UNIVERSITY OF CALIFORNIA, SAN DIEGO

The Transcription Factor VAX1 is Required for Normal Development and Function of GnRH Neurons and the SCN

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by

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The Thesis of Crystal Trang is approved and it is acceptable in quality and form for publication on microfilm and electronically:

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University of California, San Diego

2016

Dedication

I would like to dedicate this thesis to my loved ones:

To my Mom and Dad for their eternal love and support.

To Diana and Phoebe for constantly encouraging me and believing in me.

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Abbreviations

AVP	Arginine vasopressin
BMAL	Brain muscle ARNT-like
cHET	Conditional knockout
cKO	Conditional knockout
E	Embryonic day
FSH	Follicle-stimulating hormone
GnRH	Gonadotropin-releasing hormone
Gt	Ganglion terminate
H&E	Hematoxylin and eosin
HET	Heterozygous
HPG	Hypothalamic-pituitary-gonadal
IHC	Immunohistochemistry
IHH	Idiopathic hypogonadotropic hypogonadism
KO	Knockout
LacZ	Beta galactosidase
LH	Luteinizing hormone
MPOA	Medial preoptic area
Nucl acc	Nucleus accumbens
Ob	Olfactory bulb
Per1	Period 1
Poa	Preoptic area
SCN	Suprachiasmatic nucleus

VAX1	Ventral anterior homeobox 1
Vax1^{GnRH}	*Vax1*$^{fl/fl}$*:GnRH*^{cre}
Vax1$^{GnRH:LacZ}$	*Vax1*$^{fl/fl}$*:GnRH*^{cre}*:RosaLacZ*
Vax1^{syn}	*Vax1*$^{fl/fl}$*:Synapsin*^{cre}
Vax1$^{syn:LacZ}$	*Vax1*$^{fl/fl}$*:Synapsin*^{cre}*:RosaLacZ*
VIP	Vasoactive intestinal peptide
Vno	Vomernasal organ
WT	Wild type

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ABSTRACT OF THE THESIS

The Transcription Factor VAX1 is Required for Normal Development and Function of GnRH Neurons and the SCN

by

Crystal Trang

Master of Science in Biology

University of California, San Diego, 2016

Professor Pamela L. Mellon

Infertility is becoming increasingly prevalent in our society, where one in six couples are faced with difficulties in conceiving. Fertility is tightly regulated by the hypothalamic-pituitary-gonadal axis, where the hypothalamic gonadotropin-releasing hormone (GnRH) neurons and the suprachiasmatic nucleus (SCN) are most vital. Here we identify a novel transcription factor, Ventral anterior homeobox 1 (VAX1), as a critical regulator of GnRH neuron development and SCN function. By the use of transgenic mouse models, we deleted *Vax1* in GnRH neurons early in development

leading to a 99% reduction in GnRH expression, resulting in delayed pubertal onset, hypogonadism, infertility in both sexes, and atypical estrous cyclicity in females. Furthermore, the deletion of *Vax1* in the mature SCN significantly reduced expression of the key SCN peptide, VIP, which was associated with disrupted wheel-running activity. The weakened SCN output led to female subfertility, while the males were unaffected. These data together highlight the importance of VAX1 in both the developmental stage of fertility and its maintenance through adulthood.

Introduction

Fertility is Regulated by the Hypothalamic-Pituitary-Gonadal Axis and the Suprachiasmatic Nucleus

Infertility is a common health issue shared by many Americans. According to Centers for Disease Control and Prevention (CDC), infertility affects about 12% of women from the ages of 15 to 44 and about 7.5% of sexually active men in the United States (http://www.cdc.gov/reproductivehealth/infertility/). Fertility is highly dependent on circadian rhythms, which are ~24 h rhythms generated by the suprachiasmatic nucleus (SCN), an endogenous pacemaker located in the hypothalamus. Unsynchronized rhythms from irregular sleeping patterns result in critical health disorders such as reduced cognitive responsiveness, cardiovascular disease, diabetes, obesity, and infertility (Fig. 1) [1]. Over the last century, the United States has become increasingly dependent on shift work to meet socioeconomic demands [2], making this area of research of utmost importance.

Female fertility is particularly sensitive to disrupted circadian rhythms. Female shift workers are highly susceptible to irregular menstrual cycles, miscarriages, and other infertility issues [3]. As shift work becomes a more integral factor of our society, there is an increasing need to understand how circadian rhythms affect fertility and human health overall.

Circadian rhythms regulate fertility via the hypothalamic-pituitary-gonadal (HPG) axis, which is dependent on the pulsatile firing of gonadotropin-releasing hormone (GnRH) neurons [4]. Localized in the anterior hypothalamus, GnRH neurons project to

the median eminence, where GnRH release stimulates the pituitary to release luteinizing hormone (LH) and follicle-stimulating hormone (FSH) (Fig. 1). LH and FSH signal to the gonads to release sex hormones, such as testosterone, estrogen and progesterone. The rhythmicity of GnRH neurons is critical for proper LH and FSH release. Current studies suggest that GnRH neuron rhythmicity is coordinated by the SCN [5]. The SCN projects directly onto two neuronal population of the HPG axis through arginine vasopressin (AVP) neurons to kisspeptin neurons, a neuronal population activating GnRH neurons, and through vasoactive intestinal peptide (VIP) projections onto GnRH neurons (Fig. 1) [5]. Circadian signals can therefore indirectly and directly control GnRH neurons to release GnRH rhythmically. Interestingly, ablation of the SCN, absence of GnRH neurons, or uncoordinated release of GnRH can all lead to impaired fertility [6], illustrating the complexity of fertility maintenance in mammals.

