UNIVERSITY OF CALIFORNIA, SAN DIEGO

The Transcription Factor VAX1 is Required in Kisspeptin, but Not AVP, Neurons for Fertility

A Thesis submitted in partial satisfaction of the requirements for the degree Master of Science

in

Biology

by

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Professor Stuart Brody

2017

The Thesis of Jessica Sora Lee is approved and it is acceptable in quality and form for publication on microfilm and electronically:

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Co-Chair

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Chair

University of California, San Diego

2017

Dedication

I would like to dedicate this thesis to:

To my Mom and Dad for their love and support.

To Rachael and McKenna for their friendship, the fun, and the food.

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Abbreviations

AVP	Arginine vasopressin
AVPV	Anteroventral periventricular nucleus
cHET	Conditional knockout of one allele (heterozygote)
cKO	Conditional knockout
E	Embryonic day
FSH	Follicle-stimulating hormone
GnRH	Gonadotropin-releasing hormone
HET	Heterozygous
HPG	Hypothalamic-pituitary-gonadal
IHC	Immunohistochemistry
IHH	Idiopathic hypogonadotropic hypogonadism
KISS	Kisspeptin
KO	Knockout
LacZ	Beta galactosidase
LH	Luteinizing hormone
Per1	Period 1
POA	Preoptic area
SCN	Suprachiasmatic nucleus
VAX1	Ventral anterior homeobox 1
$Vax1^{KISS}$	$Vax1^{fl/fl}:KISS^{cre}$
$Vax1^{AVP}$	$Vax1^{fl/fl}:AVP^{cre}$
$Vax1^{syn}$	$Vax1^{fl/fl}:Synapsin^{cre}$

VIP	Vasoactive intestinal peptide
WT	Wild type

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ABSTRACT OF THE THESIS

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by

Jessica Sora Lee

Master of Science in Biology

University of California, San Diego, 2017

Professor Pamela L. Mellon, Chair

Professor Randolph Hampton, Co-Chair

Infertility has commonly been linked to problems in the gonads, such as the testes or ovaries; however, today it is clear that many cases of subfertility and infertility are caused by issues in the hypothalamus, an important brain structure of the neuroendocrine system that regulates fertility. The gonadotropin-releasing hormone (GnRH) and kisspeptin neurons within the hypothalamus are at the apex of the hypothalamic-pituitary-gonadal (HPG) axis, which controls fertility. Both of these neuronal populations receive

input from the suprachiasmatic nucleus (SCN), which incorporates time-of-day information and circadian rhythms into fertility regulation. In this study, we identify a novel transcription factor, Ventral anterior homeobox 1 (VAX1), as a critical regulator of GnRH neuron development and kisspeptin neuron function. Through the use of transgenic mouse models, we deleted *Vax1* within the hypothalamus using the *synapsin*^{cre} mouse, allowing deletion of *Vax1* in mature neurons. *Vax1*^{flox}*:synapsin*^{cre} (*Vax1*^{syn}) females were subfertile, but paradoxically had more GnRH neurons, and normal hormonal levels. To determine what neuronal populations within the hypothalamus were responsible for the subfertility of *Vax1*^{syn} females, we specifically deleted *Vax1* within kisspeptin neurons (*Vax1*^{KISS}) and arginine vasopressin (AVP) neurons (*Vax1*^{AVP}), one of the major neuronal populations of the SCN. Both *Vax1*^{KISS} and *Vax1*^{AVP} females had delayed first estrus. However, only *Vax1*^{KISS} had impaired fertility. Thus, *Vax1* in AVP and kisspeptin neurons is important in determining first estrus, and *Vax1* in kisspeptin neurons is required for normal fertility.

Introduction

Fertility is Regulated by the Hypothalamic-Pituitary-Gonadal Axis

Infertility, which is also referred to as sterility or the inability to conceive, affects about fifteen percent of couples [1]. A common misconception is the idea that only women can be sterile, when there are actually around at least 30 million infertile males worldwide [2]. Infertility can affect men and women of any age after pubertal onset, and reproductive ability tends to decline with age. Many causes of infertility arise from the environment, medical conditions, lifestyles, and genetics [3]. Infertility has become not only a major concern to males and females of all ages worldwide, but also an important focus of research.

Fertility is regulated by the hypothalamic-pituitary-gonadal (HPG) axis, which consists of several organs and hormones that regulate reproductive function as a single entity (Figure 1). At the top of the HPG axis sits the hypothalamus, which contains four key neuronal populations important in fertility: the kisspeptin and gonadotropin-releasing hormone (GnRH) neurons, both of which are required for fertility; in addition to two neuronal populations of the suprachiasmatic nucleus (SCN), the arginine vasopressin (AVP) and vasoactive intestinal peptide (VIP) neurons, which both play modulatory roles of the HPG axis (Figure 1). Kisspeptin stimulates release of GnRH from GnRH neurons. GnRH released at the median eminence acts upon the pituitary to release luteinizing hormone (LH) and follicle-stimulating hormone (FSH). LH and FSH signal to the gonads to release sex hormones such as estrogen, testosterone, and progesterone, as well as maturation of sperm and eggs. Correct function, signal, and release of all factors of the HPG axis are critical for normal fertility.

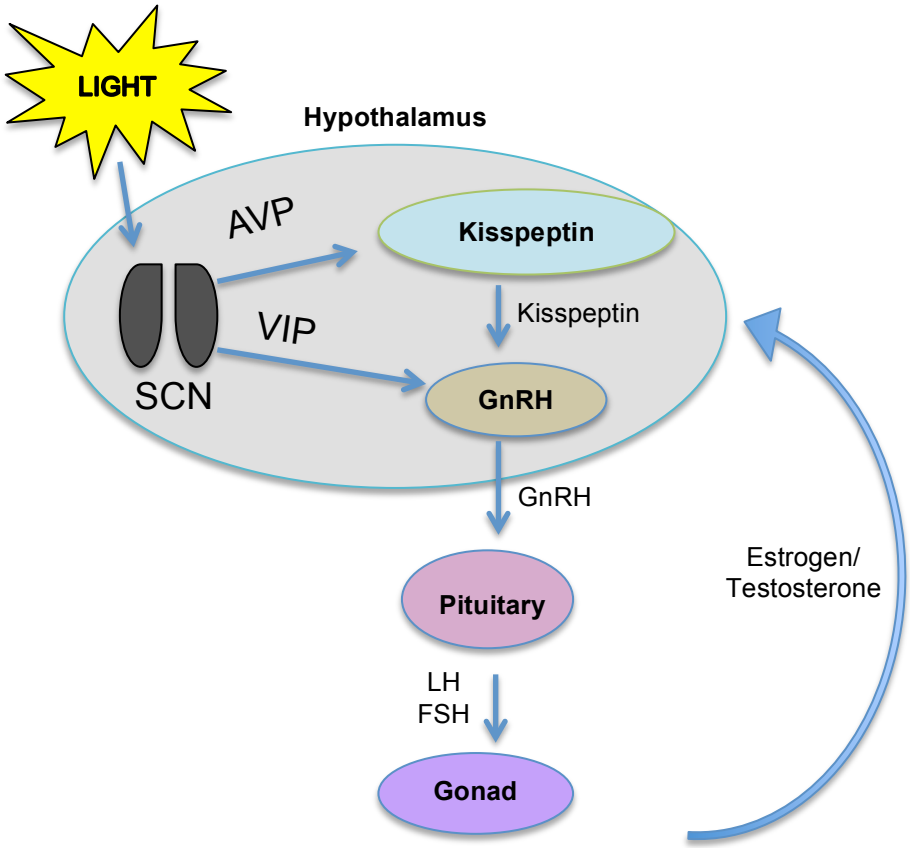

Figure 1. **The Hypothalamic-Pituitary-Gonadal (HPG) Axis**
Light and dark input enters the suprachiasmatic nucleus (SCN) through the retinohypothalamic tract. The two major neuronal populations of the SCN are arginine vasopressin (AVP) and vasoactive intestinal peptide (VIP). The AVP neurons project onto kisspeptin neurons and the VIP neurons project onto GnRH neurons. Kisspeptin stimulates the GnRH neurons to release GnRH, which stimulates the pituitary to release LH and FSH onto the gonads for the release of sex hormones such as testosterone and estrogen.

The role of circadian rhythms in fertility

In recent studies, a strong connection has been made between circadian rhythms and fertility. Circadian rhythms are rhythms with a cycle of approximately 24-hours

which allow the body to align its physiological rhythms to the length of day. Working beyond the traditional hours of 9 am to 5 pm, referred to as shift work, has a detrimental effect on human health by not only disturbing the normal circadian rhythms, but also resulting in severe gastrointestinal, metabolic, neuro-psychic, cardiovascular, and fertility disorders (Table 1) [4]. Further, in seasonal breeders, reproductive ability is determined by seasonal changes in day length, where both ovulation in the females, and/or gonadal size and sperm production in the males, can be modulated throughout the year [5]. Ovarian activity, including increased FSH secretion, larger follicle size, more ovulation, and shorter menstrual cycles, was found to be greater in the summer than the winter in women [6]. With chronic shift work, jet lag, and artificial lights at night becoming an essential part of daily lives, disruptions to the circadian rhythms have become a real issue.

The suprachiasmatic nucleus, the body's master pacemaker

The suprachiasmatic nucleus (SCN) is an endogenous pacemaker of the body that regulates circadian rhythms using external input from the environment, predominantly the light/dark cycle [7] to align internal rhythms of cells and tissues to the length of day. The SCN is a small structure of the hypothalamus within the HPG axis where two of the major neuronal populations are VIP neurons and AVP neurons. VIP neurons project directly to the GnRH neurons and the AVP neurons project directly to the kisspeptin neurons (Figure 1). Proper output of the SCN onto kisspeptin and GnRH neurons is believed to be important in timing the LH surge, which elicits ovulation [8].

Table 1. **Fertility patterns of different species**
Different species have different fertility patterns, determining the time of year that they breed. Organisms may breed all year round or are limited to a specific time of year, referred to as non-seasonal and seasonal breeders respectively. Despite being seasonal or non-seasonal breeders, fertility may still be affected by changes in the environment, such as disruption of circadian rhythms and abnormal light exposure.

