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Understanding Immunohistochemical Techniques As They Relate To Brain Sexual Dimorphisms

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ABSTRACT OF THE THESIS

Understanding Immunohistochemical Techniques

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The sexually dimorphic nucleus of the preoptic area (SDN-POA) is one of the largest known sexual dimorphisms in the brain, and its discovery led to a greater understanding of sexual differentiation in non-gonadal tissues. The SDN-POA is larger in males, mostly due to gonadal hormones' organizational effect around birth. The Four Core Genotypes (FCG) model in mice is an important model in understanding sex differences and is often used with other mouse models of disease and physiology. The FCG-like model in rats, better described as the *Sry*-modified rat model, is currently being developed in our lab. When established gonadal hormone effects are replicated in the *Sry*-modified rats, then people gain confidence in this newly developed model for sex differences. There are two components of the *Sry*-modified rat model. The first is the CRISPR-mediated reduction in dose of the testis-determining gene *Sry* on the Y chromosome (denoted as Y^{Δ}), producing XY^{Δ} and XXY^{Δ} rats with ovaries. The second component is the insertion of an *Sry* transgene onto an autosome (denoted as *Sry*TG+), producing XX(*Sry*TG+) rats with testes. These two separate modifications are bred in two breeding paradigms: transgenic and KO. The transgenic litters are produced by mating an XX

female and an XY(*Sry*TG+) male, and the KO litters are produced by mating an XY male and an XY^{Δ} female. From these two breeding schemes, six genotypes are produced: XX gonadal females, XXY^{Δ} gonadal females, XY gonadal males, XYY^{Δ} gonadal males, XY(*Sry*TG+) gonadal males, XX(*Sry*TG+) gonadal males. Furthermore, mating XXY^{Δ} gonadal females and XY(*Sry*TG+) gonadal males produces two more viable genotypes: XXY^{Δ}(*Sry*TG+) gonadal males and XYY^{Δ}(*Sry*TG+) gonadal males. In total in the *Sry*-modified rats, there are eight genotypes produced and utilized for comparison. The SDN-POA can be examined in these eight genotypes and compared across groups of different sex chromosomes, gonads, and *Sry* and Y chromosome doses. The SDN-POA size is quantified through immunohistochemical staining for calbindin immunoreactive cells, but there are many variables that influence the quality of the immunohistochemistry. These variables include but are not limited to perfusion rates during preparation of the tissues, types of reagents used during the immunohistochemistry, and the concentrations of reagents. The SDN-POA is used for comparison because the development of this nucleus is heavily influenced by organizational effects of hormones, so it serves as a bioassay for testicular hormones before and around the time of birth. Furthermore, by measuring the sex differences in the SDN-POA in the *Sry*-modified rat model, it can help validate the model so that it can be used in other models of disease and physiology.

The thesis of Tierra Chiyaka Parker is approved.

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TABLE OF CONTENTS

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INTRODUCTION

Sex determination and sexual differentiation are two important and distinct events in the development of an embryo. Sex determination is the determination of the type of gonads which can depend on different factors, such as sex chromosomes or environment, depending on the organisms. In mammals, sex determination is controlled by sex chromosomes so an individual's chance of having testes is dependent on the presence of the sex-determining region of the Y chromosome, also known as *Sry*. On the other hand, sexual differentiation is the process by which non-gonadal tissues are differentiated in the two sexes. Sexual differentiation of mating behavior in female guinea pigs was studied by Charles H. Phoenix et al. when they administered testosterone prenatally. As adults they do not exhibit typical female lordosis behavior when injected with estrogen and progesterone, but rather exhibit typical male mounting behavior when injected with androgens in adulthood. These studies suggest an “organizational” effect of testosterone prenatally that have permanent effects on the sexual differentiation of non-gonadal tissues (1). For decades, it was however thought that sex chromosomes only influenced sexual differentiation through the development of gonads that secrete sex steroid hormones.

While some tissues can be directly influenced by the organizational effects of gonadal hormones, it has been recently discovered that sex chromosomes also act directly on non-gonadal tissues to cause sexual differentiation. Each XX and XY cell can be phenotypically different because each cell shows different expression of X and Y genes which are caused by differences in the effects of XX vs. XY sex chromosomes (2). For example, in the presence of two X chromosomes, one of the X chromosomes will express *Xist*, a non-coding RNA found on the X chromosome. The expression of *Xist* on an X chromosome will silence itself in organisms with two X chromosomes to induce X inactivation so that there is an equal expression of most X genes in the XX and XY cells. X inactivation does not occur in XY cells so there is unequal expression of *Xist* in the cells of the two sexes. Moreover, some X genes escape X inactivation

so that XX cells will have higher expression of these genes compared to XY cells (3). A phenotype dependent on the sex chromosome is vasopressin expression in the septum. There were higher vasopressin-immunoreactive fibers in the septum in XY mice compared to XX mice (46). These are a few examples of sex differences found within sexual differentiation that are not dependent on gonadal hormones, which disproves the notion that sexual differentiation solely relies on gonadal hormones.

Sex differences arise during sexual differentiation during critical points of embryonic and postnatal development and puberty. As previously mentioned, these differences are not only influenced by the organizational effects of gonadal hormones but also by differences in the effects of the sex chromosomes. Sex differences are evident in many neurological and psychiatric diseases, such as Multiple Sclerosis (MS) and Alzheimer's disease (AD). They are both neurodegenerative diseases that do not affect the two sexes similarly in that women are more likely to develop MS and AD compared to men but men with MS have a worse disease progression compared to women (4-5). Consequently, it can be concluded that a particular aspect of an individual's sex is preventing them from the onset of symptoms or exacerbating the disease, but it is difficult to separate the effects of the sex chromosomes and gonadal hormones. In understanding sex differences in diseases, individuals can receive treatment specific to the pathogenesis of the disease and physicians can be aware of potential differences at the cellular level to diagnose certain individuals earlier.

While sex differences are evident, they are not well understood and are difficult to study. Studying sex differences can be challenging because gonadal hormones and sex chromosomes influence each other and separating the two is not an easy task. The Four Core Genotypes (FCG) mouse model allows the effects of sex chromosomes and gonadal hormones to be separated. There are two important components of the FCG mice model: deletion of *Sry* from the Y chromosome and insertion of the *Sry* transgene onto an autosome. Both components were discovered separately in different studies in mice. Robin Lovell-Badge discovered a

deletion of *Sry* from the mouse Y chromosome led to the development of ovaries instead of testes, while Peter Koopman inserted a transgene encoding *Sry* into an autosome to create an XX gonadal male (6-7). The FCG mouse model uses a Y chromosome deleted for *Sry*, called the Y- (Y minus) chromosome, which fails to cause testes to develop so that XY- mice have ovaries. The loss of *Sry* is complemented by an *Sry* transgene on Chromosome 3 causing testes development in mice regardless of the sex chromosome complement. FCG transgenic mice are created by mating an XX female and an XY-(*Sry*TG+) father, so that four genotypes can be created: XX gonadal female, XY- gonadal female, XX(*Sry*TG+) gonadal male, and XY-(*Sry*TG+) gonadal male. These four genotypes are then compared to each other to understand the effects of sex chromosomes and gonadal hormones independent from each other. Any phenotypic differences between XX and XY animals with the same type of gonad is interpreted as a sex chromosome effect, while any phenotypic difference between gonadal female and male groups with the same complement of sex chromosome are the result of a gonadal hormone effect. Another possibility is an interaction between sex chromosomes and gonadal hormones which would cause a difference in the effect of one variable because it is influenced by the level of the other variable (8). By using the FCG mouse model and separating the sex chromosomes and gonadal hormones, the cause of sex differences is more apparent and can be better understood for further studies examining these sex differences in other physiology and disease models.

The FCG mouse model can be used in conjunction with other mouse models of disease and physiology to better understand sex differences. For example, autoimmune diseases, such as lupus and MS, have an evident sex difference that affects females more than males. When looking at an MS mouse model in conjunction with the FCG mouse model, gonadectomized XX mice showed greater motor deficits compared to gonadectomized XY mice regardless of their gonadal type, which suggests that having two X chromosomes is disadvantageous, relative to XY (42). Other studies have examined sex differences using the FCG mouse model, such as

cardiac ischemia and reperfusion injury, alcohol consumption and relapse behavior, pulmonary hypertension, and body weight and adiposity (9-12). While many studies have been done in mice, the FCG mouse model cannot be applied to rat models of disease and physiology.

Creating the *Sry*-modified rat model is important because some models of disease and physiology are better represented in rats than in mice and studies done in mice cannot be generalized to other animals. Rats are larger so they are easier to perform surgical procedures and observe certain behaviors compared to mice. Furthermore, some diseases are better modeled in rats, such as Autism Spectrum Disorder, Parkinson's Disease, and addictive and impulsive behavior, because rats used in these models arguably show symptoms and behaviors more similar to those found in humans (13). Moreover, the FCG mouse model is the main model used to discover sex chromosome effects, although other models have been used, so results are difficult to generalized beyond mice (14). By creating the *Sry*-modified rat model, more studies can be done to understand sex differences in more diseases and phenotypes that are not only found in mice and more generalizable information can be discovered.

The production of the *Sry*-modified rat model is more complex compared to the FCG mouse model because of differences in the number of the *Sry* genes in the two animals. As previously mentioned, the FCG mouse model is made through the insertion or removal of one *Sry* gene from the Y chromosome onto an autosome. On the other hand, the rat Y chromosome contains 8-11 different *Sry* genes, but it is unclear which of these genes specifically and/or what combination of *Sry* genes lead to testes development. It was previously found by Jeremy Prokop et. al that at least three *Sry* genes (Sry 4a, 1, and 3c) are candidates for causing the formation of testes because these genes were expressed in the gonadal ridge just prior and during testis formation in developing embryos. Therefore, the *Sry*-modified rat model takes advantage of these genes in the production of the rat model discussed here (43). Since there are multiple *Sry* genes and it is unclear what combinations of genes lead to testes development,

in the *Sry*-modified rat model, currently being developed in our lab, there are complications in accurately reproducing the FCG mouse model.

The transgenic rat litters are produced by mating an XX female and an XY male which carries a transgene containing the testes-determining gene *Sry* on an autosome, indicated as (*Sry*TG+). A single Bacterial Artificial Chromosome (BAC) clone containing the three *Sry* genes (Sry 4a, 1, and 3c) is inserted onto an autosome so that an XY male rat still has the *Sry* dosage from its Y chromosome but also has an extra dose of the *Sry* genes from the BAC transgene. Here four genotypes are produced: XX gonadal female, XY gonadal male, XX(*Sry*TG+) gonadal male, and XY(*Sry*TG+) gonadal male (Figure 1). Here the XX and XY rats with testes can be compared, so that any phenotypic differences between these groups would be explained by a sex chromosome effect.

Theoretically, the KO rat litters are produced by mating an XY wild-type male and an XY gonadal female who has a “knockout” of *Sry* on the Y chromosome, indicated as XY^{Δ}. The CRISPR modification of the Y^{Δ} chromosome likely reduced the number of copies of *Sry* or eliminated specific *Sry* genes critical for testis determination. Some *Sry* copies remain on the Y^{Δ} chromosome, but these are insufficient to cause testis development. In the cross seen in Table 1, the females make 3 kinds of eggs (X, Y^{Δ}, XY^{Δ}) and males make X or Y sperm. The cross results in 5 viable genotypes: three gonadal females (XX, XY^{Δ}, XXY^{Δ}) and two gonadal males (XY, XYY^{Δ}) (Figure 2). With these groups, comparisons can be made between groups with different numbers of X chromosomes and *Sry* dosages within this litter and between the transgenic litter and the KO litter.

The nondisjunction leading to XY^{Δ} egg cells is the result of lack of pairing of X and Y^{Δ} chromosome in diploid oogonia as they prepare for meiosis I. The genes required for X-Y pairing evolved in spermatogonia, but not in oogonia where such pairing presumably never occurred during evolution. Accordingly, the unpaired X and Y^{Δ} can separate at meiosis I and enter different daughter primary oocytes, or they can enter the same oocyte. Mahadevaiah et. al

compared fertility and offspring from XY- and XXY- mice. Some differences in their breeding pattern could explain why our lab has not been able to retrieve XY^{Δ} gonadal female rats from XXY^{Δ} mothers. These mice were produced so that any individual with a Y chromosome does not necessarily have testes. The XXY- female mice had normal fertility while the XY^{-} female mice had low fertility compared to the wildtype XX female mice. These studies suggest that it is not the presence of the Y chromosome but the absence of the second X chromosome that influences fertility in these mice (15). The KO litter in the *Sry*-modified rat model show a similar breeding pattern to the previously mentioned XY- and XXY- mice. Once our lab discovered that some of our XY^{Δ} gonadal female rats were in fact XXY^{Δ} gonadal female rats, it was difficult to recover the XY^{Δ} gonadal female rats because XXY^{Δ} gonadal female rats do not produce XY^{Δ} daughters.