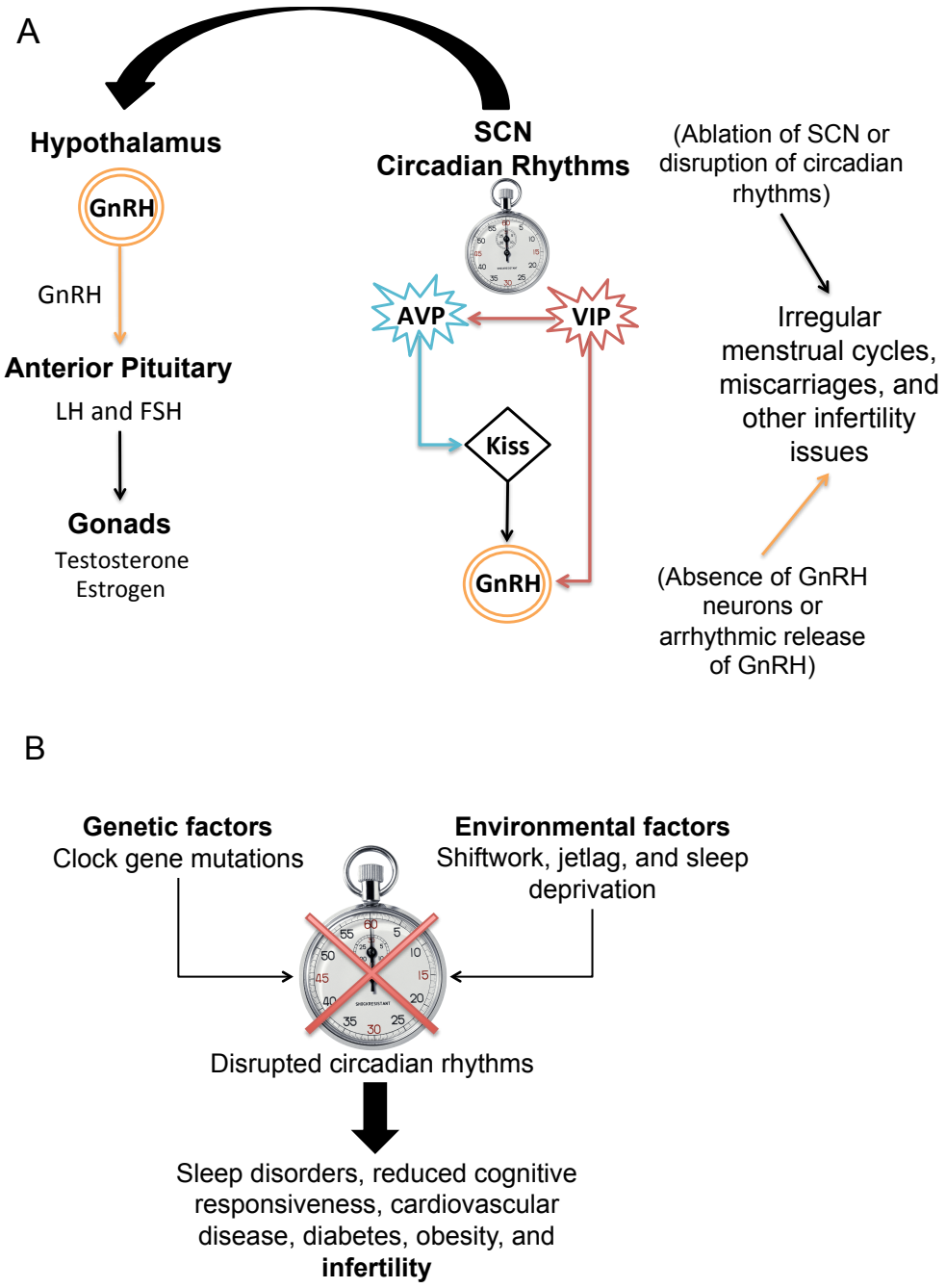

Figure 1. Fertility is highly regulated by the HPG axis and the SCN. (A) Schematic of the HPG axis. In the hypothalamus, the rhythmic release of GnRH from GnRH neurons (show in orange) triggers the pituitary to release of LH and FSH, which then target the gonads to release sex steroids. (B) Schematic of the cause and effects of disrupted circadian rhythms. Abbreviations: arginine vasopressin (AVP), vasoactive intestinal peptide (VIP), luteinizing hormone (LH), follicle-stimulating hormone (FSH), gonadotropin-releasing hormone (GnRH), kisspeptin (kiss), and suprachiasmatic nucleus (SCN). [1, 4].

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*^{tm1Grl} [7], here referred to as *Vax1* mice, were provided by Dr. Bharti (National Institutes of Health, Bethesda, MD). *Vax1* knock-out first mice were generated from the knock-out allele with conditional potential *Vax1*$^{tm1a(KOMP)Mbp}$ obtained from KOMP (UC Davis, Knock-Out Mouse Project, www.komp.org). *Vax1* knock-out first mice (*Vax1*$^{tm1a(KOMP)Mbp}$ mice) were crossed with a *flpase* mouse (http://jaxmice.jax.org/strain/003800.html) to generate a *Vax1* conditional knock-out allele, here referred to as *Vax1*^{flox} mice. *Vax1*^{flox} mice [8] were crossed with *GnRH*^{cre} [9] to generate *Vax1*^{flox}*:GnRH*^{cre} mice (*Vax1*^{GnRH}). For lineage tracing *Rosa-LacZ* [10] (http://jaxmice.jax.org/strain/003309.html) reporter mice were used. The *Vax1*$^{fl/fl}$*:Synapsin*^{cre} (*Vax1*^{Syn}) mice were generated in a similar fashion by crossing *Vax1*^{flox} mice with *Synapsin*^{cre} [11]. 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

Pubertal onset in male mice is observed by preputial separation, whereas vaginal opening is indicative of pubertal onset in female mice. Males and females were observed every day starting at approximately 23-25 days of age.

Determination of estrous cyclicity

To assess estrous cyclicity, vaginal smears were performed daily between 9-11 am on 3-5 month-old mice by vaginal lavage. Smears were collected on glass slides and counterstained with 0.1% of methylene blue (Spectrum, Gardena, CA). Cell type was observed through bright field microscopy to determine the corresponding stage of the estrous cycle.

Fertility assessment

At 12-15 weeks of age, virgin $Vax1^{flox}$ (control), $Vax1^{fl/fl}$:$GnRH^{cre}$ ($Vax1^{GnRH}$) and $Vax1^{fl/fl}$:$Synapsin^{cre}$ ($Vax1^{syn}$) mice were housed in pairs. The number of litters produced was recorded for 90 days ($Vax1^{GnRH}$) or 120 days ($Vax1^{syn}$).

Hormone levels and GnRH and Kisspeptin-10 challenges

Tail blood was collected from adult male and female metestrus/diestrus littermates between 10 AM and 12 PM. Ten min after receiving an i.p. injection of 1

µg/kg GnRH in physiological serum, or 20 min after receiving an i.p. injection of 3 mmoles kisspeptin-10 in sterile saline, a second collection of tail blood was performed. The total volume of blood collected did not exceed 100 µl. Blood was allowed to clot for 1 h at RT, then centrifuged (15 min, 2600 g). Serum was collected and stored at -20ºC before ELISA analysis of luteinizing hormone (LH) and follicle-stimulating hormone (FSH). Blood was collected between 9 am and 12 pm. Samples were run as singlet on MILIPLEX® (#MPTMAG-49K, Millipore, Baltimore). LH: lower detection limit: 5.6 pg/mL, intra-ACOV 15.2 and inter-ACOV 4.7%. FSH: lower detection limit: 24.9 pg/mL, intra-ACOV 13.7 and inter-ACOV 3.9%.

Immunohistochemistry.

Immunohistochemistry was performed as previously described [12], 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). Texas Red (Vector Labs) was used to counterstain sections as directed by manufactures instructions.

Collection of uterus, ovary, testis, and gonadal histology

Ovaries and uteri from diestrus females and testes from males were dissected and weighed from animals of 2.8-4 months of age. Ovaries and testes were fixed for 1.5 h at RT and O/N at 4ºC, respectively, in 60% ethanol, 30% formaldehyde, 10% glacial acetic acid. Gonads were paraffin embedded, serially sectioned at 10 μm, and stained with hematoxylin and eosin (H&E) (Sigma-Aldrich). Histology was examined and the number of corpora lutea in the ovaries recorded and presence of sperm in the testis assessed.

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 um, Falcon #352350) and sperm were diluted 1:10 with MQ 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: Deletion of Vax1 in GnRH Neurons Abolishes GnRH Expression Leading to Delayed Puberty, Hypogonadism, and Infertility

Introduction

Infertility can result from irregular or absent GnRH release, absence of GnRH neurons, or abnormal GnRH neuron migration during development [13]. Originating at the olfactory placode at embryonic day 11 (E11) in the mouse, GnRH neurons migrate into the brain through the cribriform plate to reach their final destination in the hypothalamus around E16 (Fig. 2) [14]. The migration of GnRH neurons is restricted to the ventral forebrain, where homeodomain transcription factors are expressed to direct the migration and maturation of GnRH neurons [12, 15-17]. Abnormal GnRH neuron development is often associated with a human disorder called idiopathic hypogonadotropic hypogonadism (IHH), which is characterized by delayed or absent sexual development, low sex steroid and gonadotropin levels, and reduced or complete loss of fertility [18]. The genetic causes of the majority of IHH cases remain unknown despite extensive research [19]. By studying the development of GnRH neurons, we hope to provide insight to the possible contributors to IHH.

Ventral anterior homeobox 1 (VAX1), a homeoprotein expressed in GnRH neurons, was recently identified by the Mellon lab as a significant regulator of GnRH neuron development, making it a promising contributor to IHH [12]. By examining the effects of the *Vax1* full body knock-out mouse (KO), Hoffmann et al. found that *Vax1* is not required for the generation of GnRH neurons, but is necessary for their development. At E13.5 (early GnRH neuron migration), *Vax1* full body knock-out (KO) mice had

normal GnRH neuron numbers when compared to the wild type (WT), demonstrating that *Vax1* is not required for the generation of GnRH neurons. However, at E17.5 (late GnRH neuron migration), *Vax1* KO had no GnRH neurons and *Vax1* heterozygous (HET) had a 50% reduction in GnRH neurons, implying that VAX1 has an effect on GnRH neuronal development and this effect is dose sensitive. Furthermore, it was identified that haploinsufficiency of *Vax1* lowers the number of GnRH neurons by >60% in adult mice, leading to subfertility of both male and female mice [12]. *Vax1* KO mice were not studied further into adulthood, because *Vax1* KO are neonatal lethal. Despite the reduced number of GnRH neurons in *Vax1* HET females, we found that these mice had higher than normal luteinizing hormone (LH) and estradiol levels in diestrus, leading to prolonged estrous cycles and reduced fertility. Furthermore, recent transfection studies with three model cells, immature GnRH cells GN11 [20], mature GnRH cells, GT1-7 [21], and mouse fibroblasts cells NIH3T3 showed that *Vax1* is only highly expressed in GT1-7 cells. Through transfection and luciferase assay, VAX1 is also determined to be a positive regulator of the *Gnrh1* promoter, by directly binding to ATTA sites of the promotor [8].