Species	Seasonal / Non-seasonal Breeders	Fertility
Women	Non-seasonal	Female fertility is impaired during shift work, jet lag, etc.
Mice	Non-seasonal	Disrupted circadian rhythms or constant light impair fertility. Female fertility is more sensitive to disrupted circadian rhythms than male fertility.
Meadow Voles	Seasonal	Short day length shuts down HPG axis and leads to small testis and male infertility.
Sheep	Seasonal	Female is fertile "in season" and is anestrus the rest of the year.

The cellular clocks within the AVP neurons have been identified to be crucial for regulating circadian behavior [9]. Interestingly, the absence of circadian input to the kisspeptin neuronal population results in disruption in pregnancy and mimics the nonpregnant state [10]. These data highlight the importance of circadian input into the kisspeptin neurons for proper fertility and reproductive functions.

Vax1 and its role in hypothalamic development

Vax1 is a homeodomain transcription factor, necessary for normal GnRH neuron maturation [11, 12]. In mice, *Vax1* mRNA is first expressed in the anterior neural plate and anterior neural ridge around embryonic day 8 (e8) and limited to the rostral area of the anterior hypothalamus in adults [13]. *Vax1* is critical for embryonic development, and *Vax1KO* mice die soon after birth due to major development defects including cleft lip [11]. In the Mellon lab, it was found that *Vax1* is required for the development of GnRH

neurons, with *Vax1* heterozygosity resulting in a >50% reduction in the number of GnRH neurons and subfertility [11]. Conditional deletion of *Vax1* from GnRH neurons, using $Vax1^{flox}$:$GnRH^{cre}$ mice, revealed that *Vax1* is required to maintain GnRH expression from embryonic day 13 (e13) onward. The absence of GnRH in these conditional KO (cKO) mice, leads to hypogonadism and complete infertility [12]. Although the role of VAX1 in mature neurons has not been addressed prior to the studies in this thesis, we initiated this work based on our finding that, in adult mice, *Vax1* is localized primarily in the hypothalamus, and very highly within the SCN (Figure 2a). It is interesting to note that we also found that VAX1 is required for the development of the SCN (Figure 2b).

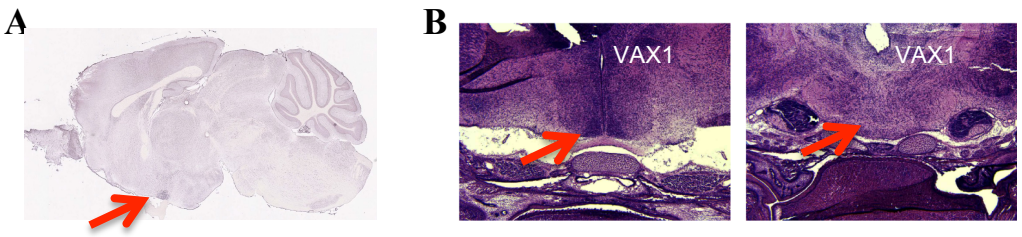

Figure 2. **VAX1 is Required for SCN Development:** (A) In situ hybridization of Vax1 RNA in adult male mouse brain (image from www.brain-map.org). Red arrow indicates *Vax1* expression in the SCN. (B) H&E staining of the brain of control (WT) and *Vax1KO* mice at birth (P0). As indicated by the red arrows, the WT mouse has proper formation and development of the SCN, leading to a structure easily recognizable by the high cell density. The VAX1 KO mouse lacks the SCN structure.

During embryonic development (e12-e15), a rapid cell proliferation in the ventral forebrain gives rise to the SCN, a brain structure characterized by its high cell density (Figure 2). Further maturation of the SCN (e17-P7) is associated with expression of numerous neuropeptides, most of which are expressed around birth and include AVP and VIP [14]. The Mellon lab identified *Vax1* as a regulator of SCN development and function (Figure 2, in preparation). *Vax1* was found to be required for proper maturation of the SCN, as well as *Avp* and *Vip* expression (not shown). Summarizing these data, our findings suggest that VAX1 might play two distinct roles in the SCN, first during development as it allows formation of the SCN, and secondly, in adulthood as its maintained high expression in this structure is potentially important for proper SCN output to the HPG axis.

The role of Vax1 in kisspeptin neurons

Within the SCN, the AVP neuronal population is the upstream regulator of kisspeptin neurons (Figure 1). When AVP was injected into mice in the morning, kisspeptin cellular activity increased, but not GnRH neuron activity [15]. GnRH constantly adjusts its responsiveness to kisspeptin, limiting the LH surge to occuring in the late afternoon, which suggests insensitivity to AVP-induced kisspeptin at all other times [15]. This finding highlighted the importance of the AVP and kisspeptin input into the GnRH neuron.

Interestingly, as stated in the section above, *Vax1* is required for *Avp* expression and SCN development (Figure 2). In addition, a prior study in the Mellon lab found that *Vax1* heterozygous females have increased kisspeptin mRNA in the female anteroventral

periventricular nucleus (AVPV), which is a kisspeptin neuronal population critical for the LH surge (Hoffmann 2014). This suggests that *Vax1* could play a dual role in regulating fertility by both modulating kisspeptin neuron development and/or function, as well as the AVP input on kisspeptin neurons, which is important in timing the LH surge. Indeed, our collaborators have found *Vax1* to be expressed in male kisspeptin cells *in vivo* in the AVPV and arcuate, a second kisspeptin neuronal population in the brain important in regulating the HPG axis (courtesy of Elisandra Sanz, Autonomous University of Barcelona, Spain). We performed *in vitro* studies in immortalized kisspeptin cell lines from the AVPV and arcuate nucleus, and found that transient transfection of VAX1 into these cell lines allows increased expression of kisspeptin-promoter driven luciferase expression, suggesting VAX1 can directly regulate kisspeptin expression in kisspeptin neurons.

Hypothesis

Proper development and function of the HPG axis is critical in maintaining and regulating fertility. This study seeks to determine the role of VAX1 in kisspeptin and AVP neurons and the resulting effects on pubertal onset and fertility. I hypothesize that VAX1 is required for normal kisspeptin and AVP neuron circuit formation, pubertal onset, and reproduction.

Materials and Methods

Mouse breeding

All animal procedures were performed according to protocols approved by University of California, San Diego Institutional Animal Care and Use Committee. *Vax1*^{flox} mice [16] were generated in the Mellon lab, *GnRH*^{cre} [17], *Lhrh*^{cre} *(REF-it is form Dulac)*, AVP^{cre} [18], Kiss^{cre} [19]. For lineage tracing *Rosa-LacZ* [20] (http://jaxmice.jax.org/strain/003309.html), or *Rosa26-TdTomato* (JAX #007909) reporter mice were used. The *Vax1*^{flox}*:Synapsin*^{cre} (*Vax1*^{Syn}) mice were generated in a similar fashion by crossing *Vax1*^{flox} mice with *Synapsin*^{cre} [21]. The *Vax1*^{flox}*:KISS*^{cre} (*Vax1*^{KISS}) mice were generated by crossing *Vax1*^{flox} mice with *KISS*^{cre}. The *Vax1*$^{foxl/fl}$*:AVP*^{cre} (*Vax1*^{AVP}) mice were generated by crossing *Vax1*^{flox} mice with *AVP*^{cre} All mice were kept on a C57BL background. Mice were killed by CO_2 or isoflurane (Vet One, Meridian, ID) overdose and decapitation.

Determination of pubertal onset

Male and female mice were observed for pubertal onset every day starting from the day of weaning, approximately 23-25 days of age. Pubertal onset was determined in male mice by the observation of preputial separation. In females, observation of vaginal opening indicated pubertal onset. A second indicator of female pubertal onset is her first estrus. Once the female mouse had vaginal opening, vaginal smears were collected daily before noon. The vaginal smears were collected on glass slides, counterstained with 0.1%

of methylene blue stain and staged based on the representative cell morphology of the 4 stages of the estrus cycle.

Fertility assessment

At ~12 weeks of age, virgin $Vax1^{flox}$ (control), $Vax1^{fl/fl}$:$KISS^{cre}$ ($Vax1^{KISS}$), and $Vax1^{fl/fl}$:AVP^{cre} ($Vax1^{AVP}$), mice were housed in pairs. The number of litters produced was recorded for 90 days.

GnRH challenges

GnRH challenges were performed on female mice in metestrus or diestrus and male mice prior to noon. A blood sample was collected from the mice through the tail vein before GnRH injection. Each mouse was weighed and injected via intraperitoneal injection with 1 μg/kg GnRH in physiological serum. 10 minutes after the injection, another blood sample was collected via the tail vein. The total volume collected from the pre- and post- injections did not exceed 100 μl. Blood was left at room temperature to clot for 1 hour, then centrifuged for 15 minutes at 2800 g. The serum was collected and stored at -20C.

Immunohistochemistry.

Immunohistochemistry was performed as previously described [11], with the only modification being antigen retrieval by boiling the samples for 10 min in 10 mM sodium citrate. Briefly, the primary antibody used was rabbit anti-GnRH (1:1000, Thermo

Scientific, # PA1-121) or rabbit anti-GnRH (1:1000, Novus, #NBP2-22444). GnRH-positive neurons were counted throughout the brain. For lineage tracing, LacZ expression was detected with a chicken anti-LacZ primary antibody (1:1000, Abcam, #ab9361). Cre expression was detected with a primary anti-cre (1:2000, Biolegend, #908001), and secondary anti-rabbit (1:300, Vector, BA-1000). The secondary antibody used was anti-rabbit (1:300, Vector, BA-1000).

Kisspeptin-10 challenges

Kisspeptin challenges were performed on female mice in metestrus or diestrus and male mice prior to noon. Basal blood sample was collected from the mice through the tail vein prior to Kiss-10 administration. 100 µL of 30 nmoles of Kisspeptin-10 in sterile saline was injected via intraperitoneal injection. 20 minutes after the injection, another blood sample was collected via the tail vein. The total volume collected from the pre- and post- injections did not exceed 100 µl. Blood was left at room temperature to clot for 1 hour, then centrifuged for 15 minutes at 2800 g. The serum was collected and stored at -20^{o}C.