Both the transgenic and KO groups allow for some separation of sex chromosomes and gonadal hormone effects and how they affect sex differences in rats. With the addition of the new genotypes with different X and Y chromosome dosages, the *Sry*-modified rat model will also provide a better understanding of the potential effects of chromosome dosages on sexual differentiation in rats. Sex chromosome dosages can be examined in mice through the XY* mouse model and the Sex Chromosome Trisomy mouse model because these models produce mice with different numbers of sex chromosomes and different X and Y chromosome dosages (XO, XXY, XYY) (11-12). Previously, these sex chromosome aneuploid genotypes have not been produced in rats. In humans, individuals can be trisomic, such as those with Klinefelter's syndrome (XXY) and Jacobs syndrome (XYY), which can have negative effects on fertility and other health and behavioral complications. With the new genotypes in the *Sry*-modified rat model (Figure 2) further studies can be done to understand the mechanisms that differentiate any phenotypic differences in individuals with sex chromosome aneuploidies (Table 2).

Klinefelter's syndrome (XXY) is the most common sex chromosome aneuploidy and genetic cause of male infertility. Individuals with Klinefelter's syndrome receive an extra X

chromosome from either parent due to nondisjunction during meiosis. These individuals are often undiagnosed because they have typical male phenotypic characteristics, but they are infertile in adulthood which raises concerns and leads to diagnosis. During puberty, these individuals are characterized with small testes and varying symptoms of androgen deficiency (16). Studies have examined Klinefelter's syndrome-like mice by mating wild-type XY male mice with female mice chimeras that receive an injection of male embryonic stem cells so that about half of their embryonic stem cell-derived male offspring will have nonmosaic aneuploidy. Another way to make these mice is through the previously mentioned XY* model. Similar to in humans, adult XXY mice are reported to have small testes, decreased plasma testosterone levels and androgen receptor expression, and elevated LH and FSH levels compared to XY mice. Adult XXY mice also showed small seminiferous tubules, fewer Sertoli cells, and more abundant Leydig cells. Sertoli cells are dependent on androgen receptor activity in the nucleus to allow for normal cell function to occur, so there are dysfunctional Sertoli cells in the XXY mice due to the few numbers of androgen receptors. There is not a clear explanation for why these events occur, but a possible explanation includes dysfunctional somatic-germ cell communication during differentiation of the testis which leads to decreased androgen receptor expression (17).

Jacobs syndrome (XYY) is another sex chromosome aneuploidy. Individuals with Jacobs syndrome will receive an extra Y chromosome from their XY parent as a result of nondisjunction during the second meiotic division. As in Klinefelter's syndrome, these individuals are often undiagnosed but behavioral problems, infertility, learning disabilities, and delayed speech development may raise concern. Few studies have been conducted in both humans and animals, because XYY individuals are often left undiagnosed and XYY mice are nearly always sterile. Because XYY individuals are typically diagnosed late in life because of cognitive or behavioral issues, most of the studies done in the past have not represented the entire XYY population. When examining XYY individuals with ages ranging from newborns to adults,

pubertal onset was generally normal and pubertal hormone levels were within normal range with some variability but there was a general trend towards enlarged testes (18). A study where the mice either were XYY*^{X} (one dose of the Y chromosome because the Y*^{X} chromosome is an X chromosome lacking most genes) or XYY (two doses of the Y chromosome) compared the fertility of mice with one or two doses of the Y chromosome. It was found that it was not the presence of an extra Y chromosome that led to infertility, but it is the difference in sex chromosome synapsis. Synapsis allows for proper chromosome segregation by aligning homologous chromosomes prior to separation. When looking at cells undergoing meiosis, the XYY*^{X} male mice sex chromosomes showed fuller pseudoautosomal region (PAR) synapsis compared to XYY male mice sex chromosomes. When there is dysfunctional PAR synapsis, there is a decrease in spermatogenic proficiency. This difference could explain why XYY*^{X} male mice are fertile and have higher testis weights and sperm counts compared to XYY male mice (19). While XXY and XYY individuals have differences in sex chromosome dosages, these differences in sex chromosome dosages in comparison to individuals with two sex chromosomes creates differences in fertility, hormone levels, and behavior. There are still some unanswered questions surrounding the fertility and physiology associated with sex chromosome aneuploidies, but the *Sry*-modified rat model should allow for investigation of these questions in the future.

The present project involved analysis of the sexually dimorphic nucleus of the preoptic area (SDN-POA) in the *Sry*-modified rat model. The SDN-POA is one of the largest known sexual dimorphisms in the brain and its discovery allowed for a greater understanding of sexual differentiation in non-gonadal tissues. Previously named the medial preoptic nucleus (MPON), the nucleus was discovered by Roger Gorski in 1977 at the University of California, Los Angeles in rats. This sex difference was also reported in humans, guinea pigs, ferrets, gerbils, Japanese quail (20). Initial studies of the nucleus examined a clear sex difference in this region of the preoptic area between the brains of male and female rats, but the mechanism behind this sex

difference was not well understood. When looking at gonadectomized adult rats, gonadectomized adult male rats displayed a larger MPON compared to the gonadectomized adult female rats (21). Male rats also treated neonatally with an aromatase inhibitor showed a smaller SDN-POA volume compared to male rats treated prenatally with the aromatase inhibitor and control male rats (22). Female adult rats given testosterone propionate and neonatally castrated males had significantly larger volumes compared to the control female group (23). These studies illustrate the influence of the hormonal environment surrounding birth on the development of the SDN-POA. Therefore, the SDN-POA serves as a bioassay because of the known organizational effects of testosterone in this nucleus. If rats with testes and different sex chromosome complements have similar SDN-POA sizes, then the *Sry*-modified rat model is valuable because an important component of the model is holding the hormone effects constant.

While the exact function of the SDN-POA is not completely understood, the preoptic area within the hypothalamus is involved in sexual behaviors and the reproductive endocrine system in both sexes (24). Some studies examined the relationship between the SDN-POA volume and typical male sexual behavior in adult rats under different conditions. Lesion studies done on the medial preoptic area in adult male rats show disrupted mounting initiations and less ejaculatory behavior in the presence of estrous females compared to sham-lesion male rats (25). Male rats treated prenatally with an aromatase inhibitor showed reduced female partner preference and lower frequency of mounts, intromissions, and ejaculations in the presence of an estrous female (22). Furthermore, neurons within the SDN-POA have been shown to express calbindin-D28K (calbindin), a calcium binding protein that functions in calcium transportation and have been shown to also be greater in male rats and activated during sexual behavior; however, the exact role of calbindin neurons in the SDN-POA is still unknown (24). These studies suggest that the SDN-POA is related to partner preference and sexual arousal and may

have a role in calcium transport, but further research needs to be done on the function of this brain region.

Previous work done in our lab examined the SDN-POA in the *Sry*-modified rat model in five genotypes: XY gonadal males, XY(SryTG+) gonadal males, XX(SryTG+) gonadal males, XX gonadal females, and XY^{Δ} gonadal females. The SDN-POA was quantified by measuring the SDN-POA total volume, total number of cells, and average cell area. The gonadal male groups had a larger SDN-POA volume, greater neuron count, and larger neuron cell size compared to gonadal female groups, which suggests a sex difference due to gonads. Moreover, there were no differences in the same-gonad groups differing in sex chromosome complement (26). After this work was performed, it was discovered that the XY^{Δ} gonadal female group also contained some XXY^{Δ} gonadal females. Thus, the previous study needs to be redone to measure the XXY^{Δ} gonadal females accurately and measure the new trisomic genotype, XYY^{Δ} gonadal males, XXY^{Δ} (*Sry*TG+) gonadal males, and XYY^{Δ} (*Sry*TG+) gonadal males. With these new groups a new comparison can be made within groups of different X and Y chromosome dosages (Table 1). Since it has been reported that gonadal males have a larger SDN-POA, we hypothesize the SDN-POA of the gonadal males in the six genotypes will be larger than the two gonadal female groups, with larger and more numerous calbindin neurons. We also hypothesize the number of X chromosomes will not cause a significant difference in the gonadal females. We do not expect any differences in the same-gonad groups differing in sex chromosome complement.

SEXUALLY DIMORPHIC NUCLEUS OF THE PREOPTIC AREA (SDN-POA)

In the first study identifying the SDN-POA, Roger Gorski examined the influence of prenatal hormone environment on the size of the nucleus in adulthood. Male and female rats were gonadectomized as young adults and were given no treatment, oil treatment, progesterone and estradiol treatment, or testosterone treatment. The rats were then sacrificed and perfused

for brain dissection and preparation for thionine staining. Among all of the groups, the male groups had a significantly larger nucleus volume independent of the hormonal environment in adulthood (23). These studies suggest that the hormones introduced in adulthood had no effect on the development on the nucleus. Therefore, the development of the SDN-POA must be prior to adulthood. The next experiment examined the hormone environment surrounding birth.

In looking at the influence of neonatal hormone environment on the size of the nucleus in adulthood, there were three female groups and two male groups. At four days following birth, the female rats were either injected with oil treatment, 90 μg testosterone propionate, or 1 mg testosterone propionate. These rats were ovariectomized at 45-50 days of age and sacrificed and stained similarly to the previous experiment. Male rats were either castrated or sham castrated at birth. The sham castrated males had a significantly greater nucleus volume compared to any other groups. Furthermore, the neonatally castrated males and the females treated with 1 mg testosterone propionate had a significantly larger nucleus volume compared to the control female group. These two studies suggest an organizational effect of androgens in the development of the nucleus at birth regardless of the hormone environment present in adulthood (23). The female groups given testosterone in adulthood did not show a significantly large volume because they were not exposed to testosterone during the critical period near birth that would allow them to develop this nucleus. Therefore, the female groups introduced to testosterone near birth showed a larger SDN-POA.

At the time of SDN-POA discovery, sexual differentiation was also gaining more recognition so many studies were examining sexual differentiation in the brain. Around this time, it was discovered that estrogens act in the brain to masculinize the brain for male sexual behavior development. Furthermore, male rats given an aromatase-inhibitor ATD (1,4,6-androstatriene3,17-dione) at various points during pregnancy and following birth showed impaired masculine sexual behavior, enhanced feminine sexual behavior, and decreased female partner preference in adulthood. Aromatase acts to convert androgens to estrogens, so

these studies suggest aromatase as an important factor in male sexual differentiation. Houtsmuller et. al examined the effects of aromatase in the development of the SDN-POA and sexual behaviors in male rats varying in ATD exposure (22). Female rats were given daily injections of ATD or propylene glycerol (control) during days 10-22 of pregnancy. Within the male pups from the ATD mothers, some were administered a subcutaneous ATD implant that was removed after 21 days, and some received no implants. With these variables, three groups were formed: males treated pre- and neonatally with ATD (pn-ATD), males treated with ATD only prenatally (pre-ATD), and males without ATD exposure. Sexual behavior and SDN-POA volume were tested and examined in adulthood. The pn-ATD groups had a significantly smaller SDN-POA volume compared to the pre-ATD and control groups and the pre-ATD group had a significantly smaller volume compared to the control group. When in the presence of an estrous female, the pn-ATD group showed a significant difference in female partner preference and did not interact with the available female partner. Furthermore, there was a significant positive correlation between frequency of mounts and intromissions and SDN-POA volume. The number of ejaculations was significantly lower in the pn-ATD compared to the other two groups and there was a positive correlation between ejaculations and SDN-POA volume. The mounts were also significantly lower in pn-ATD groups compared to the other two groups, but there was no difference between pre-ATD and control groups (22). Thus, these studies suggest an important role of aromatase and estrogen in the development of the SDN-POA around birth.