Also expressed in the ventral forebrain and eye [7], *Vax1* can potentially regulate the development of GnRH neurons from either its expression in cells surrounding migrating GnRH neurons or from its intracellular expression within the GnRH neurons. We hope that investigating the mechanism through which VAX1 affects the development of GnRH neurons will bring us closer to the genetic causes of IHH. To study the roles of VAX1 in GnRH neurons, we generated $Vax1^{fl/fl}$:$GnRH^{cre}$ ($Vax1^{GnRH}$) mice that have *Vax1* conditionally knocked out in GnRH neurons.

My hypothesis is that *Vax1* in GnRH neurons is required for normal GnRH neuronal development and the deletion of *Vax1* in GnRH neurons will lead to infertility.

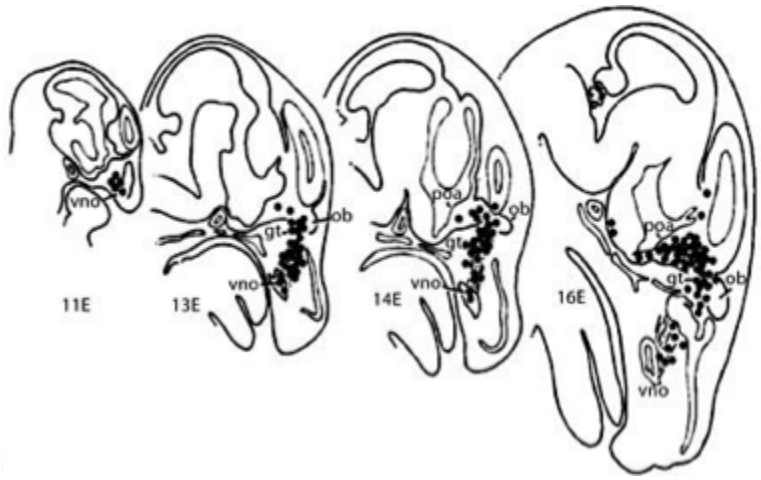

Figure 2. Migration of GnRH Neurons During Embryogenesis. GnRH neurons (represented by the black dots) originate in the olfactory placode region Vomeronasal Organ VNO at embryonic day 11 (E11) in the mouse, GnRH neurons then migrate into the brain through the cribriform plate to reach their final destination in the preoptic area (POA) of the hypothalamus around E16. Abbreviations: embryonic day (E), vomernasal organ (vno), preoptic area (poa), olfactory bulb (ob), and ganglion terminale (gt). [14].

Results

Vax1 in GnRH neurons is required for GnRH expression, but not for the survival of GnRH neurons.

To characterize the role of *Vax1* in GnRH neuronal development, we deleted *Vax1* specifically in GnRH neurons, using a conditional knockout (cKO) mouse model, $Vax1^{fl/fl}$:$GnRH^{cre}$ ($Vax1^{GnRH}$). Immunohistochemistry (IHC) for the presence of GnRH in

the hypothalamus and median eminence was performed on adult *Vax1*^{GnRH} cKO and the WT control to assess how the deletion of *Vax1* affects GnRH expression. The number of GnRH expressing neurons present in adult mice was determined and the *Vax1*^{GnRH} cKO had a significantly lower count than the control group. The entire brain of the *Vax1*^{GnRH} cKO only had a couple of GnRH expressing neurons, leading to a 99% reduction when compared to the WT (Fig. 3). The same trend was seen in both sexes, therefore there are no significant differences between males and females (data not shown).

The reduction of GnRH-expressing neurons can be caused by the death of GnRH neurons or the inability of these neurons to synthesize GnRH. As the only specific marker of GnRH neurons is GnRH, in order to determine whether VAX1 in GnRH neurons is required for the survival of these neurons or the expression of GnRH, we performed lineage tracing in *Vax1*$^{fl/fl}$*:GnRH*^{cre}*:RosaLacZ* adult mice (*Vax1*$^{GnRH:LacZ}$). The expression of LacZ, a reporter gene, is activated by recombination by the Cre driven from the GnRH promoter and therefore will indicate whether GnRH neurons are alive or not. I found that the *Vax1*$^{GnRH:LacZ}$ cKO adult mice had the same number of LacZ expressing neurons as the control group in both sexes, indicating the presence and survival of GnRH neurons in the *Vax1*^{GnRH} cKO mice (Fig. 4).

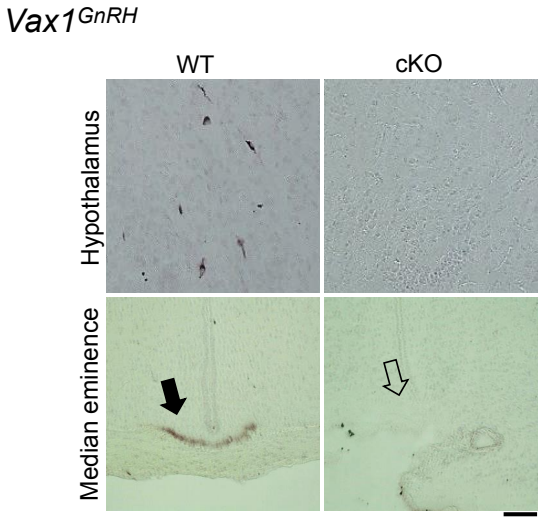

Figure 3. Vax1^{GnRH} cKO Mice Have 99% Loss of GnRH. The entire brain of adult male Vax1^{GnRH} WT and cKO were stained using immunohistochemistry (IHC) for GnRH. The black arrow indicates where GnRH staining is expected to be in the median eminence. The scale indicates 100 µm. [8].

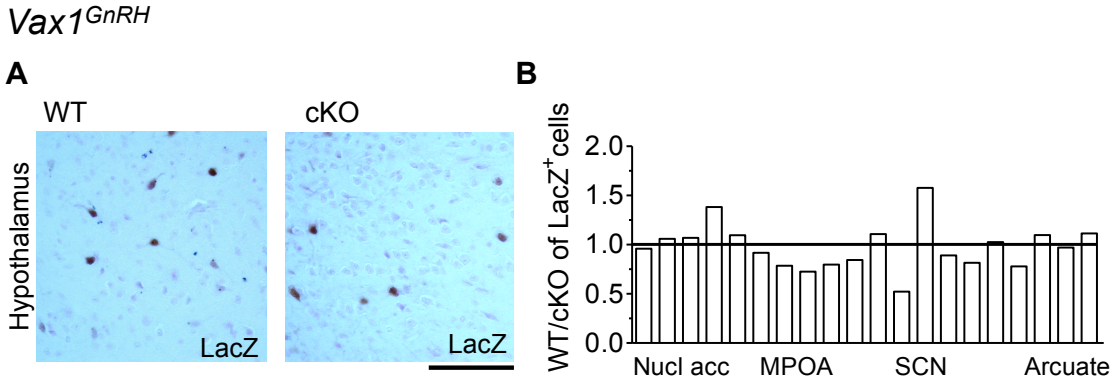

Figure 4. Vax1$^{GnRH:LacZ}$ WT and cKO Have the Same Number of LacZ Expressing Cells. (A) Lineage tracing of adult female Vax1$^{GnRH:LacZ}$ was performed by IHC staining for the LacZ reporter, which is driven by a GnRH promoter. The scale indicates 100 µm. (B) The number of LacZ expressing cells in the WT is compared to that of the cKO in the following brain areas that we would expect to see GnRH neurons: nucleus accumbens (nucl acc), medial preoptic area (MPOA), and supreachiasmatic nucleus (SCN). [8].

Vax1 in GnRH neurons is required for normal fertility

The absence of *Vax1* in GnRH neurons stops the expression of GnRH, which is required for the release of gonadotropins, pubertal onset, and normal fertility. To determine the degree to which *Vax1* in GnRH neurons affects fertility, we first examined pubertal onset by checking for preputial separation in males and vaginal opening in females. We found that adult $Vax1^{GnRH}$ cKO males have delayed puberty as well as underdeveloped gonads (Fig. 5A & B). The $Vax1^{GnRH}$ cKO males exhibited preputial separation at 56.67±9.60 days of age, while the WT littermates had preputial separation by 30.43±0.75 days of age (One way ANOVA, p=0.0002, n=3-7) (data not shown). This is an expected phenotype, because $Vax1^{GnRH}$ cKO mice have a disrupted HPG axis due to the significantly low levels of GnRH, leading to low levels of LH and FSH in the blood (see below).