Collection of uterus, ovary, testis, and gonadal histology

Mice were weighed, tailed, and smeared (if female) prior to dissection. The brain, pituitary, and gonads were collected and fixed in 60% ethanol, 30% formaldehyde, and 10% glacial acetic acid in 4^{o}C for two days. On the third day, the collected tissue was moved in 70% ethanol until paraffin embedding.

Sperm count and sperm motility

Sperm was collected from epididymis of male mice in M2 media (Sigma #M7167). Epididymis was cut in half and sperm were expelled by gently pressing down on the epididymis and then left in M2 media at room temperature for 15 min. The numbers of total and motile sperm were counted from a 1:10 dilution of the M2 media containing sperm by using a hemocytometer. The second epididymis was cut into small pieces and left 15 min at room temperature in M2 media. The solution was homogenized frequently to help liberate the sperm. The solution was filtered using a cell streamer (70 μm, Falcon #352350) and sperm were diluted 1:10 with MQ water before counting total numbers of sperm.

Statistical analysis

Statistical analyses were performed using either Student's t-test, One-Way ANOVA or Two-Way ANOVA, followed by *post hoc* analysis by Tukey or Bonferroni as indicated in figure legends, with $p < 0.05$ to indicate significance.

CHAPTER I: Comparison of the Number of Cells Targeted by Two Different GnRH^{CRE} Alleles

Introduction

Gonadotropin-releasing hormone (GnRH) is at the top of the hypothalamic-pituitary-gonadal (HPG) axis (Figure 1), which plays a critical role in development and regulation of the body's reproductive system in both males and females. GnRH neurons appear at embryonic day ~10.5 (e10.5) in the olfactory placode and migrate through the cribriform plate to localize in the anterior hypothalamic region around e15 to e18 (Figure 3). Proper GnRH neuron maturation is crucial in maintaining puberty, fertility, and reproduction. The failure of differentiation and or development of GnRH neurons is a common cause for an infertility condition called idiopathic hypogonadotropic hypogonadism (IHH), which presents symptoms of diminished or delayed sexual maturation, low gonadotropins and sex hormones, and decreased fertility or sterility [22]. Thus, GnRH neurons are required for fertility, and it is therefore important to further our understanding of GnRH neuron development and function in adulthood.

To study the importance of specific genes in GnRH neuron function, many researchers use the Cre-Lox recombinase system. The Cre-Lox system has become one of the most powerful tools in generating conditional knock-down of genes allowing to study a genes function in specific cell populations. The CRE protein is a 38 Da site-specific DNA recombinase of the P1 bacteriophage, which allows catalysis of the recombination between specific sites of DNA, known as loxP sites. The 34 bp loxP consensus sequences flank the target gene in the DNA and a single recombinase protein binds to each loxP

site, which brings the loxP sites together. Through this mechanism, the Cre-Lox recombination facilitates the modifications in mouse genomes from simple point mutations to large chromosomal aberrations, such as deletions, insertions, translocations and inversions. One of the many uses of the Cre-Lox system is to create a conditional knock-out (cKO) mouse, in which a specific gene is deleted from a target tissue or cell (Figure 4 and 5a).

Although this recombination method has been widely used in many studies for cell-specific gene deletions, it also presents many limitations including possible cytotoxicity of CRE expression, germline recombination, inefficient gene deletion, and low or non-specific CRE expression. It is therefore necessary to determine whether the recombination is successful, and where it is occurring. This can be evaluated through the use of for example PCR, southern blot, and expression of a reporter gene.

To further our understanding of specific genes in GnRH neuron function, we first wanted to validate our CRE-mice, and understand the timing of CRE-expression, as well as the number of cells targeted. For the conditional deletion of genes in GnRH neurons we have two CRE-expressing mouse lines available, here termed $Lhrh^{cre}$ and $GnRH^{cre}$. Although both transgenic mouse lines express Cre-recombinase in GnRH neurons, they were each constructed very differently (Figure 4). To create the $Lhrh^{cre}$ transgenic mouse, a cre-recombinase cassette with a poly adenylation [poly(A)] sequence was inserted into the start codon of the mouse *Gnrh* (=*Lhrh*) gene in a 212 kb bacterial artificial chromosome (BAC) [23]. The $GnRH^{cre}$ transgenic mouse was created by inserting the 3.4 kb mouse GnRH promoter to transcribe the Cre-recombinase gene [17]. These two mouse lines are used interchangeably in the literature, however, a side-by-side comparison of the

onset of CRE expression, the number of cells targeted and potential germline recombination has not been studied.

My goal is to compare and characterize the number of cells targeted by the *GnRH*^{cre} and *Lhrh*^{cre} alleles

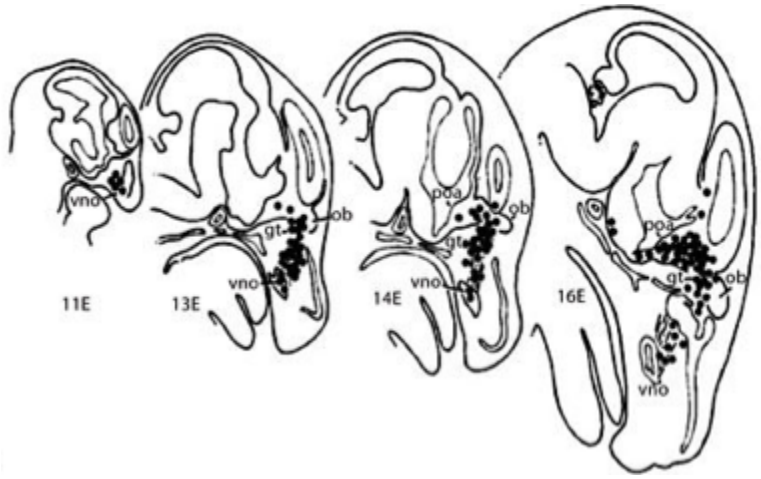

Figure 3. **GnRH Neuron Migration During Embryogenesis**
The black dots represent GnRH neurons. The GnRH neurons first appear on embryonic day 11 (e11) at the vomeronasal organ (vno) and the medial wall of the olfactory placode. At e13, the majority of GnRH neurons are localized in the vno, ganglion terminale (gt), and olfactory bulb (ob). By e14, the GnRH neurons also migrate to the preoptic area (poa). Finally, at e16, the GnRH neurons are localized throughout the forebrain. [24].

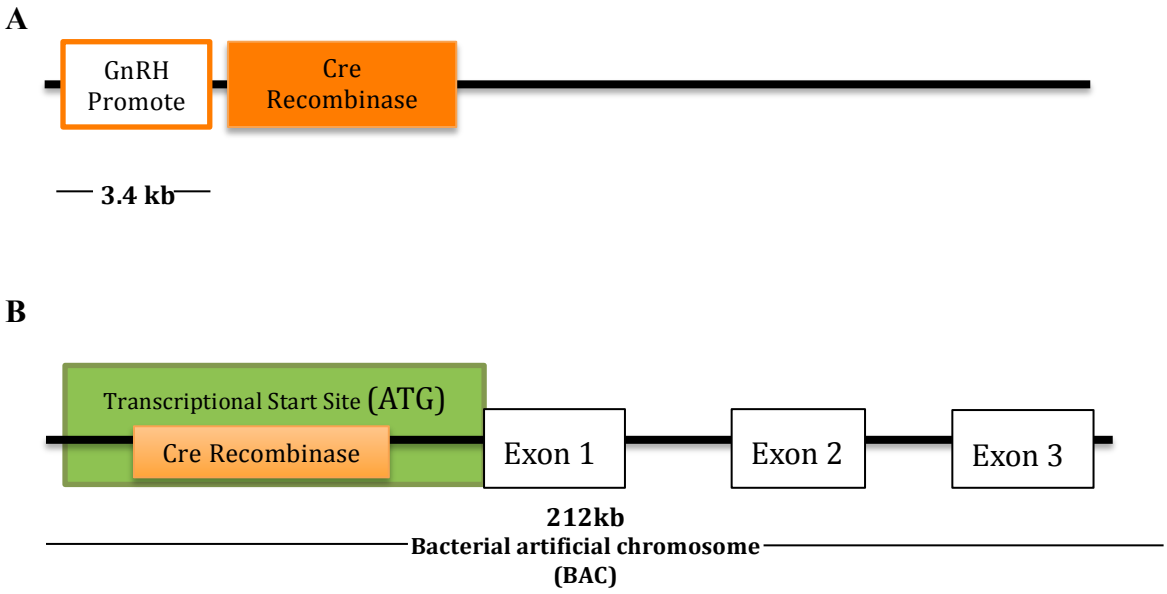

Figure 4. **Strategies to Create the GnRH^{cre} and Lhrh^{cre} Transgenes**

(A) The *GnRH^{CRE}* transgene was constructed by placing the 3.4 Kb mouse GnRH promoter into a vector with the cre-recombinase. (B) The *Lhrh^{CRE}* transgenic mouse was generated by inserting a cre-recombinase into the first exon of the mouse GnRH gene in a 212 Kb bacterial artificial chromosome (BAC).