Another phenotype sensitive to hormones around birth is the anogenital distance (AGD). The AGD is often used as a visible marker of sexual differentiation in mammals because it is sensitive to androgens around birth (52). Males have larger AGDs than females so any deviation from this trend could be the result of differences in hormone environments surrounding birth. Furthermore, AGDs have been correlated with SDN-POA volume. For example, female rats with longer AGDs had significantly larger SDN-POA volumes compared to female rats with short AGDs. These results suggest that female rats with longer AGDs have greater androgen

levels at birth compared to female rats with shorter AGDs, and that these androgens were converted to estrogens to further develop the SDN-POA. Since the SDN-POA is sensitive to estrogens around birth and there is a correlation between SDN-POA volume and AGDs, Faber et. al examined the effects of neonatal estrogens on SDN-POA volume in female rats of known AGDs (51). At birth, the AGDs were measured in female rats and the rats were separated in either long AGDs (greater than 1.4 mm) or short AGDs (less than 1.4 mm). Then, the female rats received an injection of either corn oil or diethylstilbestrol (DES), which is a nonsteroidal estrogen. At 21 days, the rats were gonadectomized and at 42 days, anesthetized and dissected. Female rats with DES injection had a significantly larger SDN-POA volume compared to control female rats. Within the two AGDs groups, the female rats with long AGDs showed a greater increase in SDN-POA volume compared to female rats with short AGDs. Interestingly, there was no significant difference between the female rats with longer AGDs that received control treatment and the female rats with shorter AGDs that received DES (51).

As seen in these studies, the aromatization of estrogens from androgens allows for the development of the SDN-POA. The aromatase acts at or near birth so that the estrogens can stimulate the development of these neurons during the critical period of sexual differentiation. The actions of aromatase are seen in the male rats pre- and neonatally treated with ATD as they have a significantly smaller SDN-POA volume and exhibit less mounting and ejaculations compared to groups with that were only introduced to ATD prenatally or not at all. Furthermore, the introduction of estrogens at birth causes the female rats to have a larger SDN-POA volume in adulthood. In the first experiments looking at the SDN-POA, the testosterone given to females near birth converted to estrogen in the brain to sexually differentiate the brain region. Thus, the SDN-POA serves as marker of sexual differentiation in the brain due to estrogens around birth and can serve as a bioassay for testicular hormones at birth to validate the *Sry*-modified rat model.

Ultimately, the function of the SDN-POA is unknown but studies suggested implications in neuroprotection and male sexual behavior. Neurons within the SDN-POA have been shown to express calbindin-D28K (calbindin) which is often used as a marker of the SDN-POA in immunohistochemistry. Similar to the sexual dimorphism in the SDN-POA, male rats have a greater number of calbindin immunoreactive neurons in the SDN-POA compared to female rats. Calbindin is a calcium binding protein that functions in calcium transportation and maintaining calcium homeostasis. Calcium is abundant in the nervous system and is thought to be important for neuronal protection, for example in Alzheimer's Disease (AD). Kook et. al generated calbindin deficient AD transgenic mice to measure changes in neuronal loss. It was found that the AD mice without calbindin had greater neuronal loss compared to AD mice with calbindin. These findings suggest that calbindin is important for neuronal degeneration in AD mouse model; however, it is unclear whether these findings are important for SDN-POA function (49).

One study examined the development of calbindin neurons in the SDN-POA to determine if the volume of this nucleus is sexually dimorphic throughout development. The experiments compared the calbindin neurons in the SDN-POA in male and female rats at different points of postnatal development (days 2, 4, 8, 12, and 26). There was not a significant sex difference between the groups until postnatal day 8 where the average male volume of calbindin neurons in the SDN-POA was about 2-3 times larger than the average female volume. Furthermore, since it is already known that the SDN-POA development is due to androgens present at birth, they looked at the effects of the hormone environment on the development of calbindin neurons in the SDN-POA near birth. When male rats were gonadectomized at birth, there was a significant difference in the volume of calbindin neurons in the SDN-POA compared to control male rats. When female rats were administered testosterone at birth, these rats had a significantly larger volume of calbindin neurons in the SDN-POA compared to control female rats (48). Similar to experiments looking at the relationship between hormones and the SDN-

POA, calbindin neurons in the SDN-POA show a similar pattern, thus it is used as a marker of the SDN-POA in immunohistochemistry.

The SDN-POA is found within the preoptic area within the hypothalamus which is heavily involved in the regulation of sexual behaviors and the reproductive endocrine system in both sexes (24). More specifically, the SDN-POA is found within the medial preoptic area (MPOA) which is thought to be responsible for sexual arousal and ejaculatory behavior. Thus, it has been suggested that the SDN-POA is related to male sexual behaviors and partner preference. To test the function of the SDN-POA, lesion studies and identifying neuronal activation in this region have been conducted.

Lesion studies in the MPOA in adult male rats examined the relationship between the SDN-POA and sexual behavior. In order to make this comparison, different types and levels of lesion studies were conducted. Adult male rats underwent surgery to implant a cannula in the MPOA for later injections of either a vehicle or excitotoxin Quinolinic acid (QA). Injections of QA destroyed the nerve cell bodies without negatively impacting the nerve fibers. Here about two-thirds of the MPOA was destroyed which led to significantly less mounting attempts and ejaculations but no difference in noncontact erections (NCEs) in the presence of estrous females compared to the control groups. The other experiments used a lower dose, higher concentration, and slower injection speed or radiofrequency to destroy neurons and axons in the MPOA, but they showed similar results to the first experiment. The results suggest that the MPOA plays an important role in mounting initiations and ejaculatory behaviors but not NCEs (25).

Studies have also directly activated the central part of the MPON, which contains the SDN-POA, and then observed c-Fos expression during different sexual behaviors across groups with different levels of sexual experience. The adult males used in the study were either sexually experienced, meaning they have ejaculated before, or sexually inexperienced, meaning they have never ejaculated. These males were placed with an ovariectomized female rat treated

with estrogen and progesterone to mimic the estrous phase of the cycle. Once the expected behavior from the male was observed, such as sniffing, mounting, intromission and ejaculation, the male rats were euthanized and dissected for staining of c-Fos immunopositive and calbindin immunopositive cells. The number of cells was determined through c-Fos-immunopositive cells immunopositive (c-Fos+/Calb+ cells) or immunonegative (c-Fos+/Calb- cells) for calbindin. The number of c-Fos+/Calb- cells and c-Fos+/Calb+ cells in the SDN-POA of male rats was significantly the greatest following ejaculation compared to control and the other expected behaviors. This difference was less in sexually experienced males compared to sexually inexperienced males (50). This study suggests that the SDN-POA is important for male sexual function, specifically in ejaculatory behavior; however, further studies need to be done to confirm this relationship.

As previously mentioned, the SDN-POA is important for the development of the *Sry*-modified rats because of its sensitivity to hormones surrounding birth. The conversion of testosterone into estrogen in the brain via aromatase is essential in the development of the SDN-POA in male rats. Thus, any gonadal male rat regardless of the sex chromosome complement in the *Sry*-modified rat model should show a greater volume in the SDN-POA because of the testicular hormones present at birth, which is reflected in the data previously done in our lab.

IMMUNOHISTOCHEMISTRY

Immunohistochemistry History

Immunohistochemistry combines physiology, immunology, and biochemistry to visualize proteins in tissue samples. There is a long history behind immunostaining and a culmination of many discoveries made from the late 1800s to the present day. In 1890, Emil von Behring and Shibasaburo Kitasato injected bacteria into a guinea pig and discovered the guinea pig would produce anti-toxins in the serum which would be later used to prevent future disease. These

anti-toxins were later renamed antibodies and are now known to be made by the immune system in response to foreign substances. Some years after these antibodies were discovered, Paul Ehrlich examined the relationship between antibodies and toxins and the specificity at which they interact with each other. In the 1920s, Michael Heidelberger discovered that antibodies were proteins, which led to a further understanding of visualizing the antigen-antibody complex with a color dye. In the 1940s, Albert Hewett Coons built upon Heidelberger's colored reaction and developed an immunofluorescent technique to label specific antibodies. Nonetheless, the fluorescent stains would fade over time, so in the 1960s, researchers started using enzymes, such as horseradish peroxidase, to locate antibodies which later led to the development of other lab techniques, such as ELISAs (enzyme-linked immunosorbent assay) and western blots (40).

Immunohistochemistry is the preferred immunostaining method because of its ability to localize specific proteins in tissue samples. Other immunostaining methods, such as western blots, can detect the presence of antigens but cannot localize these antigens. Moreover, *in situ* hybridization detect the localization of nucleic acids (40-41). Thus, immunohistochemistry is often used for cancer diagnosis, drug development, classification of neurodegenerative diseases, and brain injury detection. For example, in detecting cancer, physicians can use specific tumor markers to determine if the tissues are cancerous, the stage of the tumor, and identify the primary tumor in cases of metastasis. Furthermore, immunohistochemistry can be used to test drugs on cancerous cells and measure any effects in the target markers. In brain trauma, IHC is used to measure for beta amyloid precursor protein which can detect axonal injury shortly following head injury (32). In the case of the SDN-POA, it is important to be able to locate the presence of the cluster of neurons to measure the area and volume of the cells.

Immunohistochemistry takes advantage of the antibody specificity to detect the location of antigens in tissue sections. Antibodies or immunoglobulins (Igs) are glycoproteins produced by in response to antigen binding to a B-cell receptor on the surface of B lymphocytes. There

are many different types of Igs, but the most common used in immunohistochemistry are IgGs, which are primarily produced in the immune response to pathogens (38). Igs have a Y-shaped structure, which contains light and heavy chains, and the tail of the Y shape called Fc. The Fc region determines the function of the antibody because it allows for other molecules to bind, so it is important for immunohistochemistry. When an antigen is present, the epitope region of the antigen, which are groups of amino acids, will bind to the FC region of the antibody. The interaction between antigen and antibody will depend on the protein structure of the epitope and how the proteins are folded together. The affinity between the antigen and antibody will depend on the noncovalent bonding interactions between the two. If there is more bonding, then there is a greater affinity (29). There are many factors in deciding the appropriate antibody used for immunohistochemistry, such clonality and the host animal, and those factors will be explored later.

There are two types of immunohistochemistry techniques. One method mounts tissue sections directly onto glass slides while the other allows sections to float in solutions. The free-floating approach is used in the SDN-POA staining protocol because it allows for better antibody penetration as the solutions can interact with all sides of the tissue in the wells. Furthermore, the staining of the SDN-POA uses thicker sections (40-micrometers) and the slide mounted method is better suited for thinner sections (10-14-micrometers). Although for the SDN-POA, each well contains a single tissue section, the free-floating method generally allows for more sections to be stained in the same well which allows for less solution at a lower concentration to be used and saves time during the washing and incubation steps. One major drawback to the free-floating section is the possibility of damage to the tissue structure and quality as the sections are incubating over a couple of days and consistently washed and handled. There are different ways to remove the solutions from the wells, such as using permeable inserts to easily place and remove tissues into the solutions, but for the SDN-POA staining, the pipetting method was used. In using the pipette method, the risk of pipetting the tissue sections is considerable so

special attention was paid in between the washing and incubation steps (39). Different adjustments can be made to the protocol that can prevent any structural damage to the tissue sections which will also be explored later.

As there are many steps to an immunohistochemistry protocol, there are various factors that influence the specificity with which antibodies can bind to antigens and the ability for it to be visualized under a light microscope. The following section will explore each step of the procedure and the different factors and considerations required for each step.