$Vax1^{GnRH}$ cKO females had delayed vaginal opening, which is an external marker for pubertal onset (Fig. 6A). Vaginal opening was not seen in the $Vax1^{GnRH}$ cKO females until 49.6±5.20 days of age, while the control group had vaginal opening at 29.29±0.99 days of age (One way ANOVA, p=0.00041, n=3-7) (data not shown). $Vax1^{GnRH}$ females also had underdeveloped gonads and uterus (Fig. 6B). Similar to the males, the conditional knock females also had low LH and FSH levels (Fig. 6E). In females, LH is important for ovulation and FSH is essential for follicular development. To determine whether low levels of LH interfered with the estrous cycle and ovulation of $Vax1^{GnRH}$ cKO females, we collected and analyzed vaginal smears for 25 days. Instead of the human menstrual cycle, mice have estrous cycles consisting of 4 stages: diestrus, proestrus, estrus, and metestrus (Fig. 6C). Between the proestrus and estrus stage, a

GnRH dependent LH surge occurs to stimulate ovulation, therefore it is not surprisingly that the *Vax1*^{GnRH} cKO females have disrupted estrous cycling. The average estrous cycle for the control WT female is 5.95±0.31 days (n=7), however, in the *Vax1*^{GnRH} cKO (n=6), we only observed the diestrus stage for the 25 days we recorded, showing that the *Vax1*^{GnRH} cKO remain stuck in diestrus (Fig. 6C &D).

A fertility assessment was then conducted to confirm whether *Vax1*^{GnRH} cKO mice are truly infertile as expected from their hypogonadism. No litters were produced from either *Vax1*^{GnRH} cKO males or females after 90 days in comparison to the WT mice that produced an average of 2.25±0.48 litters in the same time frame (n=3-4). These phenotypes collectively contribute to the *Vax1*^{GnRH} cKO being completely infertile and suggest that VAX1 in GnRH neurons is required for normal fertility.

To further demonstrate that hypogonadism and infertility are caused by the lack of GnRH release in *Vax1*^{GnRH} cKO, kisspeptin and GnRH challenges were performed. In the HPG axis, kisspeptin neurons stimulate GnRH neurons to release GnRH, which then stimulates the pituitary to release LH and FSH (Fig. 2). If GnRH neurons are unable to release GnRH, then injecting *Vax1*^{GnRH} cKO mice with kisspeptin should not elicit a response from the pituitary. Instead, if GnRH is injected in *Vax1*^{GnRH} cKO mice, then there should be an increase in LH, because the pituitary should still be functioning normally. Experimental results show that kisspeptin challenges indeed did not increase LH in either male or female *Vax1*^{GnRH} WT and cKO (Fig. 5D & 6F). However, GnRH challenges did increase LH in male and female *Vax1*^{GnRH} cKO when compared to the WT control group, confirming that the pituitary is able to respond to a GnRH challenge (Fig. 5D & 6F). The same studies were performed in *Vax1*^{GnRH} conditional heterozygous

(cHET) males and females to determine whether the effect of *Vax1* is dose dependent. The *Vax1*^{GnRH} cHET mice are comparable to the WT in all of the studies. Overall, our data revealed that the deletion of *Vax1* in GnRH neurons abolishes GnRH release, which leads to delayed puberty, hypogonadism, and infertility in mice.

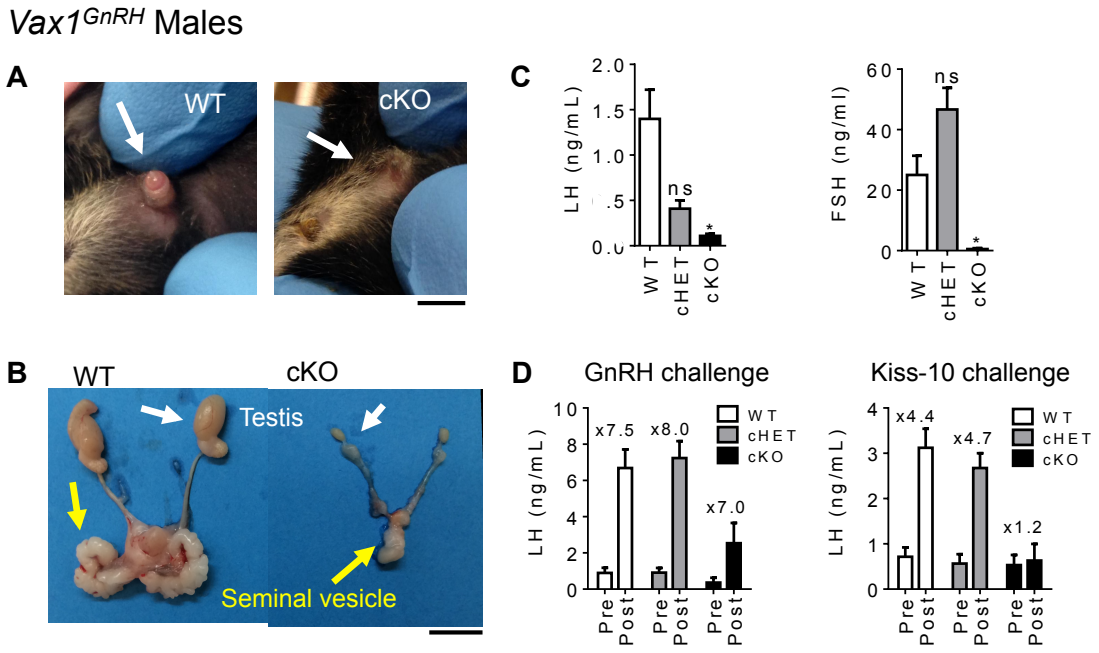

Figure 5. Vax1^{GnRH} cKO Males Have Delayed Puberty, Hypogonadism, and Low Levels of Gonadotropins. (A) Images of male Vax1^{GnRH} WT and cKO at 6 weeks of age. The WT has a well-defined penis and preputial separation, while the cKO male has a micropenis and delayed preputial separation, which is an indication of delayed puberty onset. Scale indicates 0.5 cm. (B) Testes of adult WT and cKO mice. Scale indicates 0.5 cm. (C) Plasma levels of LH and FSH (n=4-8). One-Way ANOVA as compared to control; *p<0.05. Data of the conditional heterozygous (cHET) mice are also shown. (D) Plasma levels of LH before and after challenges of either GnRH or Kiss-10. The numbers above the histogram represents fold change. [8].

