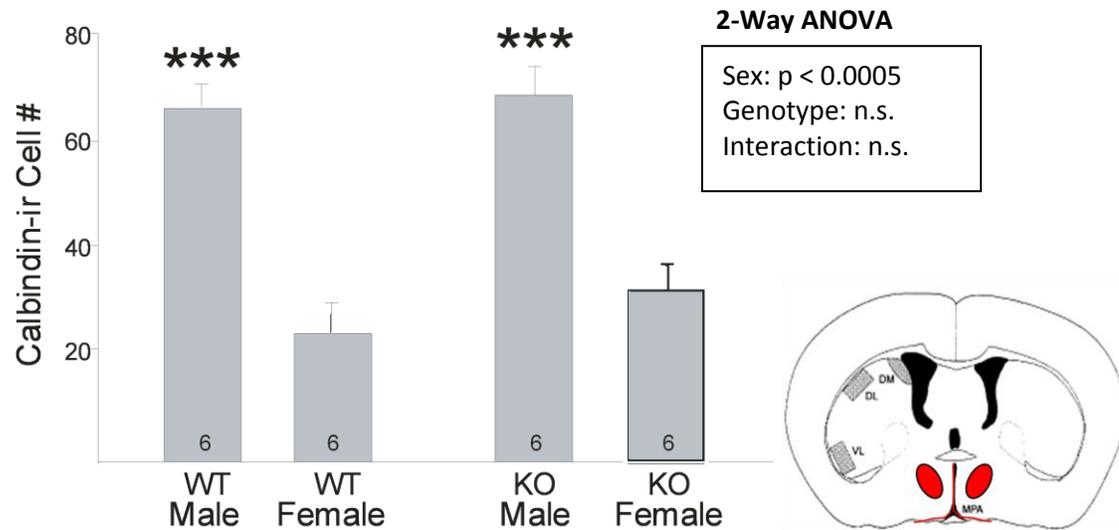


Figure 3.3 - Mean (\pm SEM) number of calbindin-ir cells in the ellipsoidal contour for *bax* +/+ (WT) and *bax* -/- (KO) male and female mice (N = 6/group). (Asterisks indicate a significant difference between males and females of the same genotype: ***, $p < 0.0005$)

Grid Contour Cell Count Analysis

The previously counted sections were next aligned with the grid contour described above. Calbindin-ir cells were counted within each of the nine grid blocks and counts analyzed via two-way ANOVA. Three animals had to be dropped from this analysis because tissue damage or other artifacts did not allow for counts within all nine grid blocks. A main effect of sex was found for the total calbindin-ir cell number (all nine grid blocks) with males once again having more cells than females (Fig 3.4; $F_{3, 17} = 12.458$; $p = 0.003$). There was no main effect of *bax* genotype or a main sex by genotype interaction on this measure.