Immunohistochemistry Methods

Immunohistochemistry is used to compare the SDN-POA size in the brain regions of eight genotypes: XX gonadal females, XXY^{Δ} gonadal females, XY gonadal males, XYY^{Δ} gonadal males, XY (Sry TG+) gonadal males, XX (Sry TG+) gonadal males, XXY^{Δ} (Sry TG+) gonadal males, and XYY^{Δ} (Sry TG+) gonadal males. Rats were anesthetized at about 2 months of age and perfused transcardially with phosphate-buffered saline (PBS) followed by 4% paraformaldehyde (PFA). Brains were post-fixed in PFA at 4ºC overnight and cryopreserved in 30% sucrose. Brains were stored at -20ºC or -80ºC for up to four weeks. 40-micrometer coronal sections were cut and processed for calbindin immunohistochemistry. Individual coronal sections were placed in 20 well plates. Following sectioning, the free-floating coronal sections underwent antigen retrieval with 0.1M glycine for 30 minutes and rinsed with 1X tris buffered saline (TBS) working solution three times for 5 minutes. Before primary antibody incubation, sections were incubated in a blocking solution which included 1% hydrogen peroxide, 10% normal horse serum (NHS), 0.4% triton-x-100, and 1X TBS working solution for 45 minutes. The primary antibody cocktail included 2% NHS, 0.4% Triton-X-100, 1X TBS working solution, and an anti-calbindin D28-K mouse monoclonal primary antibody (Sigma-Aldrich C9848) at a concentration of 1:10,000. Primary antibody incubation was for 3 nights at 4ºC. Sections were washed four times with 1X TBS working solution for 5 minutes each before secondary antibody

incubation. The secondary antibody cocktail included 2% NHS, 0.4% triton-x-100, 1X TBS working solution, and biotinylated horse anti-mouse IgG secondary antibody (Vector Laboratories BA-2000-1.5) at a concentration of 1:500. Following a 60-minute incubation, sections were washed five times with 1X TBS working solution for 5 minutes each. To identify the presence of the secondary antibody, the Avidin-Biotin Complex (ABC) detection method will be used. The ABC solution (Vector Laboratories PK-4002) includes avidin and biotinylated horse radish peroxidase. One drop of the “A” solution and one drop of the “B” solution is placed into 20 mL of 1X TBS working solution then sections were incubated for 60 minutes. Sections are washed in 1X TBS working solution four times for 5 minutes and then incubated in a DAB (3,3'-diaminobenzidine) solution to produce a colored reaction visible under a light microscope. The DAB solution includes 3,3'-diaminobenzidine, hydrogen peroxide, urea, and cobalt chloride (Sigma-Aldrich D0426). The free-floating sections are then mounted onto slides and let dry overnight. The sections were then dehydrated in 70% EtOH, 95% EtOH twice, 100% EtOH twice, and HemoDe twice for 3 minutes each. Slides are coverslipped with dibutylphthalate polystyrene xylene (DPX).

SDN-POA Immunohistochemistry Components

Tissue preparation for immunohistochemistry includes fixing the tissues with paraformaldehyde (PFA). PFA is often used for perfusion because it breaks into formaldehyde and cross-links proteins and DNA molecules for tissue preservation. The quality of the fixation is influenced by the type of fixative, fixation time and temperature, and the use of post-fixation procedures. The signs of successful perfusions include twitching and a firm, pale brain and pale liver. There is a balance in fixing tissues because over-fixation and under-fixation can harm the immunoreactivity of the target antigens in the tissues. Over-fixation can lead to excessive crosslinks and false negative results, while under-fixation can cause false positive results (29). Underfixation often leads to blood in the brain following perfusion indicating unsuccessful blood

filtering during the procedure. Underfixation can be avoided by using fresh PFA, avoiding air bubbles in the perfusion system, and allowing enough time for the saline to flush through the circulatory system. Post-fixation of the brains in PFA overnight allows for further fixation (27). If blood is still present following perfusion, it can compromise the staining because the antibodies can nonspecifically react with the blood cells or other cells in the tissue (28). To prevent stained blood vessels, the perfusion must be able to thoroughly pass the circulatory system to flush all blood from the brain.

Tissue dissection in preparation for localizing the SDN-POA in the brain requires careful dissection because of the location of the nucleus. There are different landmarks during sectioning to ensure the SDN-POA is captured in the sections. The SDN-POA is found caudal to the formation of the anterior commissure and the third ventricle. During sectioning, the optic chiasm normally keeps the hypothalamus in place, so during dissection it is important to pay close attention to preserving the optic chiasm (23). When the optic chiasm is not intact, the third ventricle is left open, and an edge effect can occur. The edge effect is a consequence of the tissue absorbing more stain on the outside edges compared to the middle of the tissue, which sometimes leads to false positive results (34). Since the SDN-POA lies near the edge of the third ventricle, when the third ventricle is left open in the absence of the optic chiasm, the tissue appears darker so it may be assumed that it is stained when it is not.

To preserve the hypothalamus during sectioning, the brains are mounted on the chuck in the cryostat so that the microtome cuts the most lateral part of the brain first and coronal sections are made. The temperature of both the tissue and the cryostat is important for sectioning and preserving the tissue. When either is too warm, it can cause chunks of tissue to be cut on the microtome or sections to crumble as the tissue hits the microtome (35). To ensure the tissue is properly frozen before sectioning, it is best to flash-freeze the tissue. When there are issues in tissue freezing, it manifests as freezing artifacts during staining.

Antibodies for immunohistochemistry are made by injecting animals with purified antigens so that these animals start producing antibodies specific to the antigens. The animals commonly used to produce antibodies are mice, rabbits, goats, and horses. There are two types of antibodies: monoclonal and polyclonal. Monoclonal antibodies are made in a single B cell species, while polyclonal antibodies are produced by multiple types of B cells. An advantage of using polyclonal antibodies over monoclonal antibodies is its ability to identify different isoforms of the target protein; however, this advantage could also lead to nonspecific staining of similar isoforms in the tissue, which will be further explored later (29). In immunohistochemistry, the primary antibody will bind specifically to the target antigen. Ideally, the primary antibody should be raised in a species other than the animal tissue used for the experiment to prevent binding with endogenous proteins in the tissue; however, it is difficult to avoid that when using rodent tissue. Thus, the secondary antibody must be raised against the primary antibody host species so that the secondary antibody can bind to the primary antibody and detect localization.

There are two methods of detecting target antigens under a microscope: direct and indirect. The direct method uses a reporter molecule, such as fluorochromes, biotin, and enzymes, to identify the presence of the primary antibody. The indirect method uses two layers to identify the primary antibody. This method uses an unlabeled primary antibody and a labeled secondary antibody that is raised against the primary antibody, which allows for a more sensitive and stronger signal for detection. Furthermore, the same secondary antibody can be used to detect different primary antibodies as long as the primary antibodies are raised in the same species. The indirect method is used to identify calbindin immunoreactive cells in the SDN-POA. Labeling the secondary antibody can be done in different ways, but for the SDN-POA, the Avidin-Biotin Complex (ABC) method is used to label the secondary antibody. The VECTASTAIN ® ABC-HRP Kit from Vector Laboratories (PK-4002) contains two solutions: A and B. The A solution contains avidin and the B solution contains biotinylated horse radish peroxidase. The two are mixed into TBS to make the ABC solution. The secondary antibody is

biotinylated so that when the tissue is incubated in the ABC solution, the avidin in the solution will bind to the biotin at a high affinity (29). The ABC solution also contains biotinylated horseradish peroxidase which will be used for the colored reaction.

High background and nonspecific staining are common problems within immunohistochemistry because many reagents will inappropriately bind to parts of the tissue that are not being examined in the experiment. One way to combat unwanted staining is to introduce a blocking solution before primary antibody incubations. Blocking strategies for immunohistochemistry prevent non-specific antibody interactions and high background staining. In the previously mentioned indirect method, one of the limitations is that the primary antibody cannot be raised in the same animal as the one of the tissues being tested. In using the same animal, the secondary antibody will be brought up against the host animal and thus endogenous immunoglobulins will also be recognized by the secondary antibody (44). Another way to prevent background staining is to introduce glycine to the sections. Following fixation, there may be aldehyde remaining in the tissues from the PFA so glycine is used to block the aldehyde groups that would otherwise bind to the primary and secondary antibodies and create background staining (33).

Diaminobenzidine (DAB) is a histological dye used to visualize the target antigen under a light microscope following prior incubations. Normally, the DAB reaction produces a brown color, but the addition of metallic ions produces different shades of blue and more intense staining. The three heavy metallic ions that modify the colored reaction are nickel, cobalt, and copper. These metallic ions produce a purplish-blue, dark blue, and grayish-blue respectively. Of the three ions, cobalt is the best option because it is stable against counterstains, creates a distinct dark blue color, and does not increase background staining (30). Different companies manufacture DAB kits and utilize different metal ions in DAB tablets. The SIGMAFAST DAB with Metal Enhancer (Sigma-Aldrich D0426) contains one DAB/cobalt tablet and one buffer/urea hydrogen peroxide tablet. These tablets are dissolved in 5 mL of ultrapure water to produce a

ready-to-use substrate solution. The hydrogen peroxide reacts with the peroxidase in the tissue introduced in the ABC step to produce products that react with the DAB and cobalt tablets for a dark blue-colored precipitate.

In most of the reagents described above, except for the DAB solutions, solutions are created by diluting into a tris-buffered saline (TBS) solution at a pH of 7.6. The pH of the buffer can influence the immunoreactivity of the antibodies. The buffer solution must have a pH near neutral or physiologic pH so that it is within the range of isoelectric points of immunoglobulins (47). The TBS solution is naturally a basic solution, so the addition of hydrochloric acid allows the solution to reach a relatively more acidic pH of 7.6. Therefore, it is important to ensure the TBS solution is at the optimal pH for maximum antibody immunoreactivity.

Many of the factors and variables explained above will be explored further in the following section outlining the immunohistochemistry troubleshooting. As previously outlined, there are many variables either before or during the staining process that can influence the success of the immunohistochemistry staining. Each of these factors were considered during the troubleshooting process and the results of those experiments will be showcased further.

SDN-POA Immunohistochemistry Troubleshooting

In the beginning of the immunohistochemistry staining trials, the main goal of the trials was to optimize different variables to stain calbindin neurons in the SDN-POA (Table 3). Experiment 0 is considered the first attempt at SDN-POA staining, but there was an absence of stained cells. There are different possible reasons for the lack of staining, but the first variable tested was the primary antibody in Experiment 1. Increasing the primary antibody concentration from 1:10,0000 to 1:1000 allows for more of the primary antibody to bind to the protein. While there were no stained cells, the tissue appears to have hole-like features that suggest insufficient freezing that could influence the immunohistochemical procedure (Figure 3). Brains at this point in the trials were placed directly into the -20ºC freezer following sucrose incubation;

however, this freezing method could prevent the brain from sufficiently freezing prior to sectioning. Following this trial, the brains were flash frozen prior to sectioning. The brains were taken from the sucrose solution and placed on dry ice until frozen then directly placed into the -20ºC or -80ºC freezer depending on how soon after freezing the brains would be sectioned.

Another variable that influences tissue quality and structure is the free-floating immunohistochemistry method used. When the sections are free-floating, multiple sections can be placed into each well, but this method has two disadvantages. One is that the solutions in the well are harder to pipette out because there are about 3-5 tissues to maneuver around. The risk of pipetting the tissue is very high with more sections. The second disadvantage is the risk of not knowing the order of the sections when mounting. A solution to this issue is placing one section per well in the order that they were sectioned in the cryostat. The free-floating method also calls for the washes to be done using a shaker to ensure proper exposure to the different solutions; however, the intensity of the shaking influenced the quality of the tissue. At first, the shaker was too strong and tissues fell out of the wells, so sections were lost over time.

Thus, in Experiment 2, the brains were flash frozen prior to sectioning and each well contained one tissue section; however, these changes did not directly influence the success of the staining. To combat the staining problem, the blocking solution was examined as well. In the SDN-POA staining protocol, the blocking solution contains hydrogen peroxide and normal horse serum. The former will react with endogenous peroxidase and the latter will react with the secondary antibody to prevent non-specific binding. So, if the hydrogen peroxide were removed from the blocking solution, then the subsequent DAB staining should react to any endogenous peroxidase in the tissue and staining should be present. Therefore, in Experiment 2, hydrogen peroxide was omitted from the blocking solution. Flash-freezing the brains eliminated the hole-like features and the hydrogen peroxide reacted with endogenous peroxidase as seen in the stained blood vessels, probably endothelial cells (Figure 4).

The first time there was SDN-POA specific staining occurred after Experiment 3, but the SDN-POA is not evenly stained. In Experiment 3, the primary antibody incubation occurred at 4ºC for 3 nights, TBS solution was kept on ice, and a new primary and secondary antibody were used. As seen in the sections, the SDN-POA is specifically stained but it is only present on one side and the third ventricle is agape. The unevenness is a result of uneven sectioning which can be solved by focusing on symmetry during sectioning. Furthermore, the optic chiasm was not intact so there was an edge effect present (Figure 5).

In an attempt to get stronger staining, a sodium citrate buffer antigen retrieval was introduced in Experiment 4. Antigen retrieval is the process in which target antigens are revealed so the antibodies can reach these antigens. Epitopes are groups of amino acids exposed on proteins that allow for antibodies to bind; however, during the fixation process, proteins crosslinking prevents antibodies from finding and binding to these epitopes. Conventionally, there are two types of antigen retrieval: proteolytic-induced epitope retrieval (PIER) and heat-induced epitope retrieval (HIER). PIER uses the digestion of proteases to remove protein crosslinks so that epitopes are revealed (37). HIER involves heating the tissue to temperatures greater than 95ºC in buffer solutions to disrupt crosslink formation during the fixation process. Heating can be done using a microwave oven, autoclave, pressure cooker, steamer, or water bath. Generally, HIER is the preferred method because the strength of the enzymes used in PIER can damage the tissue or change the epitope structure, which is why it was the chosen option for the SDN-POA (36).