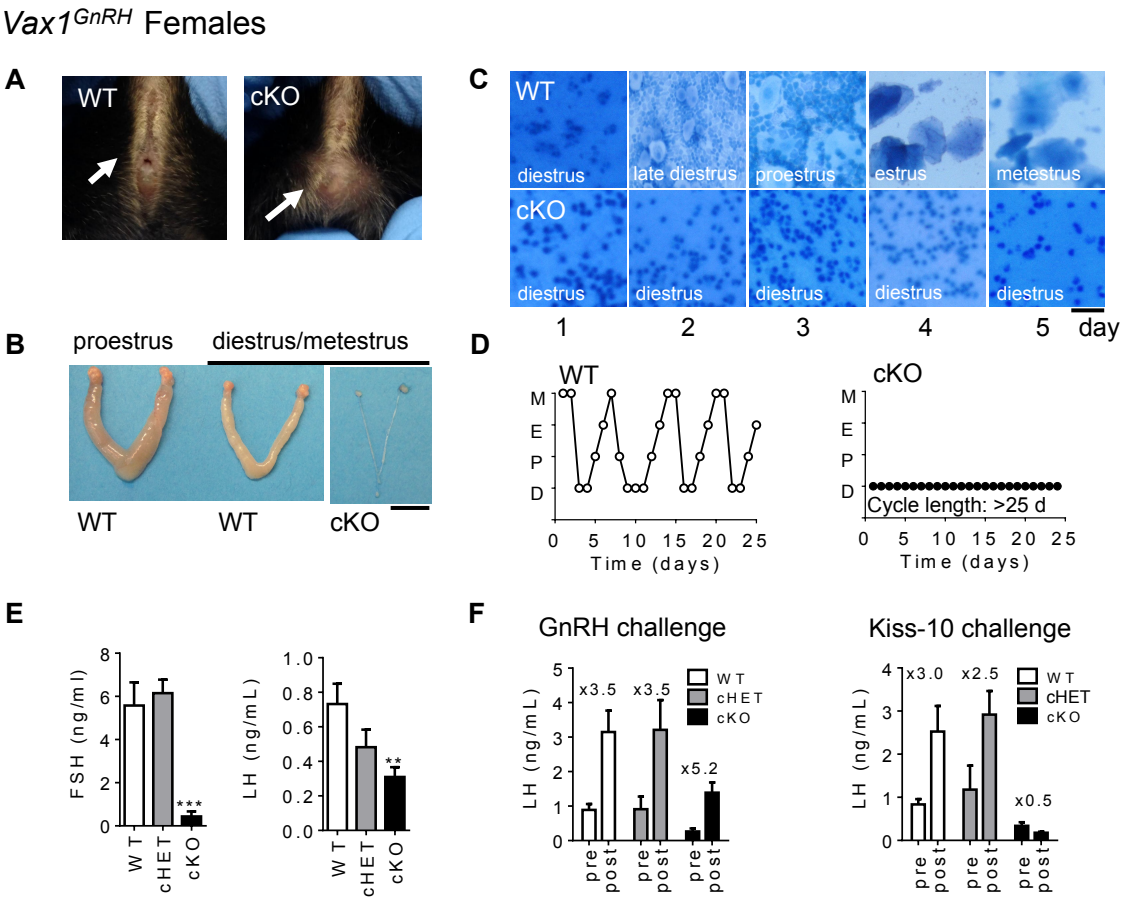

Figure 6. Vax1^{GnRH} cKO Females Have Delayed Puberty, Hypogonadism, and Low Levels of Gonadotropins. (A) Puberty onset in female mice is determined by vaginal opening. The images are of mice at 6 weeks of age. The white arrow indicates where vaginal opening is expected to be. (B) The uterus and intact ovaries were collected from proestrus (control) and diestrus females at 25 weeks of age. (C & D) Estrus cycling was monitored daily in adult WT and cKO females of 12 to 20 weeks old. The vaginal smears were stained to visual cell morphology. Abbreviations: M, metestrus; E, estrus; P, proestrus; D, diestrus. (E & F) Diestrus plasma levels of LH before and after challenges of either GnRH or Kisspeptin. One-Way ANOVA, **p<0.01; ***p<0.001. Numbers over the histograms indicate fold change after GnRH or Kiss-10 challenge. n=6-8. Data of the conditional heterozygous (cHET) mice are also shown. [8].

Discussion

To provide insight into the genetic causes of a human fertility disorder called idiopathic hypogonadotropic hypogonadism (IHH), we investigated the roles of a homeoprotein called Ventral Anterior Homeobox (VAX1) in gonadotropin-releasing hormone (GnRH) neuron maturation. IHH is associated with abnormal development of GnRH neurons, making VAX1 a promising contributor to IHH. VAX1 has been shown to directly regulate the GnRH promoter and its absence impairs fertility [8]. Hoffman, et al. demonstrated that full body knockout of *Vax1* also disrupts GnRH expression from E13.5 to E17.5 and this effect is dose sensitive. Interestingly, *Vax1* is not required for the generation of GnRH neurons, but is necessary for the correct expression of GnRH from GnRH neurons [8]. Infertility originating at the level of hypothalamic GnRH neurons is generally caused by the absences of GnRH neurons, abnormal development of GnRH neurons, or arrhythmic GnRH release [13]. To understand the effects of *Vax1* specifically in GnRH neurons, a conditional knock mouse model, $Vax1^{fl/fl}:GnRH^{cre}$ ($Vax1^{GnRH}$) was generated. I postulated that VAX1 in GnRH neurons is required for both normal GnRH neuronal development and fertility.

In our study, we found that *Vax1* in GnRH neurons is required for the expression of GnRH. The $Vax1^{GnRH}$ cKO mice had a significant decrease in GnRH expression when compared to the WT, leading to their impaired fertility. After revealing that deletion of *Vax1* in GnRH neurons eliminates GnRH expression in both adult male and female mice, we sought to determine whether this effect is actually due to the inability of GnRH neurons to secrete GnRH or due to the abnormal death of these neurons. Lineage tracing was performed in the $Vax1^{fl/fl}:GnRH^{cre}:RosaLacZ$ ($Vax1^{GnRH:LacZ}$) mouse model to study

the fate of GnRH neurons. The LacZ reporter gene, which is activated by the GnRH promoter, will allow us to detect the presence of GnRH neurons by staining for the LacZ tracer [8]. IHC staining for LacZ revealed there was no significant difference in the number of LacZ expressing GnRH neurons between the *Vax1*$^{GnRH:LacZ}$ WT and cKO groups, showing that *Vax1* in GnRH neurons is not required for their survival, but is crucial to maintain GnRH expression. Adding to our understanding of the effects of VAX1, we now know that VAX1 is not required for the generation of GnRH neurons [8, 12]. After the GnRH neurons have correctly reached their final destination in the hypothalamus, *Vax1* is then necessary for GnRH expression in adulthood. This is, to our knowledge, the first report of a transcription factor specifically being required for maintained GnRH expression. Other mouse models with transcription factors deleted in GnRH neurons as for example *Otx2* KO [17] or *Six6* KO [15] (and unpublished observations from the Mellon lab) showed that both *Otx2* and *Six6* were required for GnRH neuron survival. Thus, the role of VAX1 in GnRH neurons is distinct from these other two transcription factors, and provides us with a valuable tool to identify the transcriptional program required to maintain GnRH expression in the future.

The expression and rhythmic release of GnRH is essential to the maintenance of fertility in mice as well as humans [22]. Through the hypothalamic pituitary gonadal (HPG) axis, the GnRH released from the hypothalamus will induce the pituitary to release sex hormones, luteinizing hormone (LH) and follicle-stimulating hormone (FSH) (Fig. 1). These sex hormones will then stimulate the gonads to either secrete testosterone or estrogen. With a 99% reduction in GnRH, the *Vax1*^{GnRH} cKO are expected to be infertile. To provide further evidence that the deletion of VAX1 in GnRH neurons leads

to infertility, we found that both *Vax1*^{GnRH} cKO males and females have delayed pubertal onset and hypogonads. The cKO females also displayed arrhythmic estrous cycles, with prolonged cycles that seem to remain stuck in diestrus. Disrupted estrous cycles, which are analogous to menstrual cycles in humans, will contribute to the infertility of *Vax1*^{GnRH} cKO females. These phenotypes in both sexes of *Vax1*^{GnRH} cKO are all expected and most importantly, are in agreement with our previous finding that *Vax1* is required for the expression of GnRH [8, 12], as well as the known role of GnRH in maintaining fertility [23, 24].

The rhythmic release of GnRH from hypothalamic GnRH neurons stimulates the anterior pituitary to release two crucial sex hormones, LH and FSH (Fig. 1). LH is crucial for testosterone levels in males and ovulation in females. On the other hand, FSH facilitates the development of sperm in males and eggs in females [25]. Not surprisingly, the LH and FSH levels in *Vax1*^{GnRH} cKO were found to be significantly lower than that of the WT, because *Vax1*^{GnRH} cKO lack expression of GnRH. The low LH level in *Vax1*^{GnRH} cKO females suggests that *Vax1*^{GnRH} cKO females have abnormal ovulation [26] and supports our finding that *Vax1*^{GnRH} cKO females also have prolonged estrous cycles.

In the hypothalamus, kisspeptin neurons also influences fertility by releasing kisspeptin onto GnRH neurons, stimulating the release of GnRH [27]. To confirm that the abnormal fertility characteristics of *Vax1*^{GnRH} cKO truly arise at the level of GnRH neurons, *Vax1*^{GnRH} cKO were injected with kisspeptin and GnRH. LH and FSH levels remain low in *Vax1*^{GnRH} cKO after kisspeptin injections, showing that the GnRH neurons were unable to stimulate LH in response to kisspeptin. On the other hand, LH and FSH levels were elevated after the GnRH injections, confirming that the pituitary is still intact

and that the reproductive incompetence in *Vax1*^{GnRH} cKO is due to the inability of GnRH neurons to release GnRH.

In summary, we have identified VAX1 in GnRH neurons as a key regulator of GnRH neuron development and fertility. These findings bring us one step closer to unraveling the intricate regulation of fertility and discovering the genetic causes of IHH.