Results

Substantial differences in the number of cells targeted by the Lhrh^{cre} and the Gnrh^{cre} alleles

To compare the number of cells targeted by the *GnRH^{cre}* and *Lhrh^{cre}* alleles, we crossed the *Rosa26-LacZ* reporter mouse with *GnRH^{cre}* and *Lhrh^{cre}* mice (Figure 5A). For this study, we utilized lineage tracing, in which the *Cre* deletes a stop sequence in front of the LacZ resulting in all cells that express *Cre* to also express LacZ. Immunohistochemistry (IHC) for the presence of LacZ in the brain was performed on

adult *Lhrh^{cre}:Rosa26-LacZ* and *Gnrh^{cre}:Rosa26-LacZ* mice to assess whether the *GnRH^{cre}* and *Lhrh^{cre}* alleles allow recombination in the same number of cells. The number of LacZ expressing cells present in adult mice was determined and we found that the *Gnrh^{cre}:Rosa26-LacZ* has a significantly higher count of LacZ expressing cells than the *Lhrh^{cre}:Rosa26-LacZ* mouse. The entire brain of the *Lhrh^{cre}:Rosa26-LacZ* only had a very small number of LacZ expressing cells, comparable to the number of GnRH neurons in the brain (~800 GnRH neurons in the mouse). In contrast, we counted >10,000 LacZ expressing cells in the *Gnrh^{cre}:Rosa26-LacZ*, which is a 20-fold difference when compared to the *Lhrh^{cre}:Rosa26-LacZ* (Figure 5B). The difference between the two *Cre* alleles was observed in CRE staining as well (Figure 5C). We did not see any significant differences between males and females, so the data was pooled from both sexes.

To understand where the additional LacZ expressing cells were localized in the brain, we did a detailed comparison of the two mouse models. There were additional LacZ expressing cells found in the ventral forebrain, the septum, and the SCN of the hypothalamus.

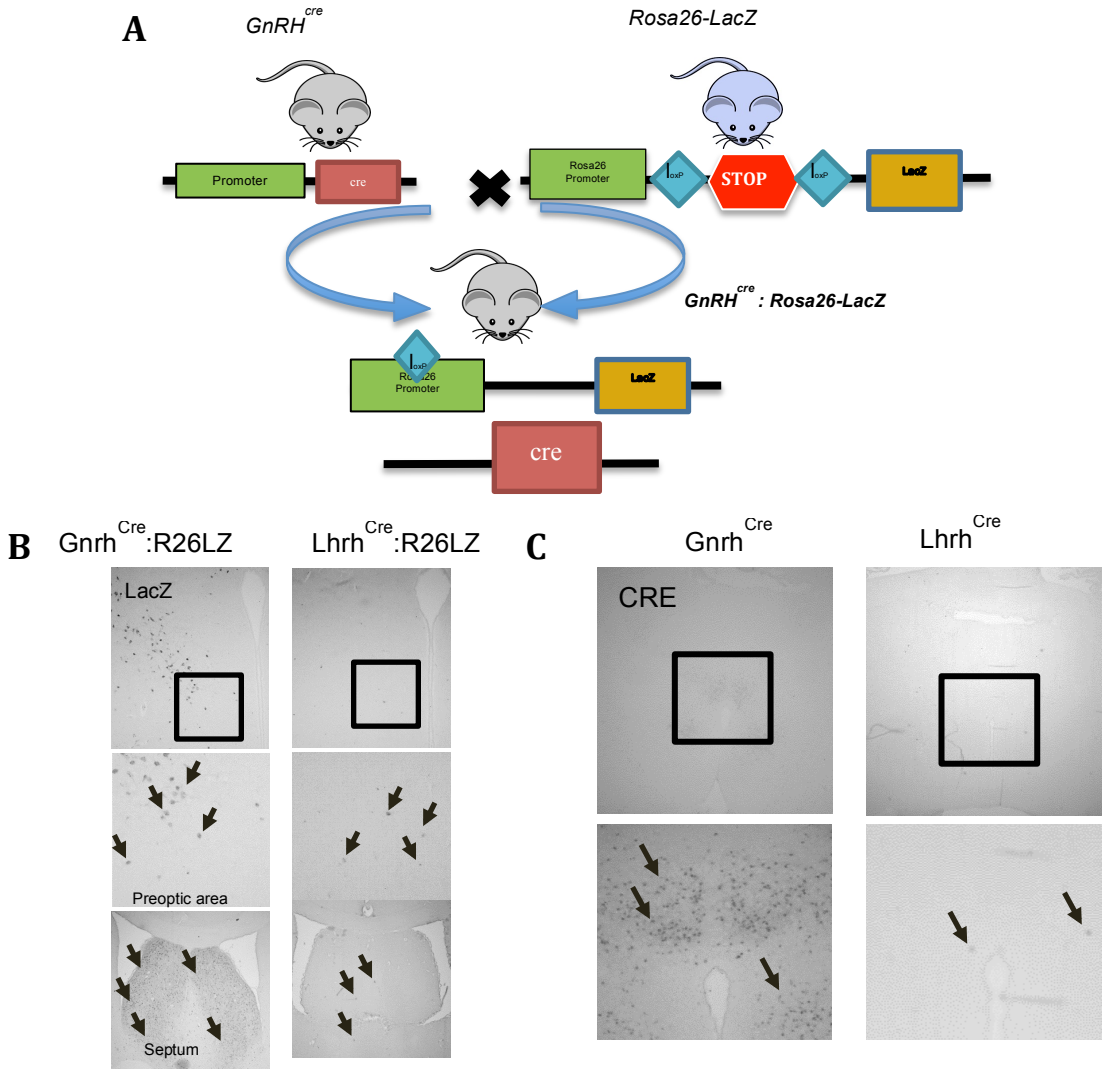

Figure 5. **Comparison of the number of cells targeted by *GnRH*^{cre} and *Lhrh*^{cre} in the brain**

(A) Lineage tracing was utilized to create the *Gnrh*^{Cre}*:Rosa26-LacZ* and *Lhrh*^{Cre}*:Rosa26-LacZ* mouse lines. Illustrative images of LacZ (B), and CRE (C) staining in adult *Gnrh*^{Cre}*:Rosa26-LacZ* and *Lhrh*^{Cre}*:Rosa26-LacZ* brains (n = 3-4). Arrows highlight LacZ or CRE expressing cells.

Germline recombination during female germ cell development in Lhrh^{cre}and Gnrh^{cre} mice

The Cre-Lox strategy comes with certain limits, such as germline recombination, which would delete the Floxed allele in all the cells of the body [25]. In our study, we found that both *Lhrh^{cre}* and *Gnrh^{cre}* were expressed in the ovary (not shown), which suggested that germline recombination could occur in the ovaries of *Lhrh^{cre}* and *Gnrh^{cre}*. This suggests that recombination can occurred when the cre-allele is transmitted from the female. For the study of the *Gnrh^{cre}* germline recombination, we used the *Rosa26-RFP* reporter, which expresses a red fluorescent protein in all the cells expressing CRE. Interestingly, when the Cre-allele was transmitted from the female *GnRH^{cre}* mouse this occasionally produced offspring that outwardly appeared pink (Figure 6). When the mice were dissected, we found that all organs, including the brain and gonads, were entirely pink. The tails of the mice were genotyped and germline recombination was confirmed.

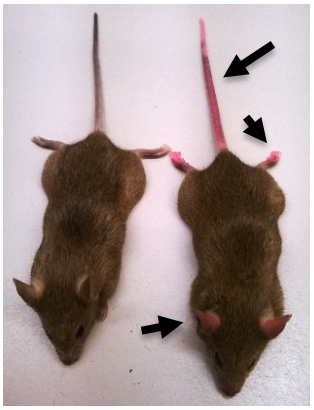

Figure 6. **Germline Recombination can Occur when the Cre-allele is Transmitted Through the Female**
Germline recombination occurred when the *Gnrh^{cre}* was transmitted by the female in the *Gnrh^{cre}:Rosa26-TdTomato* mouse line, which resulted in pinkish animals (arrows).

Discussion

Lhrh^{cre} and Gnrh^{cre} target different numbers and types of cells

Although the use of Cre-recombinase to create conditional gene deletions is widespread, there are many limitations that should be considered. *Lhrh^{cre}* and *GnRH^{cre}* are important tools in studying GnRH neuron development, which plays a major role in the HPG axis and regulating fertility. This study explores and investigates the limitations of the *Lhrh^{cre}* and *GnRH^{cre}* alleles.

To understand the destiny and number of cells targeted by *Lhrh^{cre}* and *GnRH^{cre}*, we decided to use a Rosa26 reporter approach as well as staining for the expression of the CRE protein. This is due to the fact that CRE staining only marks cells that are alive and at present expressing CRE, which indicates active transcription of the promoter. In the Rosa-LacZ cells, the CRE physically recombines the Rosa allele, which allows LacZ expression in all cells, which at some time point prior to the study, expressed the Cre allele. This recombination allows the cells to express LacZ even when CRE is no longer transcribed, so the LacZ staining marks all cells that are alive and recombined the *Rosa26-LacZ* allele at some time during development.

The main differences between the two studied CRE mouse lines is the overall size of the fragment of the GnRH gene inserted into the mouse genome. The *Lhrh^{cre}* mouse has a considerably larger fragment size of 212 kb compared to the *GnRH^{cre}* insert of 3.4 kb. The size of the insert and where the insert integrates into the genome determines how accurately and precisely timing and patterning of the Cre expression is. It was confirmed by Yoon et al., that in the *Lhrh^{cre}*, nearly all (~96%) of the CRE expressing neurons also expressed GnRH. Wolfe et al., found that in *GnRH^{cre}*, there was CRE expression in all

GnRH neurons and some ectopic expression in additional cells. These differences were clearly observed in our study of the *Lhrh^{cre}:Rosa26-LacZ* and *Gnrh^{cre}:Rosa26-LacZ*. The two mouse lines varied significantly in the number of cells targeted by Cre. There were ~20 times more LacZ expressing cells in the *Gnrh^{cre}:Rosa26-LacZ* than the *Lhrh^{cre}:Rosa26 LacZ*. The *Gnrh^{cre}:Rosa26-LacZ* mouse had additional LacZ expressing cells in the ventral forebrain, septum, preoptic area, and SCN. This finding is important to take into consideration because LacZ expressing cells were found in the SCN, an area of the brain that lacks GnRH neurons. From these data, it is evident that, in our hands, the ectopic expression of the *GnRH^{cre}* is more pronounced than previously described [17].