The tissue was incubated in 10 mM sodium citrate buffer (pH 9.0) preheated to 80ºC for 30 minutes in a water bath before the blocking solution incubation. Ultimately, there was not a significant difference in the staining; however, the tissue was noticeably weaker and more delicate compared to tissues that did not undergo sodium citrate antigen retrieval (Figure 6). A possible explanation for the weakened tissue was using a water bath to boil the tissue sections for some time. Another variable introduced in this experiment was DAB incubation times. When

there is a lack of color development, there are different adjustments to troubleshoot either DAB kits. For example, determining the activity of the enzyme conjugate and increasing staining time. Testing the enzyme conjugate involves making either DAB kits then adding one drop of “B” solution from the ABC kit. The “B” solution contains horseradish peroxidase so it should react with the hydrogen peroxide in the DAB kit once they come into contact. The amount of staining time can determine the intensity of the staining. It is difficult to determine how intense the DAB will stain the section and the instructions do not specifically explain the time it takes. For example, in a trial with different DAB incubation times, it is noticeably darker with longer incubation times. Within the sections that underwent sodium citrate antigen retrieval, each section also underwent a different DAB incubation time: 15 minutes (Figure 6a), 13 minutes (Figure 6b), or 11 minutes (Figure 6c). Since the sodium citrate antigen retrieval did not cause a significant difference in the staining but also had a negative effect on the tissue quality, it was eliminated in future experiments.

Since the SDN-POA was stained in Experiment 4, the main focus of Experiment 5 and 6 was to replicate past stainings and focus on sectioning the brains evenly so that the SDN-POA appears bilaterally (Figure 7 and 8). As seen in Experiment 6, the SDN-POA is present across five sections in the gonadal male brain. When analyzing the SDN-POA, the area of each section with the SDN-POA is measured using the image software, ImageJ, multiplied by the thickness of the section to calculate, and summed across all sections to calculate the total volume of the SDN-POA. At this point, the staining protocol has been optimized and future studies can be conducted using the previously outlined immunohistochemistry protocol.

When the SDN-POA staining was successful, the DAB tablets were the SIGMAFAST DAB with Metal Enhancer, but the company stopped manufacturing these tablets. As a replacement, the SIGMAFAST 3,3’-Diaminobenxidine tablet set was used. The difference between these two tablets is the presence of cobalt to produce a dark blue solution. The SIGMAFAST 3,3’-Diaminobenxidine tablet set (Sigma-Aldrich D4293) contains one DAB tablet

and one urea hydrogen peroxide tablet. These tablets are dissolved in 5 mL of ultrapure water to produce a ready-to-use substrate solution. Since these tablets do not contain metal ions, when the tissue reacts with the solution, it produces a brown-colored precipitate. In attempt to replace the SIGMAFAST DAB with Metal Enhancer tablet set, the SIGMAFAST 3,3'-Diaminobenxidine tablet set with the addition of cobalt chloride was used in Experiment 7. However, this experiment resulted in no staining of any cells which was apparent to the naked eye.

While Sigma-Aldrich manufactures a solid form of DAB, Vector Laboratories manufactures a liquid form of DAB in the DAB Substrate Kit with nickel (SK-4100) which was used in Experiment 8. The kit contains four reagents. Reagent 2 contains acetonitrile, cyanomethane, and 3,3'-Diaminobenzidine tetrahydrochloride hydrate and Reagent 3 contains 1-3% of hydrogen peroxide. Information about Reagent 1 is not released to the public. If a gray-black reaction product is desired, then the DAB Reagent 4 which contains nickel can be added to the solution. Since the DAB kits from the two different manufacturers are produced in different forms, there are different characteristics between the two kits. The Sigma-Aldrich tablets with metal enhancers produce a dark blue colored solution and when it reacts with the tissue, the solution colors it in a short amount of time, about 15 minutes. On the other hand, the Vector Laboratories DAB kit produces a colorless solution. In Experiment 8, the DAB incubation time was tested again but this time in relation to the Vector Laboratories DAB kit. The tissue sections were placed in the DAB solution and watched carefully for any changes in tissue section color. After 45 minutes, the solution colored the tissue but not enough to be visible under the microscope. Even with the longer amount of time, the Vector Laboratories DAB kit does not produce an intense dark blue color like the Sigma-Aldrich tablets with metal enhancers.

Between each step the sections are washed in TBS, so Experiment 9 tested if the washing had a negative effect on the staining process. Without the washing step right after the ABC solution incubation, the tissue would possibly have more horseradish peroxidase on the

sections to react with the DAB solution. The blocking solution was also omitted in this experiment, similar to previous experiments, to test the other immunohistochemical components. In this experiment, the blood vessels are stained but in comparison with the previous successful experiments, the staining is fairly weak (Figure 9).

Following all of the troubleshooting experiments, it was concluded that the SIGMAFAST DAB with Metal Enhancer tablet set is the strongest and most successful DAB staining solution for SDN-POA immunohistochemistry. While it is possible that the other components of the staining procedure could have been impaired when working with the two other DAB kits, it is unlikely that is the case. As seen in Experiment 8, the SDN-POA was stained and in following experiments after that the main variable different was the DAB kit utilized. Further experiments need to be done to optimize the Vector Laboratories DAB kit in relation to the SDN-POA immunohistochemistry. Different methods include using a different antibody that is known to work in specific tissue types or testing the timing and concentration of the DAB kits. Immunohistochemistry is a delicate process that requires many different components to work together in unison which takes time to perfect.

CONCLUSION

The SDN-POA is an important brain region for understanding sexual differentiation, but there is more to be discovered about the SDN-POA and its function in sexual behavior. Using the SDN-POA will serve as validation for the *Sry*-modified rat model because of its sexual differentiation development during the critical period near birth. In doing so, the *Sry*-modified rat model can serve as an important model in understanding sex differences in physiology and disease and also uncover a better understanding of sex chromosome aneuploidies, which is a currently an under-studied field. Further studies need to be conducted to optimize the SDN-POA immunohistochemistry techniques following the troubleshooting outlined here.

FIGURES

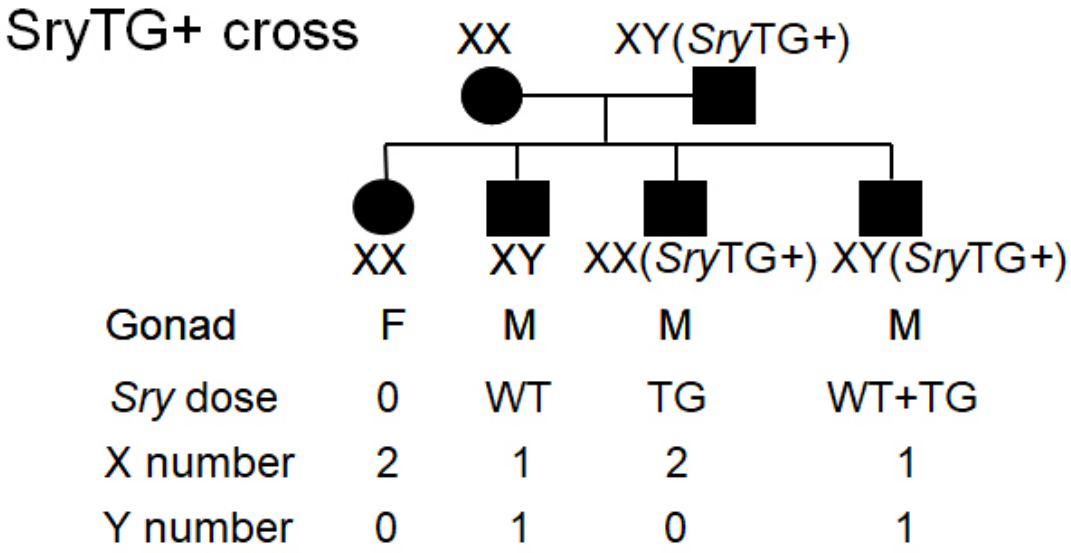


Figure 1: Transgenic breeding scheme in the *Sry*-modified rat model

The transgenic rat litters are produced by mating an XX female and an XY (*Sry*TG+) male who carries a transgene containing the *Sry* gene on an autosome. Four genotypes are produced: XX gonadal females, XY gonadal males, XX(*Sry*TG+) gonadal males, and XY(*Sry*TG+) gonadal males.

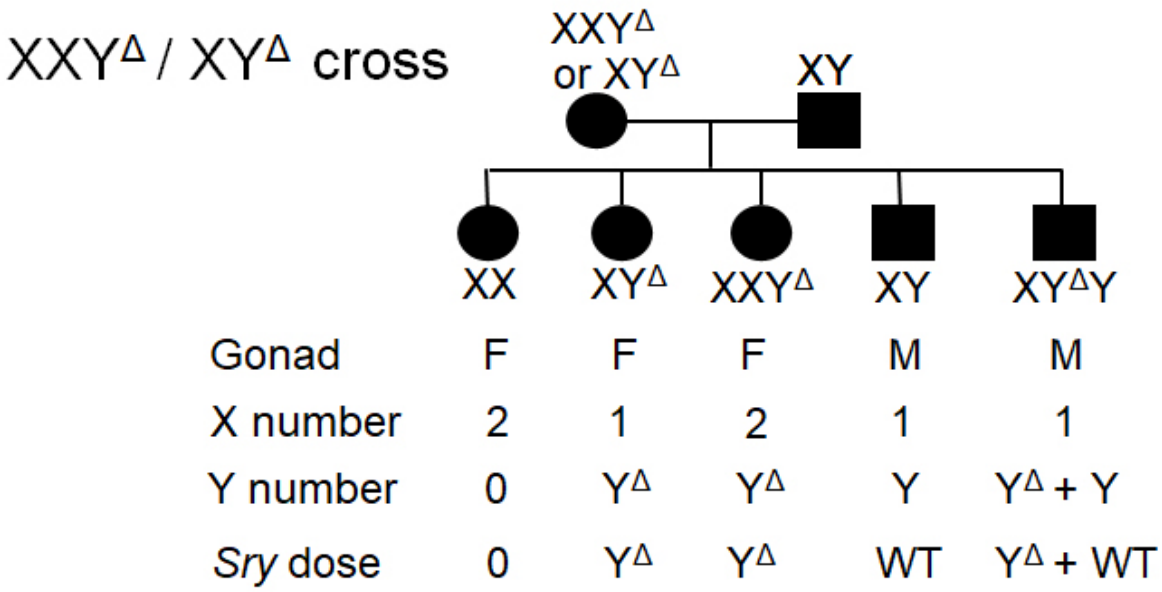


Figure 2: KO breeding scheme in the *Sry*-modified rat model

The transgenic rat litters are produced by mating an XY gonadal male and an XY$^{\Delta}$ gonadal female who has a “knockout” of some copies of *Sry* on the Y chromosome, such that this Y chromosome does not cause testis development. Five genotypes are produced: XX gonadal females, XY$^{\Delta}$ gonadal females, XXY$^{\Delta}$ gonadal females, XY gonadal males, XYY$^{\Delta}$ gonadal males, except that XXY$^{\Delta}$ mothers do not produce XY$^{\Delta}$ daughters.