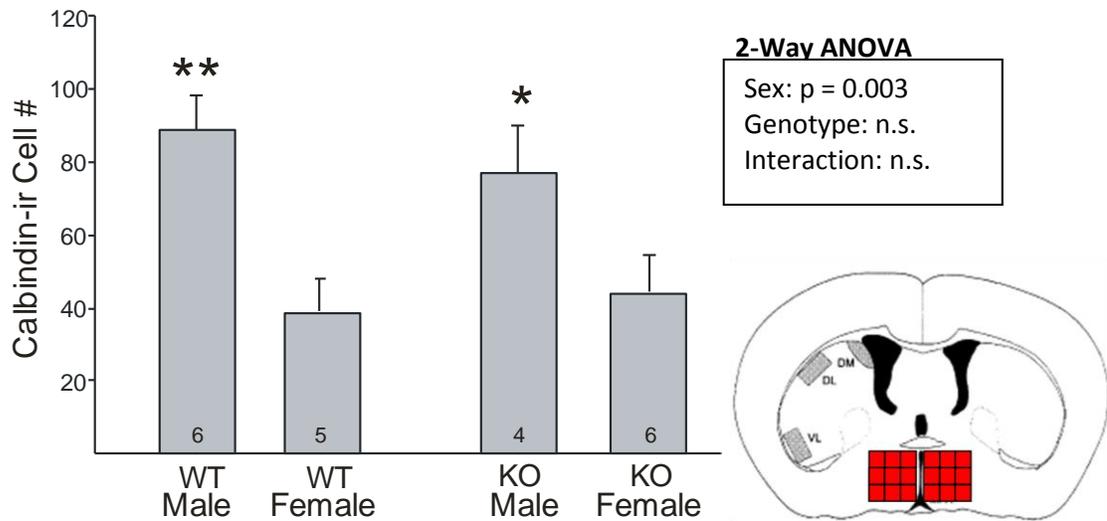


Figure 3.4 - Mean (\pm SEM) number of calbindin-ir cells in the total grid contour (blocks #1-9) for *bax* $+/+$ (WT) and *bax* $-/-$ (KO) male and female mice (N = 4-6/group). (Asterisks indicate a significant difference between males and females of the same genotype: *, $p < .05$; **, $p < .01$)

As in the previous experiment, the number of calbindin-ir cells in grid block #5 (the central block) was also compared between test groups. Once again there was a main effect of sex: males had significantly more cells than females (Fig. 3.5; $F_{3, 17} = 27.640$; $p < 0.0005$), yet no significant effect of *bax* genotype or a sex by genotype interaction was found.

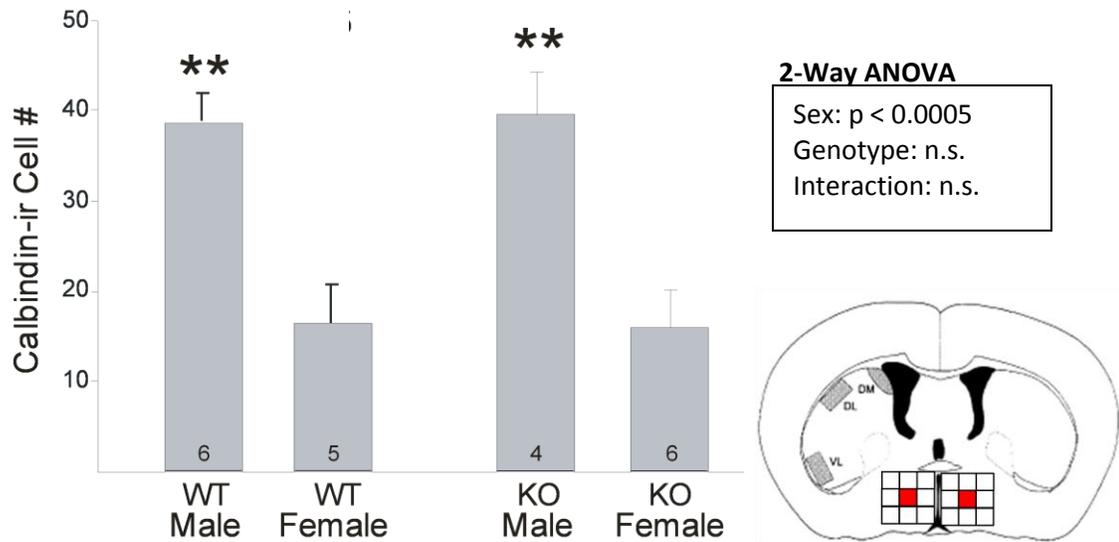


Figure 3.5 - Mean (\pm SEM) number of calbindin-ir cells in block #5 of the grid contour for *bax* $+/+$ (WT) and *bax* $-/-$ (KO) male and female mice (N = 4-6/group). (Asterisks indicate a significant difference between males and females of the same genotype: **, $p < .01$)

Similarly as in the previous experiment, even after removal of the counts from the central block of the grid (sum of blocks 1-4 and 6-9), there was still a main effect of sex with males having more calbindin-ir cells than females (Fig. 3.6; $F_{3, 17} = 4.503$; $p = 0.049$). There was once again no main effect of *bax* genotype. If post hoc tests are performed, WT males have significantly more calbindin-ir cells than females ($p = 0.011$; data not shown), but not in *bax* KO mice. However, the interaction (sex by genotype) is not significant. Finally, when the cell totals from blocks #1 and #5 were both removed from the grid total as in the first experiment, there was no main effect of sex present for the remaining calbindin-ir cells in the grid ($p = 0.31$), although males were (non-significantly) higher than females.