Chapter I, in full, has been published and the material can be found in Hoffman, et al. 2016. I would like to thank the following co-authors for their instrumental contributions: Hanne M. Hoffmann, Ping Gong, Ikuo Kimura, Erica C. Pandolfi and Pamela L. Mellon. The thesis author was the second author of this paper.

CHAPTER II: Deletion of Vax1 in the Mature SCN Impairs Female Fertility Due to a Weak SCN Output

Introduction

The suprachiasmatic nucleus (SCN), an endogenous master clock located in the hypothalamus, generates ~24 h rhythms that maintain homeostasis and coordinate our sleep wake cycles. Reproduction is heavily dependent on circadian rhythms, especially in female fertility [28, 29]. Previous studies have shown that the ablation of the SCN eliminates the pre-ovulatory luteinizing hormone (LH) surge in female rats [30]. The LH surge is required for ovulation in rodents and humans and therefore disrupted circadian rhythms will lead to infertility [31]. The SCN controls fertility via the hypothalamic-pituitary-gonadal (HPG) axis, but the exact mechanism remains unknown (Fig. 1). We know that there are two major populations of neurons in the SCN that either release vasoactive intestinal peptide (VIP) or arginine vasopressin (AVP). The VIP-expressing SCN neurons are located at the ventral part of the SCN and receive direct light input from the eye through the retinohypothalamic tract [32]. The VIP-expressing SCN neurons project to the dorsal part of the SCN and activate AVP-expressing neurons. VIP neurons also projects to and activate GnRH neurons [5]. On the other hand, the AVP-expressing SCN neurons project to and activate kisspeptin neurons [33]. In the hypothalamus, kisspeptin neurons stimulate GnRH neurons to secrete GnRH in a pulsatile fashion, which then stimulates the anterior pituitary to release sex hormones, LH and FSH (Fig. 1).

The development of the SCN and GnRH neurons share many similar characteristics in that they both depend on homeodomain transcription factors, such as Ventral Anterior Homeobox (*Vax1)*. In Chapter I, the first part of our project provided evidence that *Vax1* is required for GnRH neuronal development [8, 12]. The conditional knockdown of VAX1 in GnRH neurons abolished expression of GnRH by 99%, leading to abnormal reproductive development, such as delayed pubertal onset, hypogonadism, and disrupted estrous cycles [8, 12]. We now seek to address the role of VAX1 after development, which has never been studied before.

In adulthood, *Vax1* is expressed almost exclusively in the SCN (Fig. 7A). Our preliminary data with *Vax1* full body knockout (*Vax1* KO) at P0 confirm that *Vax1* is required for SCN formation. Through H&E staining, no SCN morphology was found in *Vax1* KO at P0 (Fig. 7B). *Vax1* KO mice also have no *Avp* expression in the SCN, a peptide required for normal SCN function. In addition, *Vax1* KO lacks the mRNA expression of SCN *Six3*, which is a transcription factor important for SCN development and function [34]. Through luciferase transfections with the mature GnRH cell line (GT1-7), *Vax1* is found to regulate transcription of two key clock genes required for generation of a 24-h rhythm, the transcription factors period 1 (Per1) and brain muscle ARNT-like (BMAL) (Fig. 7C) [31].

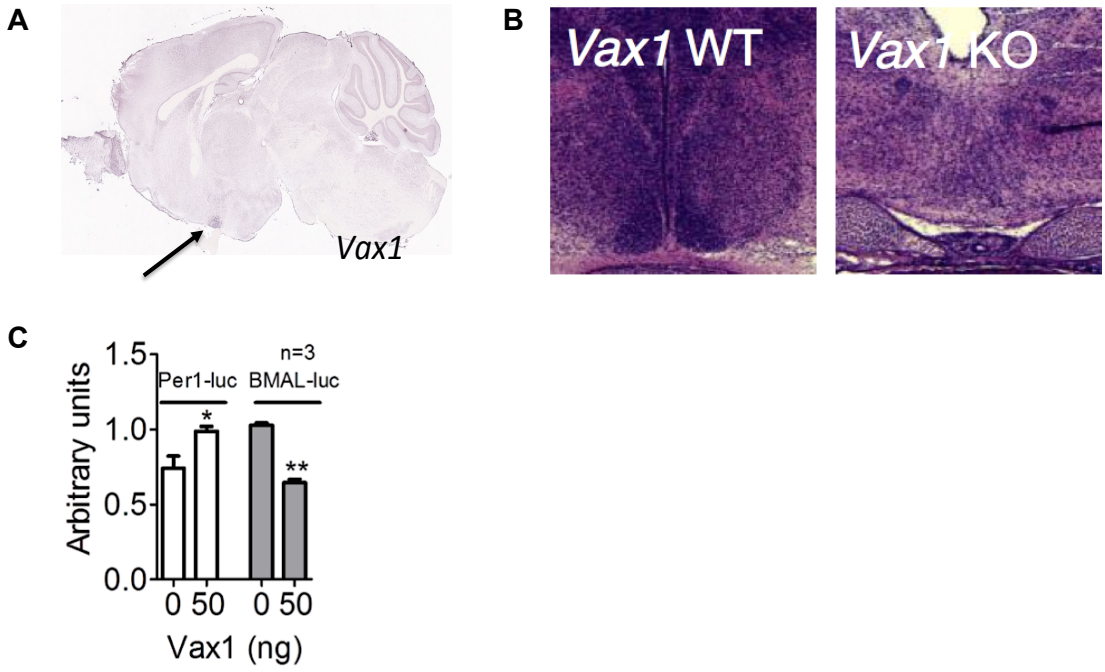

Figure 7. Expression of *Vax1* in Adulthood and Its Effect on SCN Development. (A) In situ hybridization of *Vax1* RNA in adult male mice brain. Black arrow indicates Vax1 expression in the SCN (www.brain-map.org). (B) H&E staining of the brain of *Vax1* full body knockout and control at P0. (C) The mature GnRH cell line, GT1-7, was transiently transfected with or without Vax1 and the Per1 or Bmal promoter driving the expression of luciferase. Statistical analysis was done by Student's t-test as compared to 0ng of Vax1. *, p>0.05; **, p>0.01.

The role of VAX1 in the SCN has not yet been investigated. For that reason and because *Vax1* full body KO mice die shortly after birth, the Mellon laboratory developed the $Vax1^{fl/fl}$:$Synapsin^{cre}$ ($Vax1^{syn}$) model that allows for the specific knockdown of *Vax1* in the mature SCN to study the effects of *Vax1* in adulthood. Preliminary IHC data from lineage tracing of LacZ expression in the $Synapsin^{cre}$:*RosaLacZ* mice show that $synapsin^{cre}$ targets the SCN, but not GnRH neurons, as evidenced by LacZ-positive cells in the SCN, but an absence of colocalization of GnRH and LacZ (Fig. 8). To evaluate the circadian behavior and output of $Vax1^{syn}$ cKO, wheel running activities were performed in 12 hours of light and dark cycles (LD12:12) and then in constant darkness (DD). In

LD12:12, *Vax1*^{syn} WT and cKO were both able to entrain to light, which is demonstrated by a 24 hours free-running period. When switched to constant darkness, both *Vax1*^{syn} WT and cKO mice have comparable free running periods of <24 hours, showing *Vax1*^{syn} cKO had a normal free-running period in these conditions (Fig. 9). However, *Vax1*^{syn} cKO mice did show more activity bouts in constant darkness (DD), a behavior indicative of abnormal SCN function and output (Fig. 9).

My hypothesis is that deletion of *Vax1* from the mature SCN impairs female fertility due to a weak SCN output.

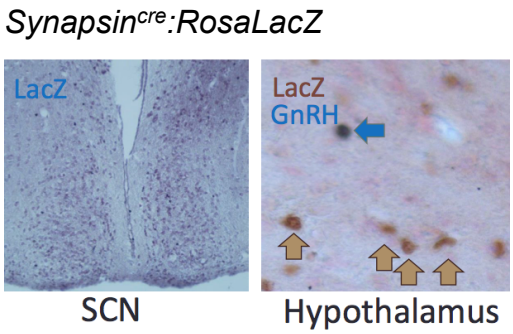

Figure 8. *Synapsin*^{cre} Targets the SCN and not GnRH Neurons. Immunohistochemistry of LacZ and GnRH in *synapsin*^{cre}*:RosaLacZ* adult brain. The orange arrow shows LacZ staining and the blue arrow shows GnRH staining.