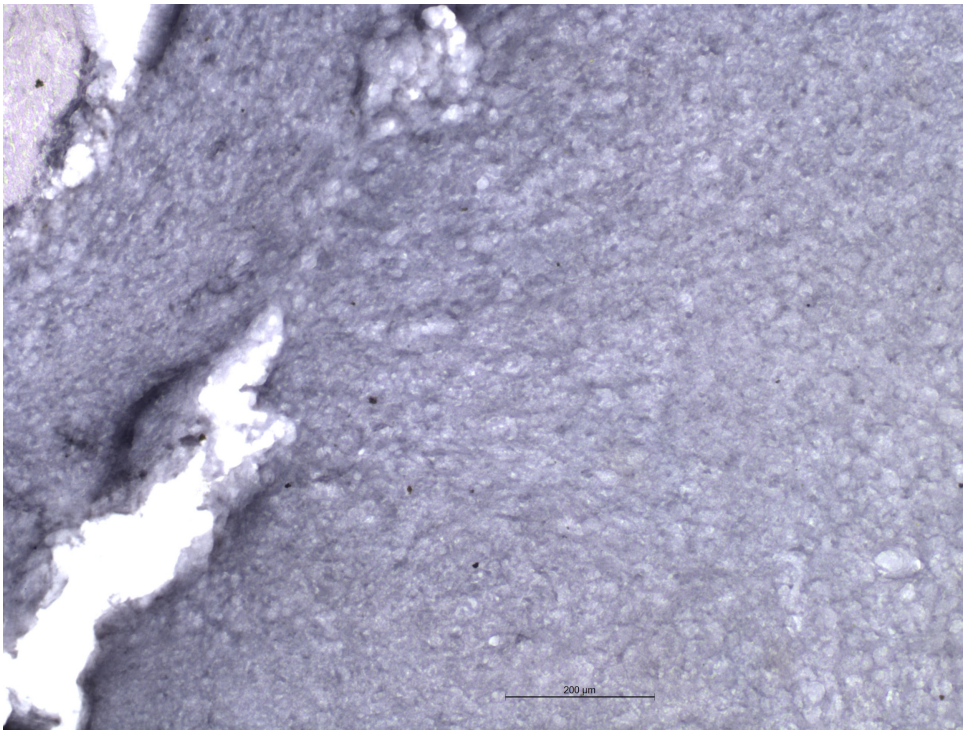


Figure 3: Experiment 1 - Hole-like features present due to freezing artifact

Brain tissue section from Experiment 1 underwent a higher primary antibody concentration incubation (1:1000) which is 10x higher than what is instructed on the protocol. There are hole-like features present in the tissue which is the result of poor freezing conditions.

Scale bar: 200 um

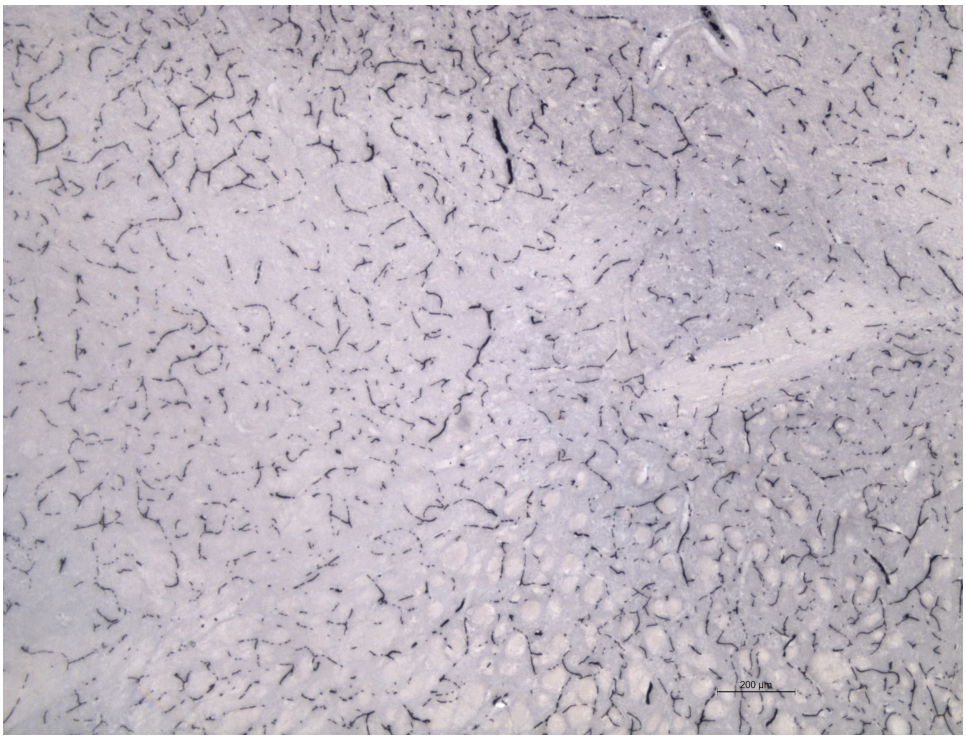
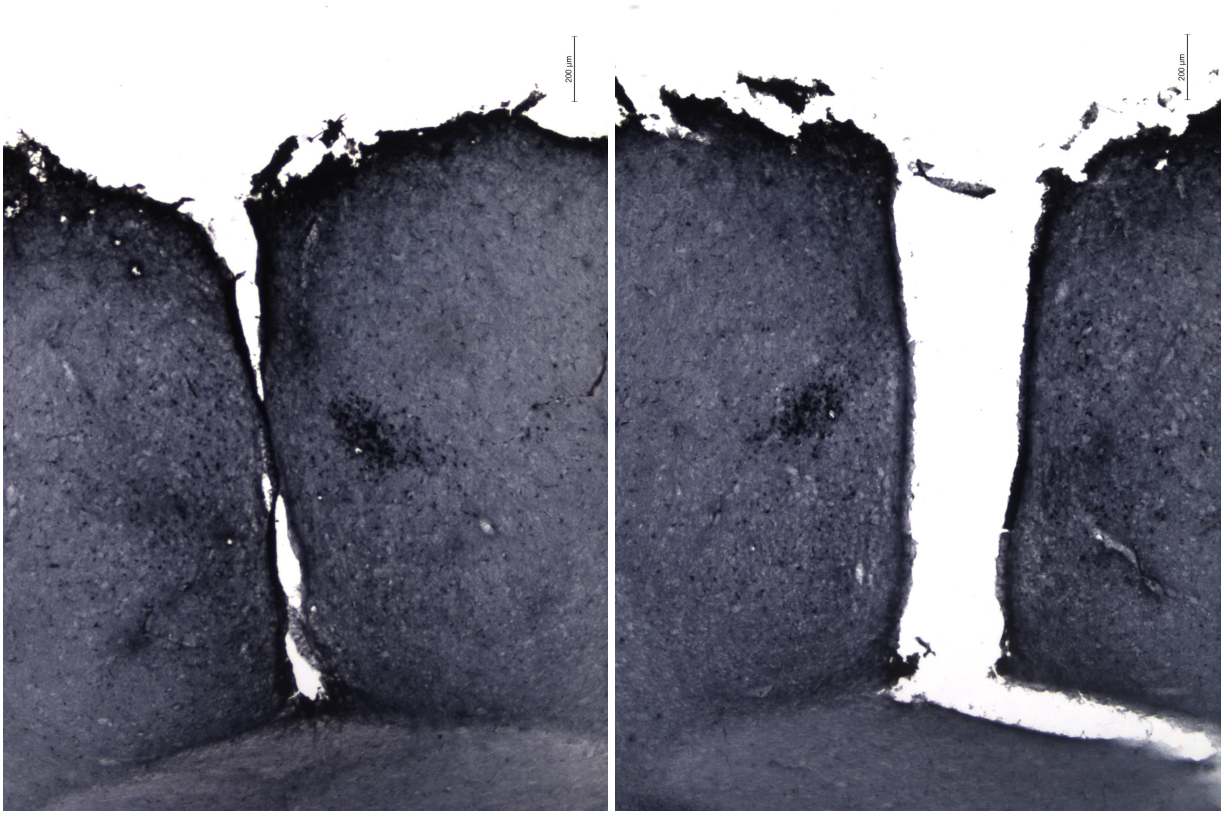


Figure 4: Experiment 2 - Stained blood vessels due to endogenous hydrogen peroxide

In Experiment 2, hydrogen peroxide was omitted from the blocking solution. Normally the hydrogen peroxide prevents non-specific staining with endogenous peroxidase. The result is stained blood vessels which suggest the staining procedure works but cannot specifically label the calbindin neurons in the SDN-POA.

Scale bar: 200 um

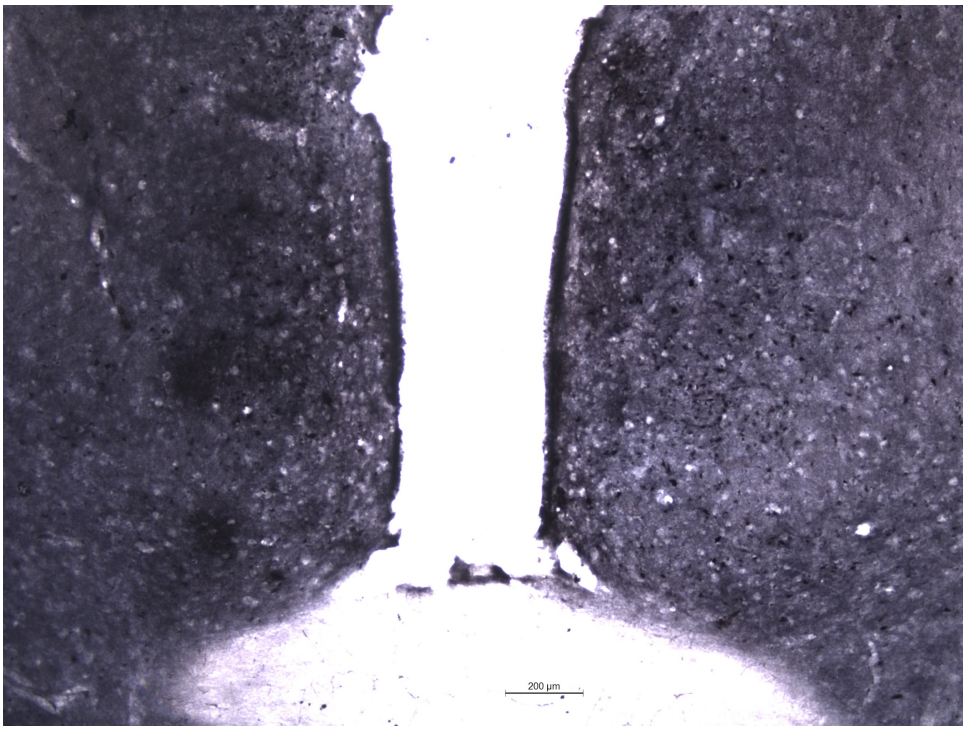


A B

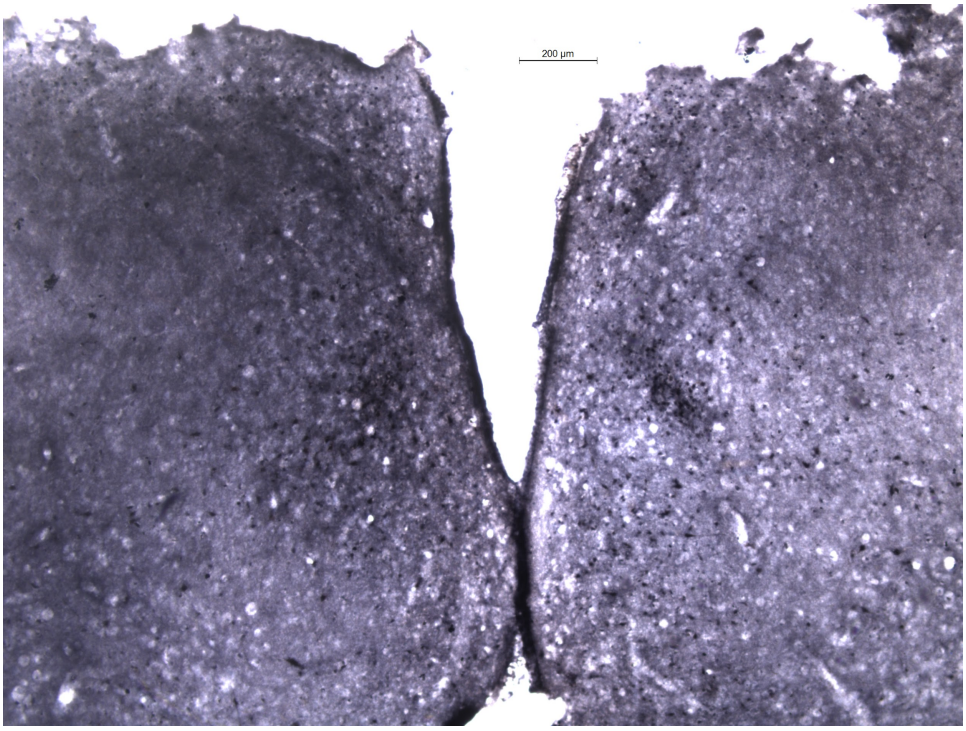
Figure 5: Experiment 3 - First result with positive SDN-POA staining

Figure 5A and 5B are tissue sections in succession (5A is found more rostral than 5B). The optic chiasm is not intact which results in an agape third ventricle and edge effect along the edges of the tissue sections.