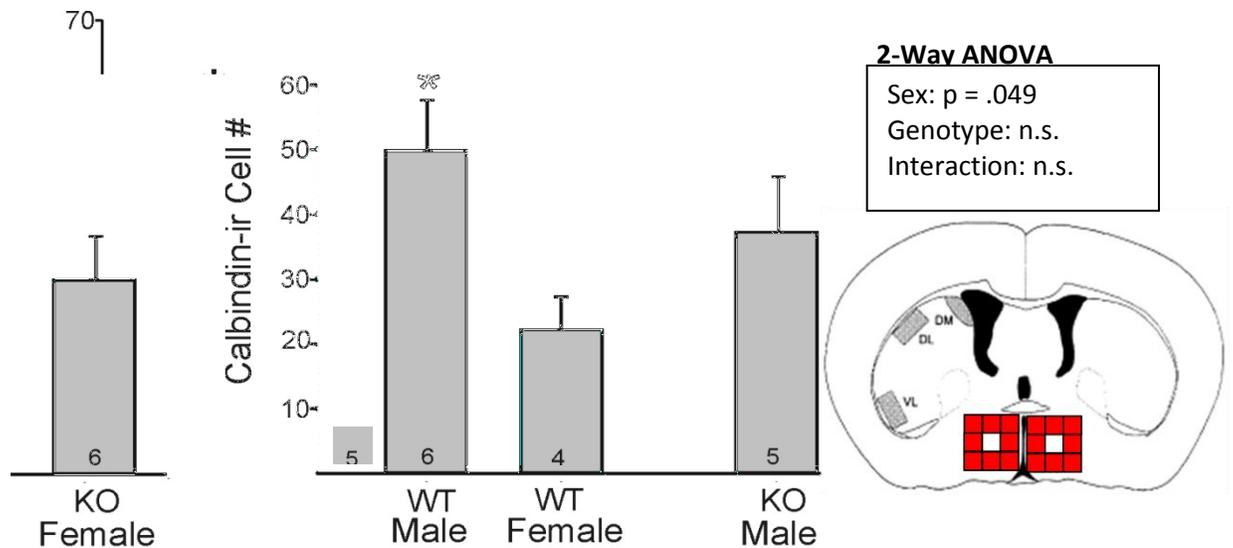


Figure 3.6 - Mean (\pm SEM) number of calbindin-ir cells in all blocks of the grid contour except for block #5 for *bax* $+/+$ (WT) and *bax* $-/-$ (KO) male and female mice (N = 4-6/group). (Asterisks indicate a significant difference between males and females of the same genotype: *, $p < .05$)

Total Cell Counts in Thionin Counterstained Tissue

Previous studies have demonstrated increased neuron numbers in many brain regions of *bax* $-/-$ mice (Deckwerth et al., 1996, Sun et al., 2003). To test for the effect of *bax* deletion on total cell number in the SDN-POA region, the same ellipsoidal cluster contour was positioned as before on sections that were immunoreacted for calbindin and subsequently counterstained with thionin. When all cells were considered (calbindin positive plus negative), two-way ANOVA showed a significant main effect of *bax* genotype: *bax* KO mice have more cells than wildtype (WT) mice (Fig. 3.7; $F_{3,18} = 5.244$; $p = 0.015$). No effect of sex or sex by genotype interaction was observed. Similarly, when only calbindin negative cells were considered, *bax* KO mice were also found to have significantly more non-calbindin cells than WT mice ($F_{3,18} = 4.073$; $p = 0.042$), with no effect of sex and no sex by genotype interaction.