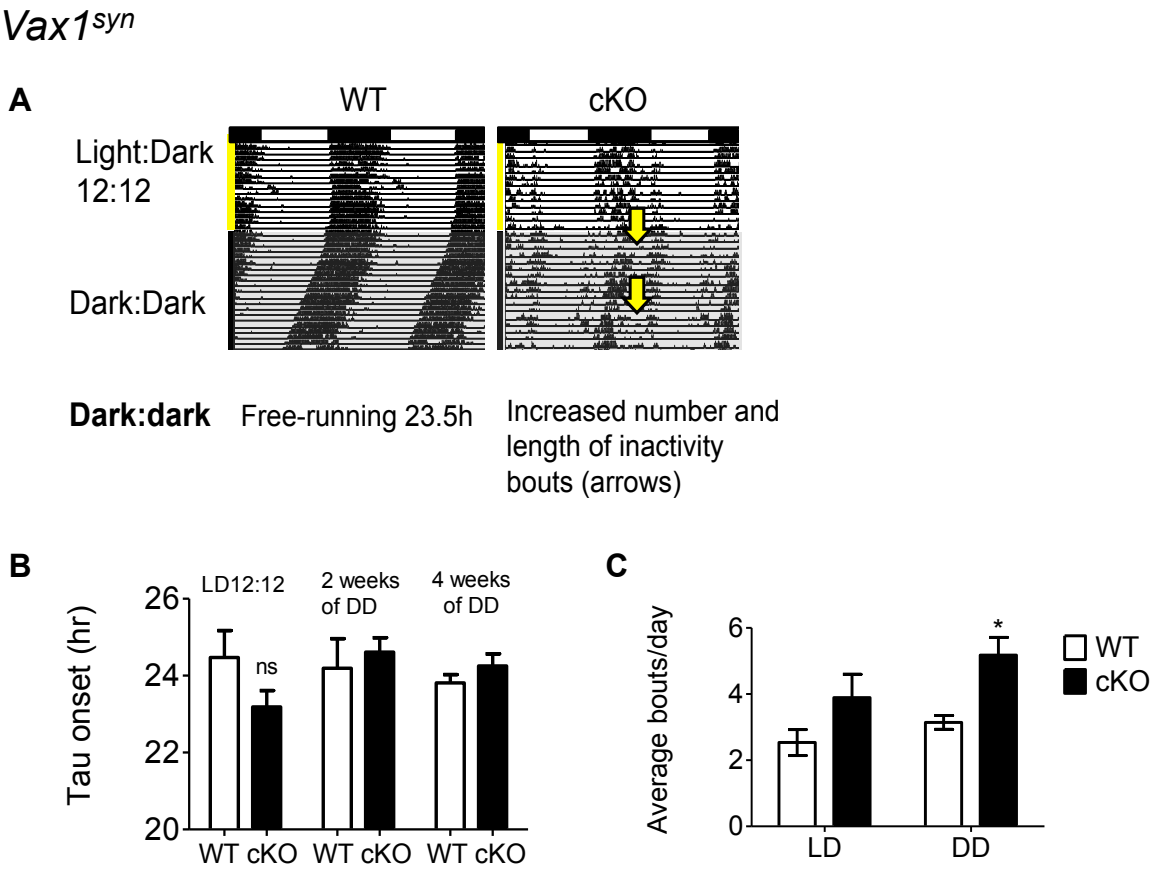

Figure 9. *Vax1*^{syn} cKO Have Disrupted Wheel Running Activity. (A) Actogram of Vax1^{syn} control and cKO in LD12:12 and constant darkness (dark:dark). Yellow arrows indicate activity bouts. (B) Histogram of the free running period of the SCN (tau) of WT and cKO in LD12:12, and 2 weeks and 4 weeks of dark:dark. Data analyzed by two-way Anova. (C) Histogram of average bouts per day in LD12:12 and constant darkness. Data analyzed by two-way Anova.

Results

Vax1 in mature SCN neurons is required for normal SCN function

To further our understanding of how the suprachiasmatic nucleus (SCN) maintains fertility, we investigated the impact of the deletion of *Vax1* in mature SCN neurons. To delete *Vax1* from mature neurons, we generated *Vax1*$^{flox/flox}$*:synapsin*^{cre}

(*Vax1*^{syn}). As *Vax1* and *synapsin*^{cre} only overlap in the SCN in the mature brain, this allowed us to specifically delete *Vax1* in the mature SCN. We know that SCN VIP neurons can directly stimulate GnRH neurons (Fig. 1). Immunohistochemistry for VIP was performed in the SCN area of the brain in both the *Vax1*^{syn} WT and cKO. A dramatic decrease in staining of VIP can be observed in the *Vax1*^{syn} cKO when compared to the WT (Fig. 10).

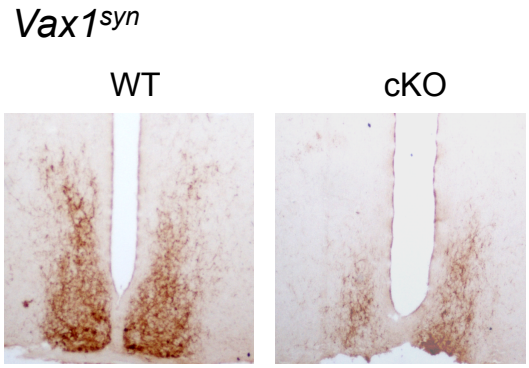

Figure 10. Adult *Vax1*^{syn} cKO Have Decreased VIP Expression in the SCN. Immunohistochemistry of VIP in adult male *Vax1*^{syn} WT and cKO.

Deletion of VAX1 in mature SCN neurons specifically impairs female fertility

Knowing that fertility is highly regulated and maintained by circadian rhythms from the SCN (Fig. 1), we determined whether the deletion of VAX1 in the mature SCN would lead to disrupted fertility. We first characterized whether the *Vax1*^{syn} cKO mice were fertile by determining the number of litters produced in 120 days. The *Vax1*^{syn} WT had an average of 3 litters within 120 days. The *Vax1*^{syn} cKO males had a comparable number of litters as the control (Fig. 11A). However, the *Vax1*^{syn} cKO females had

significantly fewer litters, averaging about only 1 litter in 120 days (Fig. 11A). We then asked whether this phenotype seen in the *Vax1*^{syn} cKO females is due to a delayed time to first litter when compared to the control and *Vax1*^{syn} cKO males. Interestingly, the control and the *Vax1*^{syn} cKO males and females had no significantly different time to first litter (Fig. 11B). It should be noted however, that it appeared we had a semi-penetrant phenotype as three *Vax1*^{syn} cKO females had litters at a comparable time point to WT, whereas three other *Vax1*^{syn} cKO females had a delay to first litter, or did not generate any litters in 120 days. We next analyzed litter size, because the number of pups in a litter is important, in addition to litter number, in determining reproductive competence. We found that the control and *Vax1*^{syn} cKO males and females had approximately the same litter size across three litters (Fig. 11C). These data taken all together suggest that the deletion of VAX1 in the SCN only disrupts female reproduction, leading to subfertility.