Ideally, mouse lines that are generated through the use of Cre-recombinase will express or delete specific genes in targeted cell types, but the preferred Cre expression patterns are not always achieved [26]. Through this study, we identified that germline recombination occurs when Cre is bred through the female for *Lhrh^{cre}* (not shown) and *GnRH^{cre}*. Thus, it is very important to know the correct genotype of mice before strategically setting up matings to avoid undesired germline recombination. There are also many types of Cre-recombinase systems with different expression and recombination patterns that must be evaluated and investigated prior to use [25]. In addition, the progeny of the matings should be genotyped for germline recombination to confirm the floxed gene was only deleted in the desired cell populations before studying the mice.

Our findings provide further insights and understanding that must be taken into consideration when utilizing the Cre-recombinase system. Both the *Lhrh^{cre}* and *GnRH^{cre}* mouse lines present differences, but are both very valuable in studying GnRH neuron development.

Chapter I, has been submitted for publication. I would like to thank the following co-authors for allowing me to share our data: Hanne M. Hoffmann, Rachel Larder, Rachael J. Hu, Crystal Trang, Daniel D. Clark, and Pamela L. Mellon. The thesis author was the third author of this paper.

CHAPTER II: Determine the Role of *Vax1* in the Hypothalamic Circuit Regulating Fertility

Introduction

Synapsin-Cre deletion of Vax1 in the hypothalamic region results in more GnRH neurons and, paradoxically, female infertility

The transcription factor, *Vax1*, plays a critical role in brain development [27]. Full-body knockout of *Vax1* (*Vax1KO*) results in neonatal death due to developmental defects, while *Vax1* heterozygosity leads to subfertility in both male and female mice [11]. Interestingly, we found that there is an absence of the formation and development of the SCN in the *Vax1KO* mice (Figure 2)

Circadian rhythms have been shown to play a critical role in regulating and maintaining fertility in both humans and mice. In both, circadian rhythms are generated by the SCN. When the SCN is ablated in mice, it abolishes the LH surge, which impairs female fertility [28]. Through the use of *Synapsin-cre* mice, the $Vax1^{flox}$ allele was conditionally deleted ($Vax1^{syn}$) from the mature ventral forebrain and hypothalamus. The $Vax1^{syn}$ mice have proper formation and development of the SCN (Figure 7). The $Vax1^{syn}$ female, but not male, mice were subfertile.

Despite this subfertility, the $Vax1^{syn}$ male and female mice responded with double the LH release in response to kisspeptin challenges (Figure 8A). Interestingly, IHC staining revealed that the WT mice and $Vax1^{syn}$ mice had a ~2 fold difference in the number of GnRH neurons (Figure 8B, C). These data suggested that the reason $Vax1^{syn}$ mice release more LH in response to kisspeptin is due to a higher number of GnRH

neurons. These data identified a paradoxical relationship between the subfertility and increased number of GnRH neurons; and suggest that GnRH neurons are not responsible for the subfertility of the *Vax1*^{syn} females, and thus, the neuronal population responsible for the subfertility has yet to be identified.

The brain area where *synapsin-cre* overlaps with *Vax1* expression is principally within the hypothalamus, an area within the brain that includes two major SCN neuronal populations: AVP, and VIP neurons, as well as the hypothalamic kisspeptin and GnRH neurons.

AVP neurons are required for proper LH surge and fertility

In the hypothalamus, the AVP neurons within the SCN act as the upstream regulator to kisspeptin (Figure 1). The AVP neurons control the timing of the preovulatory GnRH surge that later gives rise to the LH surge [15] . The LH surge is critical for ovulation and overall for fertility. This suggests that the AVP neurons could potentially be the neuronal population responsible for the impaired fertility observed in the *Vax1*^{syn} female mice.

Kisspeptin is critical for pubertal onset and fertility

Kisspeptin plays an important role in determining sexual development. The transition from youth to adulthood is marked by sexual development, known as pubertal onset. Pubertal onset is defined by the increased hypothalamic secretion of GnRH that activates the HPG axis [29]. We found that *Vax1HET* females have a ~30 fold increase in kisspeptin expression in the AVPV [11] and that *Vax1* is expressed in immortalized kisspeptin cells, where *Vax1* activates expression of the kisspeptin promoter driving luciferase expression (not shown).

Study aims and goals

Based on the data from the *Vax1*^{syn} mice, which had female infertility, normal SCN morphology, a 2-fold increase in GnRH neurons (Figure 8B,C), and impaired SCN output (as evaluated by wheel running behavior, not shown), our goal is to determine what cellular population(s) in the *Vax*^{syn} female caused the observed subfertility. Based on the absence of SCN morphology and *Avp* in *Vax1KO* SCN, as well as the expression of *Vax1* in adult male kisspeptin neurons, we focused on what kind of modulatory role AVP neurons play when regulating kisspeptin neurons, specifically by deleting *Vax1* within each of these neuronal populations. To study the role of *Vax1* in AVP neurons, we conditionally deleted *Vax1* in AVP neurons with the use of *AVP*^{cre} (these mice will be referred to as *Vax1*^{AVP} mice). We also studied the role of *Vax1* within the downstream neuronal population of kisspeptin neurons. To do this, we used the *Kiss*^{cre} mouse to delete the *Vax1*^{flox} allele. We refer to these mice as *Vax1*^{kiss}.

My hypothesis is that *Vax1* in Kisspeptin or AVP Neurons is Required for Female Fertility

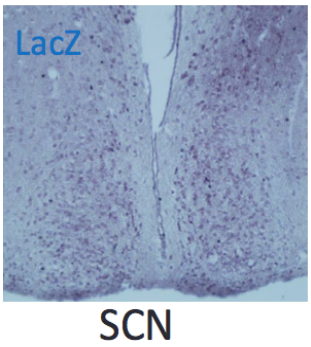

Figure 7. ***Vax1^{SYN} mice have proper SCN formation and development***
When staining for the expression of LacZ in the lineage tracing of Synaspin^{cre}:RoasaLacZ mice, proper morphology of the SCN was observed. The synapsin-cre targets the SCN, as indicated by the dark purple dots.

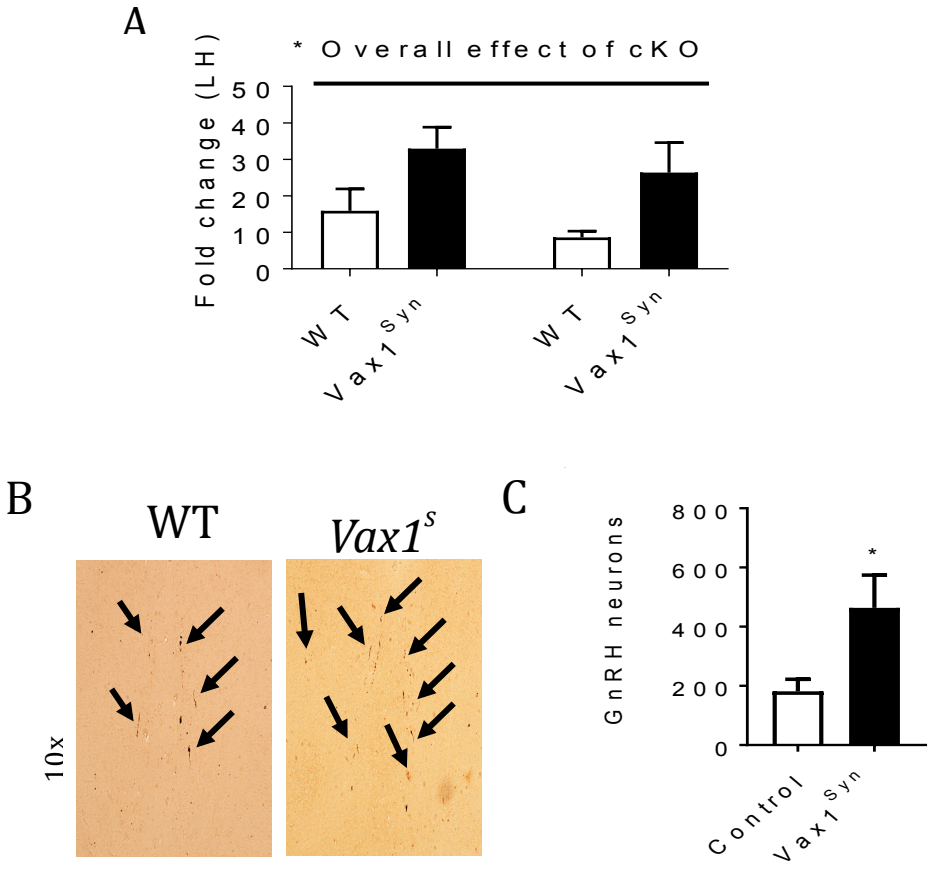

Figure 8. ***Vax1*^{SYN} mice have more GnRH neurons**
(A) Kisspeptin challenge increases luteinizing hormone (LH) response by two-fold in *Vax1*^{SYN} mice compared to the controls. (B) Immunohistochemistry (IHC) staining for GnRH expression in control (WT) and *Vax1*^{SYN} mice. (C) Counts of GnRH neurons from the IHC staining in both control and *Vax1*^{SYN} mice (n=2). Statistical analysis by Two-way ANOVA, * $p<0.05$).

Results

Vax1 in AVP neurons is required for first estrus

To further our understanding of the role of *Vax1* within AVP neurons in relation to fertility, we investigated the impact of the specific deletion of *Vax1* in AVP neurons. To delete *Vax1* from AVP neurons, we generated $Vax1^{flox/flox}$:AVP^{cre} ($Vax1^{AVP}$) mice. We first measured pubertal onset in the $Vax1^{AVP}$ mice. To determine pubertal onset, vaginal opening was determined in females, and preputial separation in males. The weight at vaginal opening and preputial separation was also noted, as weight is associated with pubertal onset. Both control female and male mice go through puberty around 30 days of age and 15 g of weight, which was comparable to $Vax1^{AVP}$ females and males (Figure 9). Puberty is a long process, and associated with numerous physiological changes. A second marker of pubertal onset in females is first estrus. Thus, after vaginal opening, vaginal smears were collected daily in female mice to determine the first estrus. Control female mice reached first estrus around 35 days of age. Interestingly, the $Vax1^{AVP}$ females reach first estrus around 40 days of age, which is significantly delayed compared to the control females (Figure 10). These data overall suggest that the $Vax1^{AVP}$ mice have delayed first estrus, and thus delayed pubertal onset.