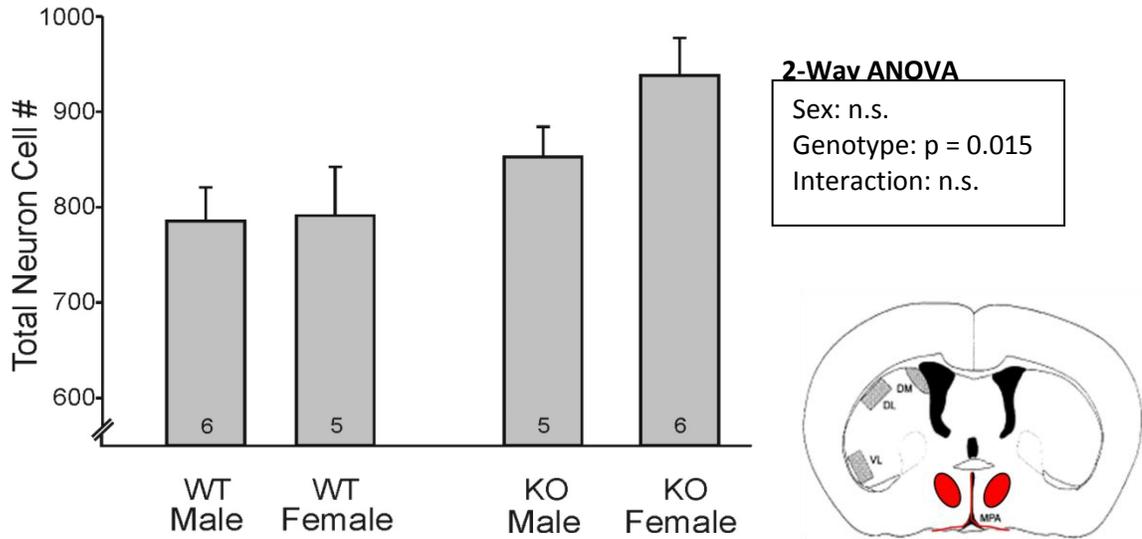


Figure 3.7 - Mean (\pm SEM) values of total cell number in the ellipsoidal contour for *bax*^{+/+} (WT) and *bax*^{-/-} (KO) male and female mice (N = 5-6/group).

Discussion

The purpose of this experiment was to test the hypothesis that *bax*-dependent cell death plays a role in the sex difference in calbindin-ir cell number in the mouse SDN-POA. Since males have more calbindin-ir cells in the SDN region than females, it is possible that females lose these cells during development. Indeed, a study of Nissl-stained SDN-POA of rats indicates that females undergo more apoptosis than males in this area (Davis et al, 1996). Since neonatal estradiol exposure produces a masculine cell cluster, it is possible that estradiol could play a role in this process by blocking a step in the cell death signaling pathway.

The results from this experiment, however, do not support this hypothesis. *Bax* gene deletion did not have any effect on the sex difference observed in calbindin-ir cell number in the ellipsoidal contour or the grid contour, with the exception of the loss of the significant main effect of sex observed in the grid after the removal of counts from blocks #1 and #5. Although subtracting the cell counts from these two grid blocks eliminated

the significant effect of sex, *bax* KO females still did not have significantly more calbindin-ir cells than WT females in this area. Thus, it cannot be concluded that the sex difference in calbindin-ir cell number in the area around the central cluster is attributed to *bax*-dependent cell death. These results indicate that neuronal apoptosis does not play a role in the development of the sex difference in the calbindin SDN-POA cluster. *Bax* deletion also did not have an effect on grid counts, suggesting no developmental apoptosis on calbindin-ir cells peripheral to the cluster. One limitation to this conclusion, however, is the possibility of a compensatory non-*bax* mechanism of cell death.

As far as I am aware, non-calbindin cells in this area had never been counted in mice due to the fact that a distinct sexually dimorphic SDN-POA cell cluster is not detected in a Nissl-stain in mice (Young, 1982). In contrast to calbindin-ir cell counts, total cell counts in the ellipse contour demonstrate no main effect of sex, but a significant effect of *bax* genotype. In other words, *bax* gene deletion causes an increase in total cells in this area in mice, but the number of cells is not sexually dimorphic for either genotype. One possible explanation of why *bax* deletion causes an increase in total cell number but not calbindin-ir cell number is that the calbindin-ir cells are not the ones being affected by cell death in development. This could make sense since calbindin-D28k is known to have anti-apoptotic properties, so it may actually help protect these cells from cell death. Thus, only the non-calbindin-ir cells would be rescued by *bax* gene deletion. These results also indicate that calbindin-ir cells of the mouse SDN-POA account for less than 10% of the total cell number in this area. This finding may weaken the argument for calbindin-D28k immunoreactivity as an accurate marker for the study of cell number in the mouse SDN-POA.