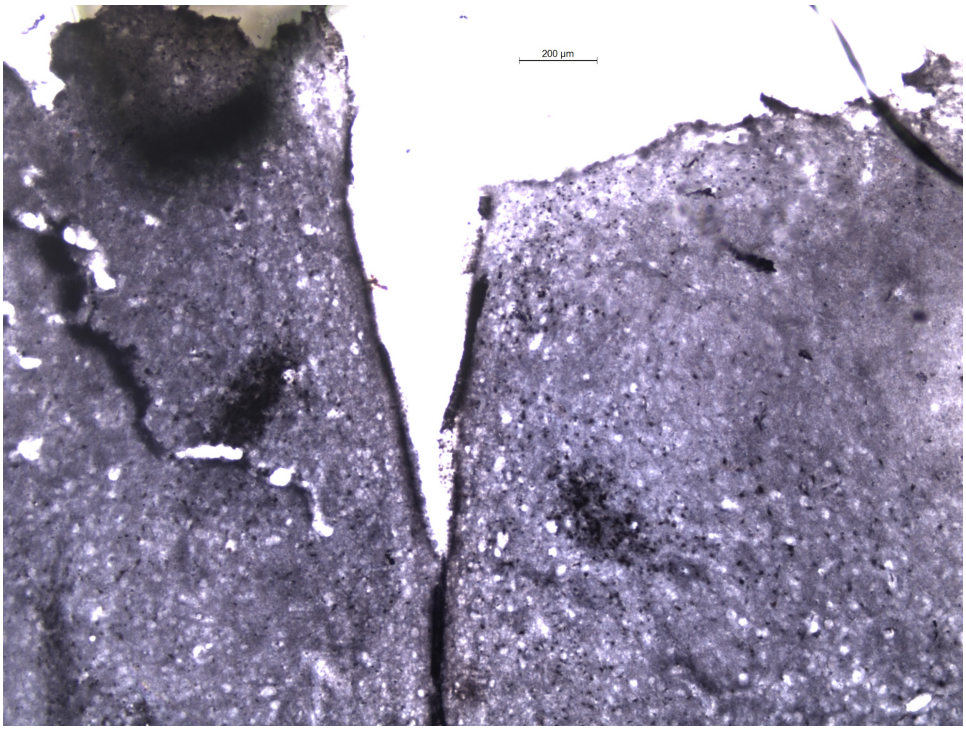
Scale bar: 200 um



A



B



C

Figure 6: Experiment 4 - Sodium citrate antigen retrieval and DAB incubation times

Experiment 4 tested sodium citrate antigen retrieval which resulted in weaker tissue sections as seen in the various tears and absent optic chiasm. Furthermore, different DAB incubation times were tested to optimize the tissue staining. The tissue sections were incubated in DAB for 15 minutes (Figure 6A), 13 minutes (Figure 6B), or 11 minutes (Figure 6C).

Scale bar: 200 um

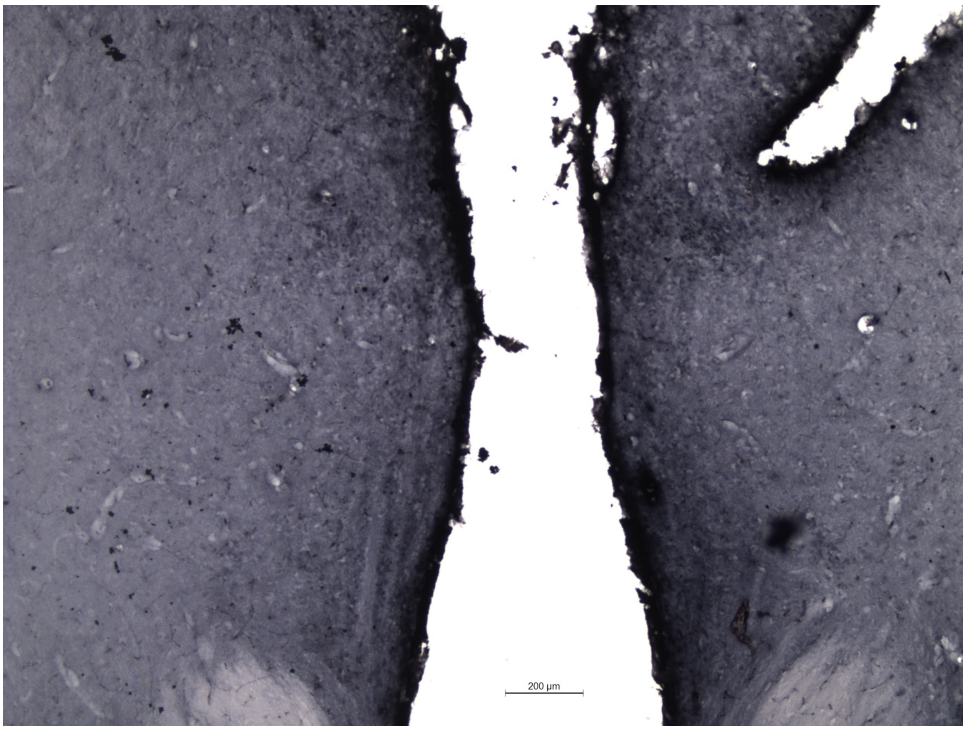
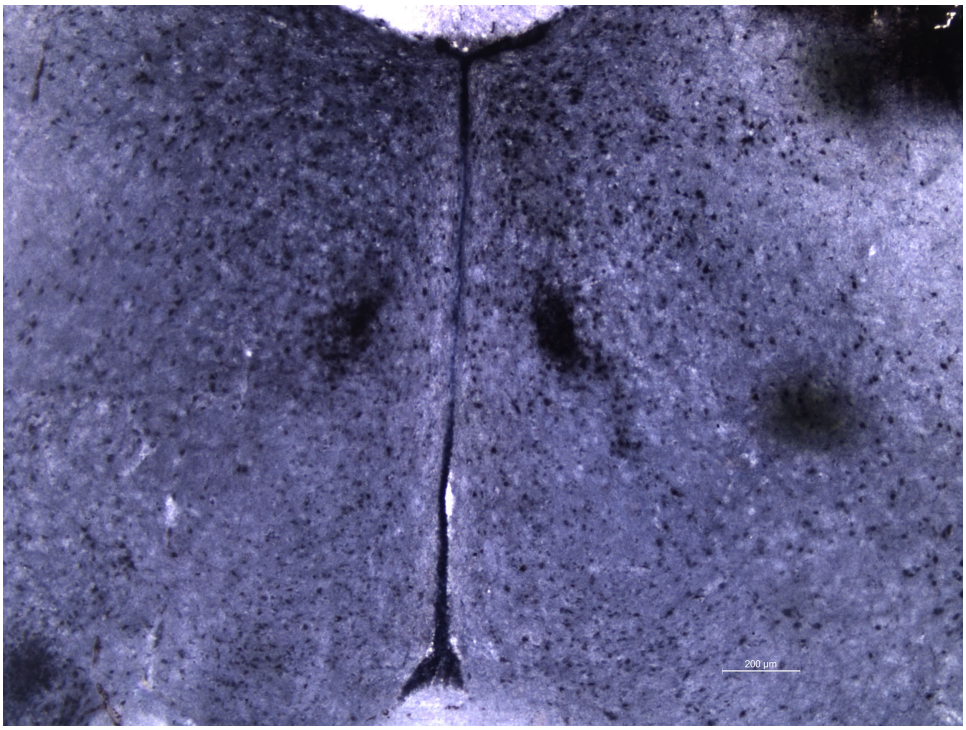


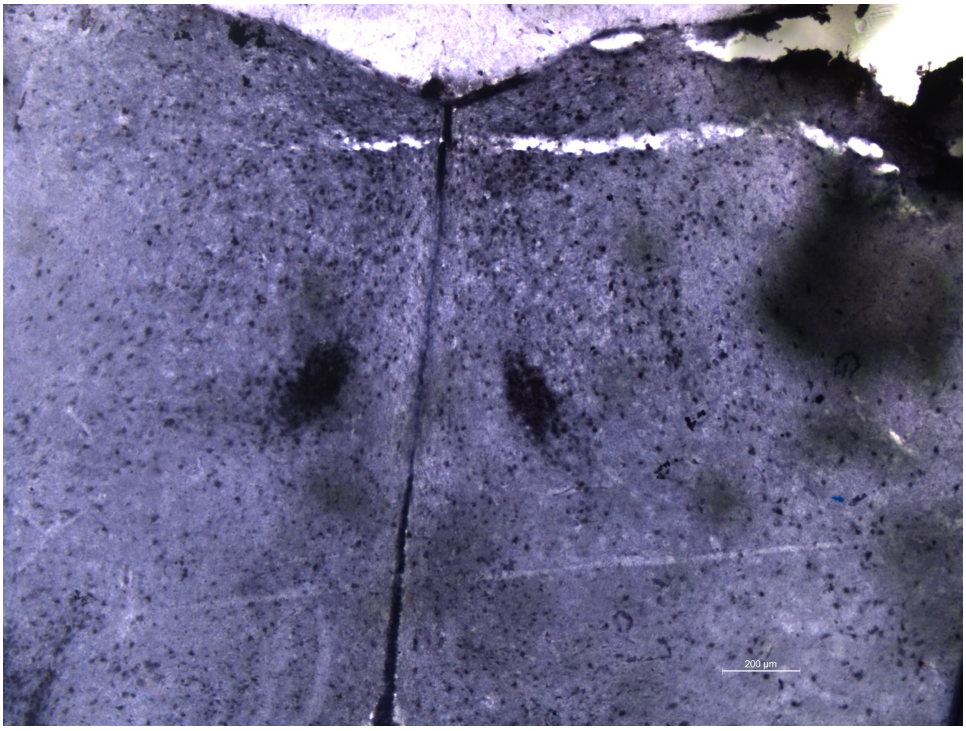
Figure 7: Experiment 5 - Replicating previous trials for stronger staining

Following Experiment 4, Experiment 5 was performed to replicate previous experiments to obtain more visible staining of the SDN-POA. Here the optic chiasm is absent, which lead to the third ventricle to remain agape and edge effect to occur.