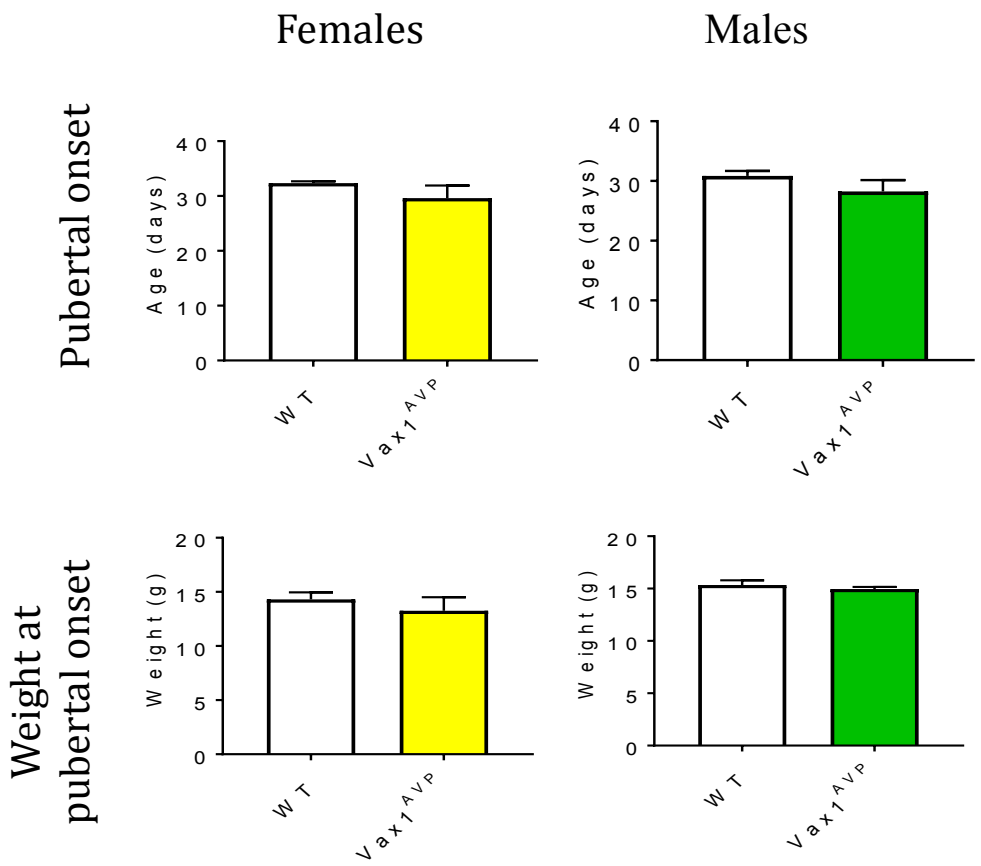

Figure 9. **Vax1^{AVP} Males and Females Have Normal Pubertal Onset**
The age and weight at vaginal opening (females) and preputial separation (males) was determined in *Vax1^{AVP}* mice. N= 5-6, statistical analysis by Students t-test, p>0.05.

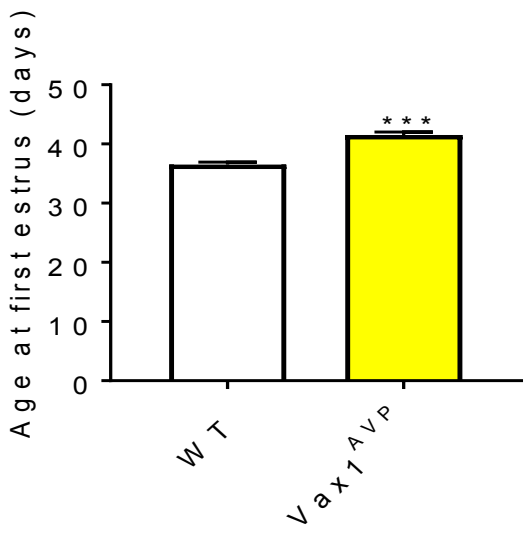

Figure 10. ***Vax1^{AVP}* females have delayed first estrus**
After vaginal opening, a second marker of pubertal onset in females, the age of first estrus, was recorded. N= 4-6, statistical analysis by Students t-test, *** $p<0.001$.

Vax1^{AVP} mice have normal fertility

The delayed first estrus in the female *Vax1^{AVP}* cKO mice suggested the possibility of impaired fertility. To determine the fertility of the *Vax1^{AVP}* mice, we set up matings of wild-type with wild-type, wild-type females with *Vax1^{AVP}* cKO males, and wild-type males with *Vax1^{AVP}* cKO females for 90 days and monitored the number of litters generated in this time frame as well as time to first litter. The control mice produced an average of 2-3 litters within 3 months, with the first litter on average 22 days after the matings were set up. The *Vax1^{AVP}* cKO male matings produced on average 3 litters within 3 months, with the first litter on average 25 days after the matings were set up (Figure 11A, B). The female *Vax1^{AVP}* cKO matings produced an average of 3 litters within 3 months, with the first litter on average 23 days after matings were set up (Figure 11A,B).

These data suggest that the *Vax1*^{AVP} mice have normal fertility, if not better fertility than the control mice.

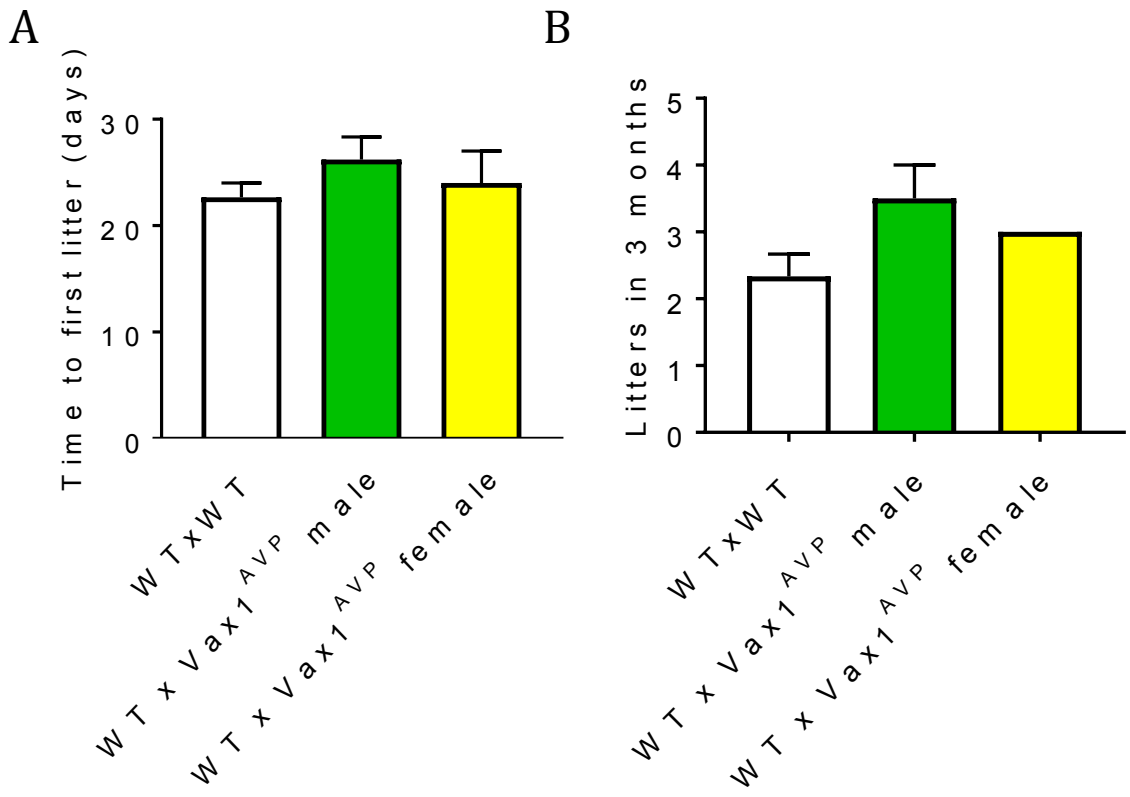

Figure 11. ***Vax1*^{AVP} male and female mice have normal fertility**
Fertility assays of control matings (WTxWT), *Vax1*^{AVP} cKO female x WT male, and *Vax1*^{AVP} cKO male x WT female were set up for 90 days. The matings were observed for the (A) time to first litter and (B) number of litters within the 3-month period. N= 1-3, no statistical analysis was done due to low "n".

Summary of the impact of deletion of Vax1 in AVP neurons

In the hypothalamic area of the HPG axis (Figure 1), we have deleted *Vax1* from the AVP neurons and found that the *Vax1*^{AVP} male mice have normal pubertal onset and

Vax1^{AVP} female mice have a delayed first estrus. Interestingly, fertility was not impaired. The deletion of *Vax1* from the AVP neurons was identified as not responsible for the infertility observed in the *Vax1*^{syn} females, so we next studied the deletion of *Vax1* in the downstream neuronal population of kisspeptin neurons.

Deletion of Vax1 in kisspeptin neurons delays first estrus

To determine if *Vax1* within kisspeptin neurons is important in maintaining fertility, we investigated the impact of the deletion of *Vax1* in kisspeptin neurons. To delete *Vax1* from kisspeptin neurons, we generated *Vax1*$^{flox/flox}$*:KISS*^{cre} (*Vax1*^{KISS}) mice. We first observed that the age and weight of vaginal opening was comparable between control and *Vax1*^{KISS} cKO female mice (Figure 12). Similar data were observed in the males, where there was no difference at age and weight at preputial separation (Figure 12). In comparison, the *Vax1*^{KISS} cKO females reached first estrus around 35 days of age, which is significantly delayed compared to the control females (Figure 13). Overall, the data show that *Vax1*^{KISS} females have delayed first estrus.