Although the current results suggest that the sex difference in calbindin-ir cell number in the SDN-POA is not due to cell death, we cannot rule out the possibility that the cells are rescued from cell death by *bax* deletion, but fail to make calbindin protein. We would then not detect a change in the sex difference with calbindin staining. Other studies have also shown similar effects in known sexually dimorphic areas in mice. Forger et al, (2004), show that *bax* deletion causes an increase in total cell number of the AVPV, eliminating the sex difference (WT females have more cells than WT males). However, this study also reports no increase in tyrosine hydroxylase (TH) positive cells in the AVPV in *bax* KO mice of either sex, and no effect on the sex difference. While a more recent study also reports this finding (Semaan et al, 2010), they also report an increase in the number of kisspeptin-positive cells in the AVPV in *bax* KO males and females, although the sex difference is not eliminated. De Vries et al, (2008), report an increase in vasopressin-labeled cells in the BNST in mice, but no effect on the sex difference in vasopressin cell number. Holmes et al, (2009) report that *bax* deletion eliminates the sex difference seen in total cell number in the mouse BNSTp, and also at least partially eliminates the sex difference in androgen receptor positive and total neuron number. Other than the TH report, these findings suggest that labeled cells are usually rescued from cell death in *bax* KO mice. Our results suggest that calbindin expression is not rescued in the supernumerary cells in the SDN-POA of *bax* KO mice, similar to what is seen in the TH cells in the AVPV.

CHAPTER IV

GENERAL DISCUSSION

The results of these studies show that a sexually dimorphic calbindin-ir cell cluster exists in mice, similar in position to that observed in rats. Furthermore, male mice demonstrate a greater number of calbindin-ir cells in this cluster than females based on analysis with a defined ellipse contour, and the cell number is masculinized in females with a single injection of EB on the day of birth. As a possible mechanism of development of this hormone-dependent sex difference, differential cell spread was investigated via use of a larger grid contour, yet male mice still demonstrated significantly greater numbers of calbindin-ir cells than females throughout the grid. Thus, more diffuse expression in females does not appear to contribute to this sex difference in mice, although it cannot be ruled out that female calbindin SDN-POA cells migrate rostrocaudally or to areas beyond the parameters of the grid contour.

Due to previously reported effects of *bax*-dependent cell death on other known sex differences in the brain, *bax* knockout mice were compared to wildtype mice in order to investigate the role of apoptosis in the establishment of this sex difference. *Bax* gene deletion did not have a significant effect on the sex difference in calbindin-ir cell number in the ellipse contour or grid contour. This suggests that cell death does not significantly contribute to the sex difference in calbindin-ir cell number observed in the POA in mice.

The increase in total cells in the ellipsoidal SDN-POA area in *bax* KO mice, yet no difference in calbindin-ir cell number, suggests that the cells being rescued from *Bax*-dependent cell death may be unable to express calbindin. Previous studies have reported similar findings in the AVPV (Forger et al, 2004), yet others in the AVPV and BNSTp

have been able to demonstrate rescue of labeled cells (De Vries et al, 2008; Homes et al, 2009; Semaan et al, 2010). Based on these findings, future studies investigating cells in the mouse SDN-POA region labeled with other markers may shed more light on the nature of these rescued cells, and may also lead to the discovery of other sex differences in expression in cells in this area.

Also, any role of calbindin-D28k itself in the mechanism of development of this sex difference can only yet be assumed. It is known, however, that calbindin has many anti-apoptotic properties that could potentially contribute to the sex difference, such as inhibition of caspase-3 (Bellido et al, 2000). This could explain why the number of calbindin-ir cells is not greater in *bax* KO mice, since these calbindin cells would be protected from developmental cell death. Future studies could use calbindin siRNA treatment during development in order to test this hypothesis.

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