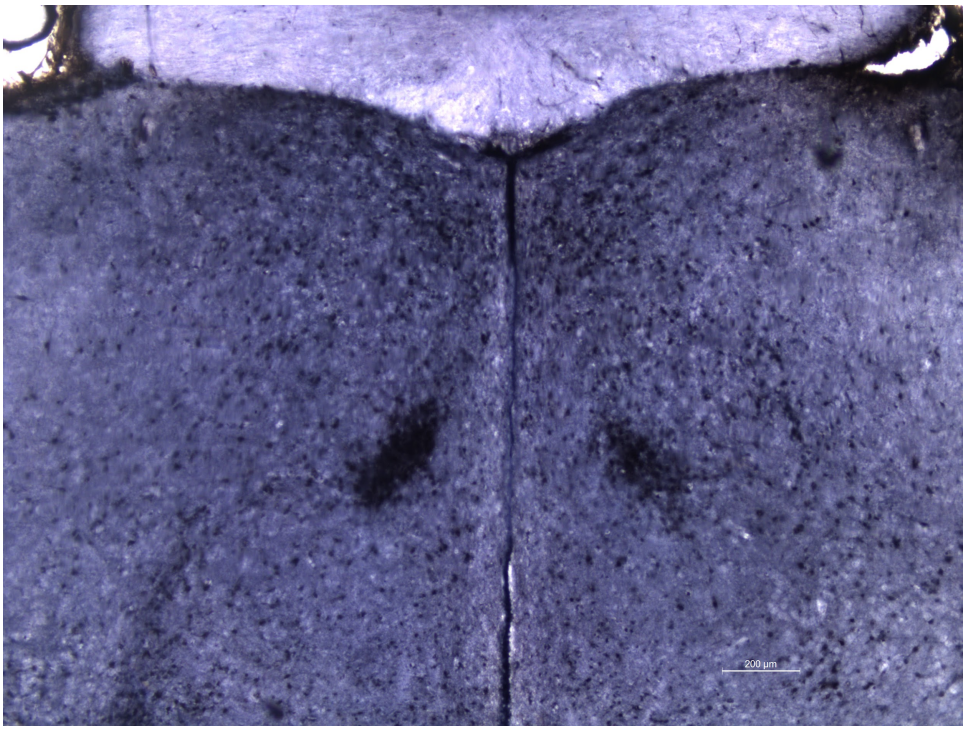
Scale bar: 200 um



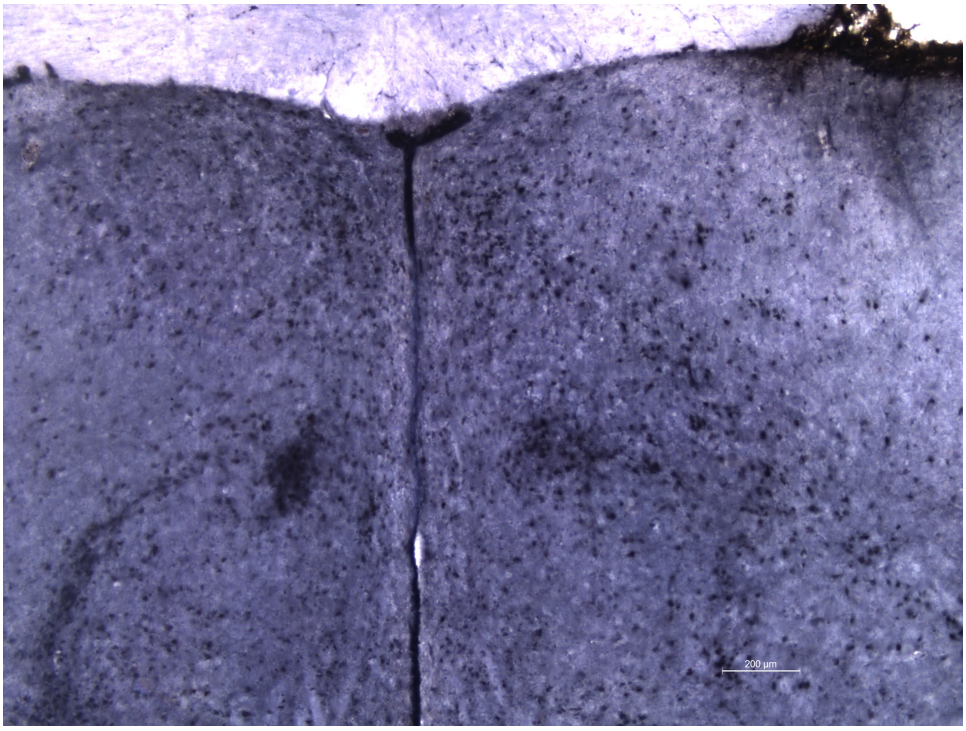
A



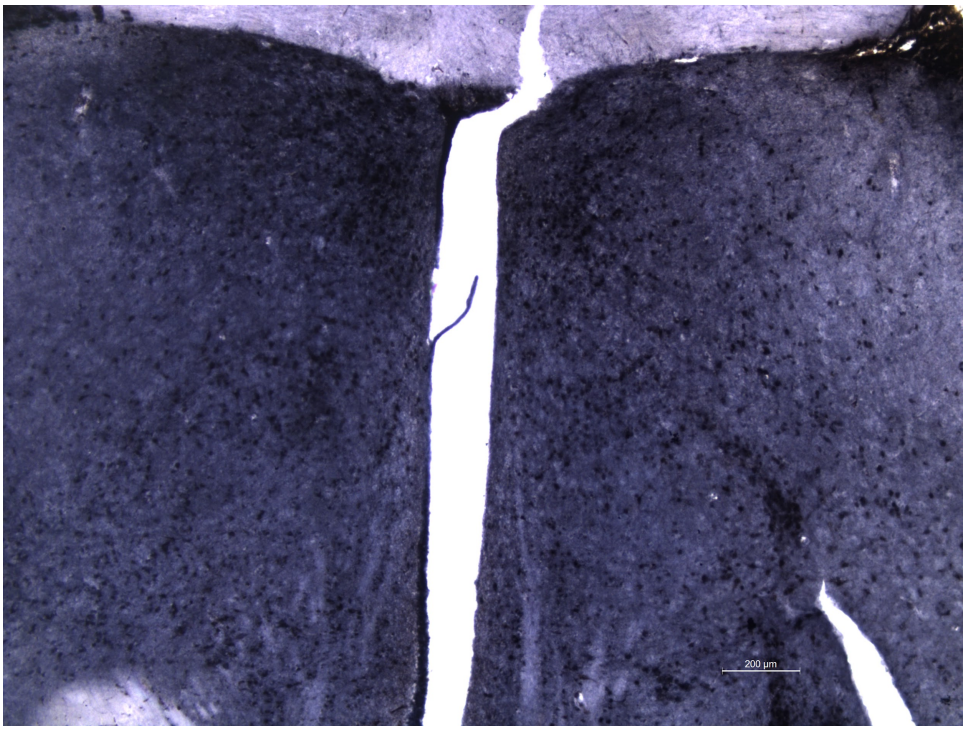
B



C



D



E

Figure 8: Experiment 6 - Successful SDN-POA staining

Experiment 6 is the most visible and apparent SDN-POA staining following the troubleshooting experiments. The tissue sections are present across five sections in order from most rostral (Figure 8A) to most caudal (Figure 8E).

Scale bar: 200 um

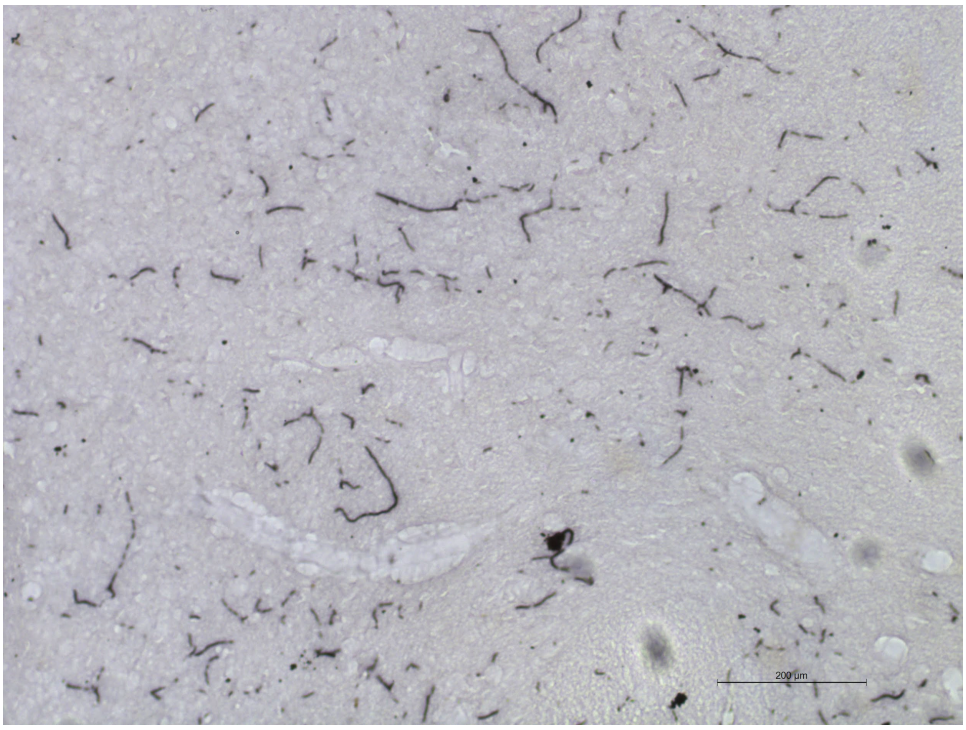


Figure 9: Experiment 9 - Testing DAB kits

Following the discovery that the SIGMAFAST DAB with Metal Enhancer was no longer manufactured, different DAB kits were tested. In Experiment 9, the Vector Laboratories DAB Substrate Kit with nickel was tested alongside omitting the hydrogen peroxide from the blocking solution. Similar to Experiment 2, the blood vessels were stained. The sections are noticeably lighter compared to previous experiments when the staining was successful.

Scale bar: 200 um

TABLES

Table 1: KO Litter Cross

Types of sperm XY father produces		Types of eggs XYΔ mother produces		
		X	**YΔ**	**XYΔ**
	X	XX	XYΔ	XXYΔ
	Y	XY	YYΔ	XYYΔ
			Lethal	

Table 1: KO Litter Cross

The types of eggs the XY^{Δ} mother can produce and the types of sperm the XY father can produce is represented. A cross between these two parents results in five viable genotypes which is used for comparison in the KO litter portion of the *Sry*-modified rat model.

Table 2: Contrasts Among Genotypes								
Cross	**Progeny**	**Genotype**	**Gonad**	**PAR**	**NPX**	**NPY**	***Sry***	**Contrasts**
Transgenic	XYWT	XY WT	M	2	1	1	WT	XXTG vs XYWT shows effects of sex chromosome complement and *Sry* dose
XX x XYTG	XYTG	XY(*Sry* TG+)	M	2	1	1	WT + TG	XXTG vs XYTG shows effects of sex chromosome complement and *Sry* dose
	XXTG	XX(*Sry* TG+)	M	2	2	0	TG	XYTG vs XYWT shows effects of *Sry* dose
	XXWT	XX WT	F	2	2	0	0	XXTG vs XXWT shows effects of gonads and *Sry*
								XXWT vs XYWT shows effects of sex chromosome complement and gonad
Knockout	XXWT	XX WT	F	2	2	0	0	XYWT vs XYYM shows effects of Y chromosome dose (1 vs 2) in males
XXY∆ x XYWT	XXYF	XXY∆	F	3	2	Y∆	Y∆	XXWT vs XXYF shows effects of Y chromosome dose (0 vs 1) in females
	XYWT	XY WT	M	2	1	Y	Y	XXWT vs XYWT shows effects of sex chromosome complement and gonad
	XYYM	XYY∆	M	3	1	Y + Y∆	Y + Y∆	XXYF vs XYWT shows effects of gonad and *Sry* dose and different Y chromosome
Transgenic x Knockout	XXYTG	XXY∆(*Sry* TG+)	M	3	2	Y∆	Y∆ + TG	XXYTG vs XYYTG shows effects of *Sry* dose (2 vs 3) in males
XXY∆ x XYTG	XYYTG	XYY∆ (*Sry* TG+)	M	3	1	Y + Y∆	Y∆ + TG + Y	XXYTG vs XYTG shows effects of X chromosome dose (1 vs 2) in males
								XYYTG vs XYTG shows effects of Y chromosome dose (1 vs 2) in males

Table 2: Contrasts Among Genotypes

The different comparisons and contrasts available among the 8 genotypes used in the *Sry*-modified rat model: XX gonadal females, XXY$^{\Delta}$ gonadal females, XY gonadal males, XYY$^{\Delta}$ gonadal males, XY(*Sry*TG+) gonadal males, XX(*Sry*TG+) gonadal males, XXY$^{\Delta}$(*Sry*TG+) gonadal males and XYY$^{\Delta}$(*Sry*TG+) gonadal males. Columns represent different characteristics of each genotype such as gonad type, number of PAR regions, and *Sry* dose types.

SDN-POA Staining Trials				
Experiment	**Hypothesis**	**Variable**	**Result**	**Next Hypothesis**
0			No staining	Increase primary antibody concentration
1	Lack of staining due to low primary antibody concentration	Increase primary antibody concentration	No staining but tissue sections were delicate and had hole-like features	Different freezing method and place fewer sections per well to increase tissue quality
2	Poor tissue quality due to insufficient freezing	Flash freezing brain; No hydrogen peroxide in the blocking solution	Blood vessels stained; Tissue quality improved - no holes	Make and obtain new solutions
3	New solutions clear of contaminants	New primary and secondary antibody	SDN-POA stained but high background staining and only stained on one side	Improve sectioning and eliminate background staining
4	Antigen retriveal method will imrpove staining; DAB incubation times	Sodium citrate antigen retrival; DAB incubations times (15 min vs 13 vs 11 min)	Longer DAB incubation times leads to darker sections	
5	Replicating previous trials to get stronger staining	No variables to test	Faint SDN-POA staining	
6	Same as Exp 5	No variables to test	SDN-POA specifically stained	
7	Test different DAB kits	SigmaFast DAB tablets	Tissue is not stained	Try different DAB kits
8	Test Vector Lab DAB kit with longer DAB incubation	No blocking solution with Vector DAB solution	Tissue is not stained	No washing following ABC step
9	Same as Exp 9; Less washing	Same as Exp 9	Blood vessels stained; No neurons stained	
All brains were gonadal males				

Table 3: SDN-POA Staining Trials

The table outlines the different experiments done during the SDN-POA staining troubleshooting process.

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