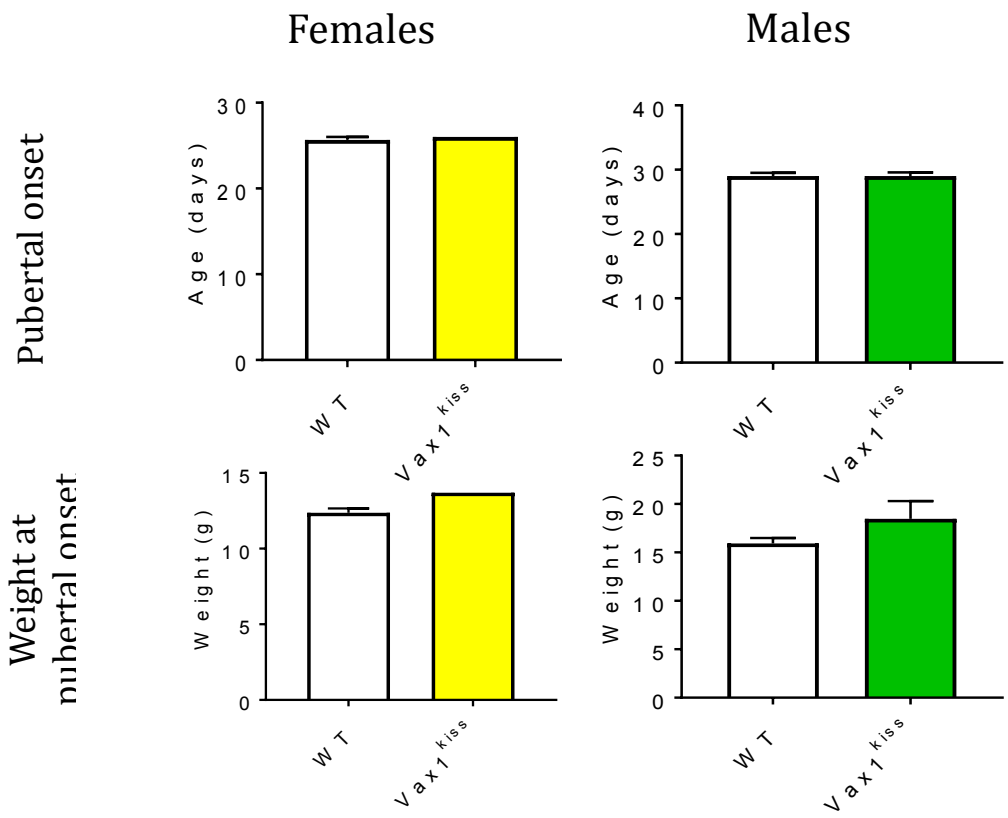

Figure 12. **Vax1^{KISS} males and females have normal pubertal onset**
The age and weight at vaginal opening (females) and preputial separation (males) was determined in *Vax1^{AVP}* mice. No statistical test was run yet due to low "n". N= 1-4 (females), 3-5 (males), Statistical analysis by Students t-test in the males, $p>0.05$.

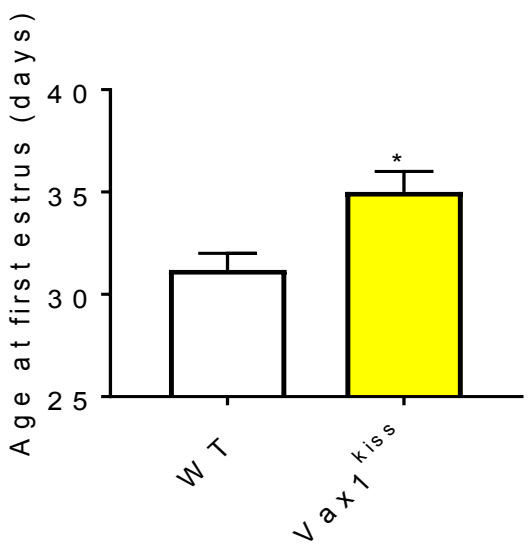

Figure 13. ***Vax1^{KISS}* females have delayed first estrus**
Once puberty was reached in the females, the age for first estrus was recorded. N= 2-6, Statistical analysis by Students t-test, * $p<0.05$.

Deletion of Vax1 in kisspeptin neurons impairs fertility

The delayed first estrus suggested impaired fertility, so we next observed reproductive ability through fertility assays. In the time period of 90 days, the control matings of wild-type and wildtype mice produced an average of 2 to 3 litters. In comparison, the *Vax1^{KISS}* cKO male matings produced on average less than 0-1 litter and the single *Vax1^{KISS}* cKO female mating currently studied did not produced a litter within the 90 days (Figure 14A). These data suggested that the *Vax1^{KISS}* cKO male and female mice were almost completely infertile.

The control matings produced the first litter on average around 25 days after the mating cages were set up. The *Vax1^{KISS}* cKO male matings produced the first litter on average around 50 days after the mating was set up and the *Vax1^{KISS}* female matings did not produce a litter within the 90 days (Figure 14B). The longer time to first litter in the

Vax1^{KISS} males and the absence of litters in the *Vax1*^{KISS} females suggest these mice are subfertile.

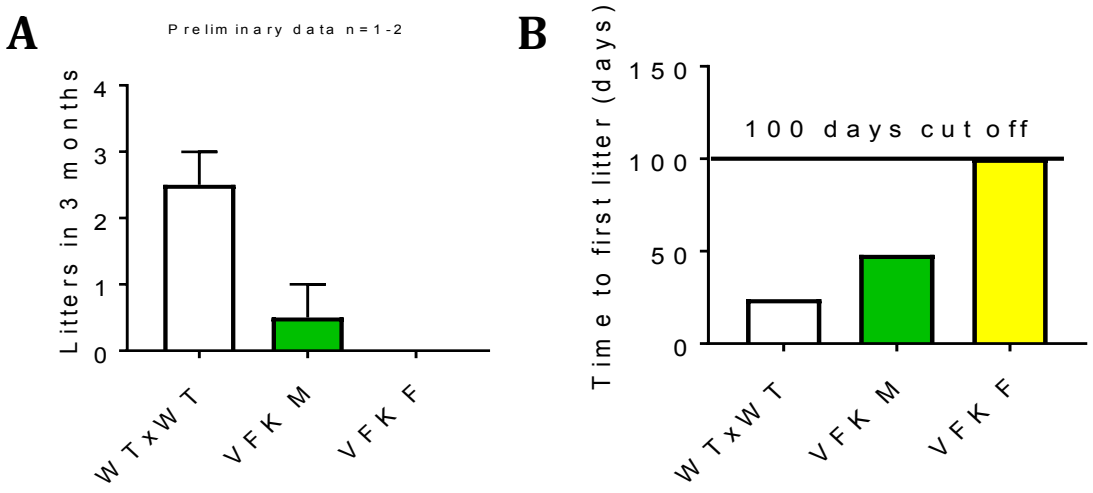

Figure 14. ***Vax1*^{KISS} males and females are subfertile**
Fertility assays were set up with control matings (WTxWT), *Vax1*^{KISS} males with WT, and *Vax1*^{KISS} females with WT. These mating cages were monitored for the (A) number of litters and the (B) time to first litter during 90 days. No statistical test was run yet due to low "n", n=1-2.

Vax1^{KISS} *males have low levels of LH, but normal sperm motility and sperm count*

The cKO *Vax1*^{KISS} males were determined to be subfertile from the fertility assays. Interestingly, the *Vax1*^{KISS} cKO males had comparable levels of FSH to the control, but lower levels of LH (Figure 15A). These data suggested that the low LH in the cKO *Vax1*^{KISS} males may be responsible for the impaired fertility.

FSH is one of the many hormones utilized for spermatogenesis and LH is responsible for continuing the spermatogenesis. The very low LH levels in *Vax1*^{Kiss} males raised the possibility of impaired spermatogenesis or impaired sperm motility. We collected the epididymis of control and cKO *Vax1*^{KISS} males and determined the total

sperm count and percent of motile sperm. The control males have approximately 25 million sperm per epididymis, with 60% of them being motile. The cKO *Vax1*^{KISS} males have approximately 30 million sperm with 40% motility, which is comparable to the controls (Figure 15B). Despite the significantly lower levels of LH, the number of sperm and amount of motile sperm were unaffected in the cKO *Vax1*^{KISS} males.

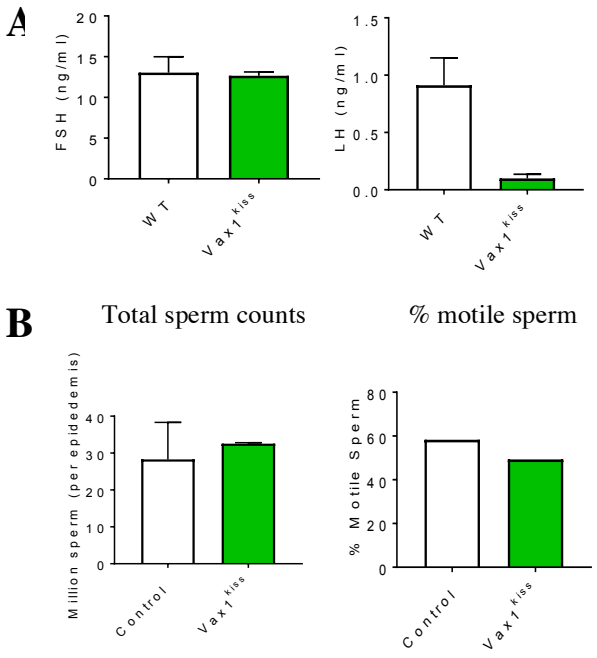

Figure 15. ***Vax1*^{KISS} males have low levels of LH, but normal sperm motility and count**
(A) The FSH and LH levels of the *Vax1*^{KISS} males were measured. (B) The epididymis was collected in male WT and *Vax1*^{KISS} cKO for sperm motility and total sperm counts. No statistical test was run due to low "n", n=1-2.

Discussion

Vax1 is expressed in the hypothalamus of the adult brain and by the use of *synapsin-cre*, *Vax1* was deleted specifically from mature neurons. Interestingly, it has been identified that the *Vax1*^{SYN} females have delayed pubertal onset and are subfertile

(Hoffmann et al, in preparation). To determine the origin of impaired fertility, hormonal challenges revealed that the *Vax1*^{SYN} mice have increased sensitivity to kisspeptin (Figure 8A). The increased LH release in response to kisspeptin may have been due to the increased number of GnRH neurons in the *Vax1*^{SYN} mice compared to the control mice. However, these data do not explain the almost complete infertility of the female mice, thus, the goal of this project was to identify the specific neurons responsible for the impaired fertility of the *Vax1*^{SYN}. The *synapsin-cre* overlaps with *Vax1* expression in the hypothalamic region of the mouse brain, and within this region, there are four major neuronal populations important in fertility regulation: AVP, VIP, kisspeptin, and GnRH neurons (Figure 1). From the four neuronal populations, we focused on the AVP and Kisspeptin neurons, as we had evidence showing that *Vax1* is required for development of AVP neurons/the SCN, *Vax1* is expressed in adult kisspeptin neurons (not shown), and *Vax1* can regulate both the *Avp* promoter driving luciferase expression (*in vitro* transfection studies, not shown), and the *kisspeptin* promoter driving luciferase expression (*in vitro* transfection studies, not shown).

In our study, we found that *Vax1* in both kisspeptin and AVP neurons is required for the proper onset of first estrus, but neither of these mouse lines (*Vax1*^{AVP} and *Vax1*^{KISS}) had impaired preputial separation or vaginal opening. The delayed first estrus in both *Vax1*^{AVP} and *Vax1*^{KISS} mouse lines highlights an important role of *Vax1* within these two neuronal populations in determining the first estrus. These data suggest that the deletion of *Vax1* causes impairment of the AVP and kisspeptin neuronal populations. When AVP and kisspeptin neurons are impaired, this may result in a disruption in the kisspeptin→GnRH neuronal connection resulting in mis-timed or improper hormone

release from the pituitary (Figure 1). Proper release of hormones, such as estrogen, is critical in determining vaginal opening in female mice. However, it is important to note that the first estrus is not representative of sexual maturity [30], which supports the maintained fertility of the $Vax1^{AVP}$ females. Next, we found an interesting difference in fertility between the two studied mouse lines, where $Vax1^{AVP}$ male and female mice both had normal fertility, whereas fertility was almost completely impaired in $Vax1^{KISS}$ males and females.

While GnRH and kisspeptin are required for pubertal onset and fertility, AVP neurons have a modulatory role of the kisspeptin neurons and HPG axis. AVP neurons are part of the SCN, and are thought to play a role in regulating when kisspeptin is released. When injected with AVP, kisspeptin cellular activity increases [15], suggesting that AVP sends a circadian signal to kisspeptin neurons facilitating the LH surge. Our data suggest that *Vax1* within AVP neurons is not required for the LH surge, or controlling fertility, and only is important in determining first estrus. On the other hand, the strong impact on fertility when deleting *Vax1* within kisspeptin cells ($Vax1^{KISS}$ mice) has for the first time associated VAX1 within kisspeptin neurons with fertility. This is very interesting as very little is known about the role of transcription factors within kisspeptin neurons, and how transcription factors can play a role in kisspeptin neuron development or function.

$Vax1^{KISS}$ males have decreased basal LH levels, which could possibly be the cause of the male subfertility. The lack of LH has been associated with impaired testicular descent, which can result in low sperm numbers and infertility [31]. To study whether the

low LH levels of the male *Vax1*^{KISS} mice and subfertility were correlated, we assessed total sperm and sperm motility. We found that *Vax1*^{KISS} mice had similar total sperm counts and percent motile sperm as the control mice. These data suggest that poor sperm quality is not the origin of the subfertility in the male *Vax1*^{KISS} mice.

Chapter II, in part, is currently being prepared for publication. I would like to thank the following co-authors for allowing me to share our data: Hanne M. Hoffmann, Joseph A. Breuer, Crystal Trang, Brittany Hereford, Kapil Bharti, Michael R. Gorman, David K. Welsh, and Pamela L. Mellon. This thesis author was the second author of this paper.

General Discussion

Fertility is tightly regulated by the HPG axis

The HPG axis of the neuroendocrine system tightly regulates fertility. It receives circadian input from the SCN, where two major neuronal populations, AVP and VIP (Figure 1), project to the kisspeptin and GnRH neurons, respectively. In response to GnRH, the pituitary releases luteinizing hormone (LH) and follicle-stimulating hormone (FSH), which stimulate the gonads to secrete sex hormones and produce sperm and eggs.

Different Cre Alleles Target Different Types and Numbers of Neurons

One of the key neuronal populations in fertility is the GnRH neurons. As part of my thesis, I have been involved in validating two different cre-alleles targeting GnRH neurons, the *Gnrh*^{cre} and the *Lhrh*^{cre} alleles. We started this work after observing different reproductive phenotypes due to deleting the transcription factor *Otx2* using the *Gnrh*^{cre} [32], and the *Lhrh*^{cre} (submitted). I initially studied the limitations of using the cre-lox recombinase system prior to creating the transgenic mouse lines for conditional knockout (cKO) of *Vax1* presented in chapter 2. First, we observed that germline recombination occurs when the *GnRH*^{cre} allele is transmitted through the female, and not the male. Further, we found that the both the cre-alleles that were designed to target GnRH neurons, presented with substantial differences in the number of neurons targeted. The *Lhrh*^{cre} had a more stringent expression in the brain than the *Gnrh*^{cre}. Further, studies not shown in this thesis, made us conclude that the ectopic expression of the *Gnrh*^{cre} allele in the septum (required for sexual behavior) and the SCN, could cause the differences in fertility between the two studies, and highlighted the precautions needed when

interpreting data obtained from cKO studies, and the importance of understanding what neuronal populations are targeted.

Vax1 is important for fertility

The complete deletion of *Vax1* (*Vax1KO*) resulted in absence of GnRH neurons, hypogonadism and infertility [12], whereas *Vax1HET* males and females were subfertile, and females presented with abnormal LH levels [11]. Understanding the role of *Vax1* within the HPG axis and identifying the root of these key characteristics of *Vax1HET* would provide better insight into the neuroendocrine regulation of fertility. Through the *Vax1HET* mice, subfertility was determined to originate in the hypothalamus, which is at the apex of the HPG axis (Figure 1) [11]. When *Vax1* was deleted from the hypothalamic region through the use of *synapsin-cre*, the $Vax1^{SYN}$ female mice were subfertile, whereas male fertility was normal. In these $Vax1^{SYN}$ mice, we identified an increase in the luteinizing hormone (LH) response to kisspeptin compared to the control mice. Interestingly, these $Vax1^{SYN}$ mice had two-fold more GnRH neurons than the control mice as well. This finding suggested the possibility of altered sensitivity to kisspeptin due to increased abundance of the GnRH neurons. Changing the GnRH neurons' sensitivity and response to the upstream regulator, kisspeptin, could possibly contribute to improper hormonal release for estrous cycling and ovulation.

Deletion of Vax1 from kisspeptin, but not AVP, neurons impairs fertility

Within the area of the HPG axis targeted by the *synapsin-cre*, the AVP, VIP, GnRH, and kisspeptin neuronal populations are present (Figure 1). Interestingly, *Vax1* was found to be expressed in male kisspeptin cells *in vivo*. First, we observed the effects

of deleting *Vax1* within the upstream neuronal population of AVP. The $Vax1^{AVP}$ male and female mice reached pubertal onset at an age and weight comparable to the control mice. Interestingly, the $Vax1^{AVP}$ female mice have a delayed first estrus, but $Vax1^{AVP}$ male and female mice had normal fertility. These data suggested that *Vax1* within AVP neurons plays a role in determining first estrus. Interestingly, AVP has been identified to control the timing of *Kiss1* mRNA expression, which has been determined required for proper timing of puberty [33], [34]. Overall, the deletion of *Vax1* within the AVP neurons may be disrupting the signaling between AVP neurons and kisspeptin neurons, and in this manner control first estrus in the female.

After determining that deletion of *Vax1* within AVP neurons had little effect on fertility, we next studied how *Vax1* deletion in kisspeptin neurons would impact pubertal onset and fertility. We found that $Vax1^{KISS}$ males had normal pubertal onset, whereas females had delayed first estrus. Both the $Vax1^{KISS}$ male and female mice were subfertile to completely infertile, which indicated that the kisspeptin neuronal population might be involved in the subfertility of the $VAX1^{SYN}$ females. This finding suggests that *Vax1* within the kisspeptin neuronal population plays an important role in regulating fertility. Yet, not all questions have been answered, as only $Vax1^{SYN}$ females were subfertile, while fertility was reduced in both male and female $Vax1^{KISS}$ mice.

Although we determined that *Vax1* within the kisspeptin neurons plays a role in fertility, we have not yet identified the exact mechanism behind its role. In future studies, we hope to identify the approximate timing of when the $synapsin^{cre}$ and $KISS^{cre}$ expression starts in the brain. If the expression of the cre recombinases is active during

earlier stages of development, the deletion of *Vax1* may potentially have a bigger impact on neuronal development than anticipated.

Our characterization of *Vax1* in relation to the function of kisspeptin neurons and the SCN, brings us one step closer to deciphering the complex neuronal circuit regulating fertility, and how light potentially can feed into this system through AVP neurons, timing first estrus and potentially the LH surge in females.

This study provides *Vax1* as a novel candidate gene for infertility conditions that are not yet completely understood. Infertility has a variety of possible causes, of which we specifically studied the importance of *Vax1* within different neuronal populations. Time changes, jet lag, artificial light, and shift work all cause disruptions in circadian rhythms, which has been identified as a contributor to infertility [35, 36].The subfertility of the $Vax1^{syn}$ female mice supports numerous other studies showing that female reproduction is more vulnerable to disruptions in circadian rhythms. In conclusion, I have shown that the transcription factor *Vax1* specifically within kisspeptin neurons is required for fertility.

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