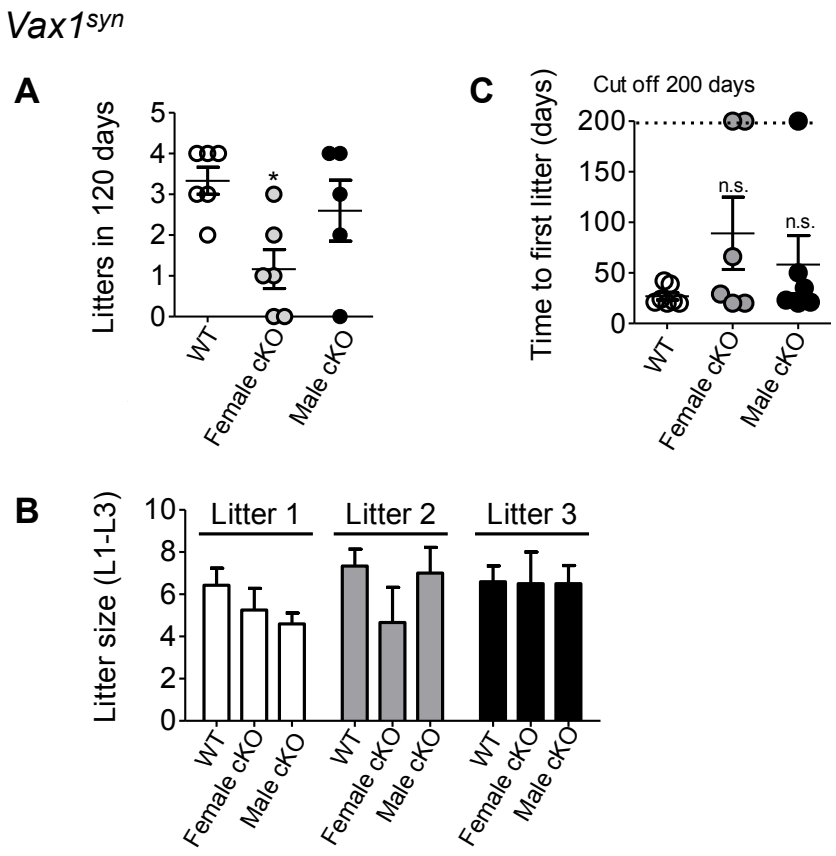

Figure 11. *Vax1*^{syn} cKO Females Have Fewer Litters, but Normal Time to First Litter and Litter Size. (A) Litters in 120 days were analyzed by one-way Anova (Krushkal-Wallis test), followed by post hoc Dunns. N=6-7. The *Vax1*^{syn} cKO females have significantly fewer litters than the control ($P<0.05$). There is no statistical difference between the WT and *Vax1*^{syn} cKO females. (B) Time to first litter was analyzed by one-way Anova (Krushkal-Wallis test), followed by post hoc Dunns. No significance was found. Two hundred days was used as a cut off for analysis. N=6-7. (C) Litter size was analyzed by two-way Anova, n=2-7. There were fewer animals in litter 3, because not all mice had 3 litters. All of the female cKO and male cKO matings were set up with WT counterparts.

Vax1^{syn} *cKO females have prolonged estrous cycle, but normal numbers of corpora lutea*

The fertility of *Vax1*^{syn} cKO females was furthered investigated to confirm the subfertility phenotype seen in the reduced number of litters in 120 days (Fig. 11A). The estrous cycle of the females was observed for 25 days. The estrous cycle of mice is

analogous to the menstrual cycle of in human females and they are both highly dependent on circadian rhythms. The *Vax1*^{syn} control had cycles that average to about 5.6 ± 0.3 days (Fig. 12A), while the *Vax1*^{syn} cKO had prolonged cycles averaging to about 10.3 ± 1.2 days (Fig. 12B). The percent of time spent in each stage of the estrous cycle within the 25 days is not statistically significant between the control and *Vax1*^{syn} *cKO* (Fig. 12C). To determine if the prolonged estrous cycles affected ovulation we counted corpora lutea in the ovary. The corpus luteum is the endocrine structure that remains in the ovary after the ovum has been released [35] and therefore can be used as an indication of ovulation. Through staining of the ovaries, we found that *Vax1*^{syn} *cKO* have comparable numbers of corpora lutea as the WT (Fig. 12E).

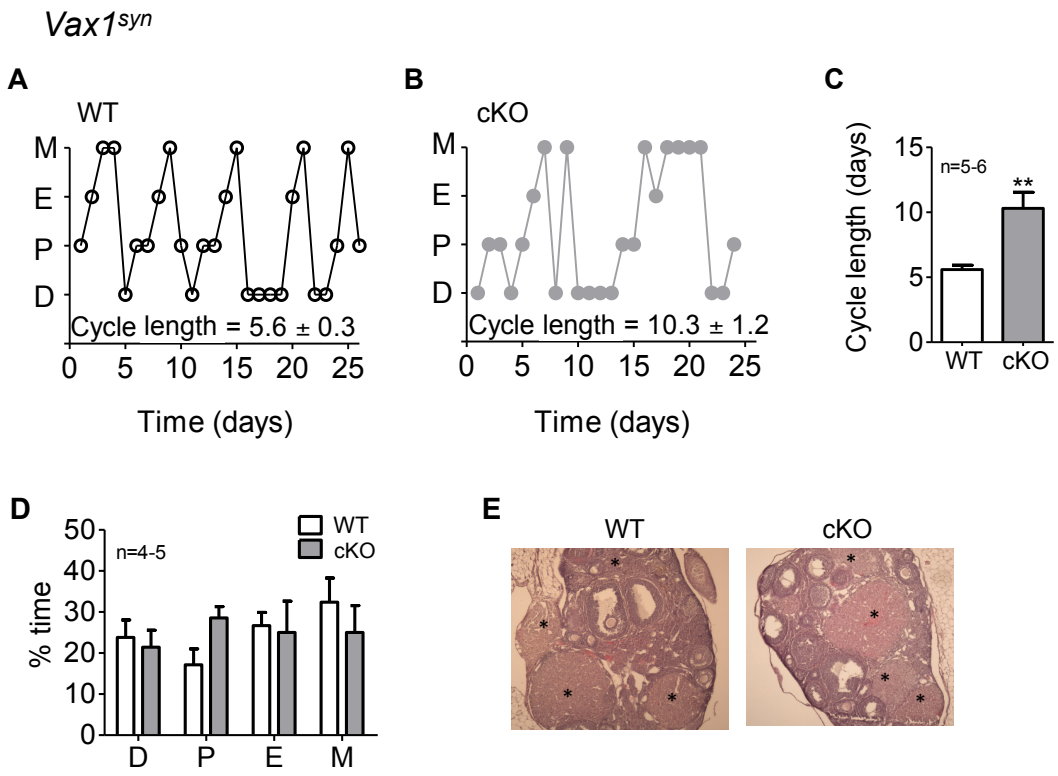

Figure 12. *Vax1*^{syn} cKO Females Have Prolonged Estrous Cycle, but Normal Number of Corpora Lutea. (A) Estrous cycle of adult control females (12 to 20 weeks old) over 25 days. (B) Estrous cycle of adult *Vax1*^{syn} cKO (12 to 20 weeks old) over 25 days. (C) Quantification of the cycle length, which was analyzed by student's t test. P<0.01, n=5-6. (D) Percent of time spent in each stage within 25 days was analyzed with two-way Anova of repeated measures. No significance was found. (E) H&E staining of adult WT and *Vax1*^{syn} cKO (12 to 20 weeks old) ovaries. The corpora lutea are labeled with asterisks. Abbreviations: Diestrus (D), proestrus (P), estrus (E), metestrus (M).

Vax1^{syn} *cKO males have normal fertility*

To determine how the deletion of VAX1 in the SCN of males impacted their gonads, the morphology of the testes, sperm count, and motility of the *Vax1*^{syn} cKO were analyzed. Through H&E staining, the testes of the WT and *Vax1*^{syn} cKO were comparable, suggesting that the testes of the *Vax1*^{syn} cKO matured normally. We further confirmed their reproductive competence by analyzing sperm motility and total count.

Not surprisingly, the control and cKO both had approximately 20 million/mL total sperm and about 40% of those sperm were motile, which is comparable to WT.

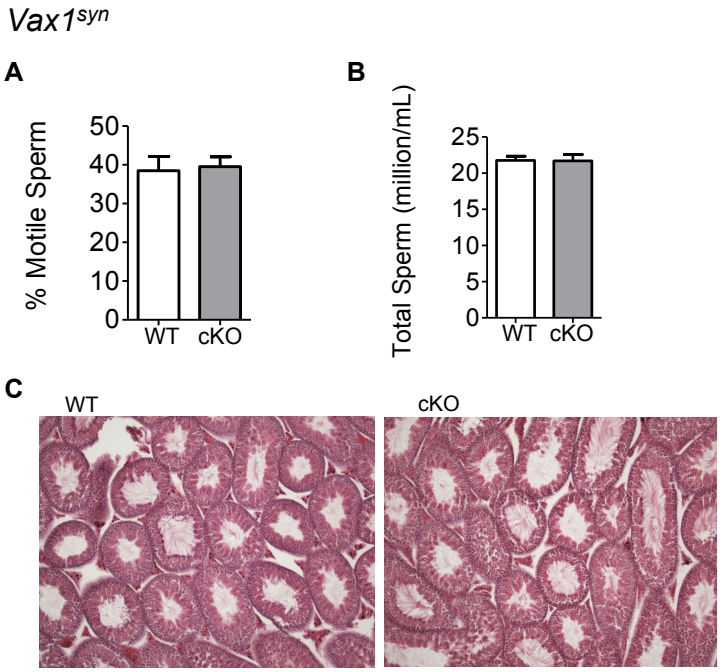

Figure 13. *Vax1*^{syn} cKO Males Have Normal Sperm Count and Motility and Testes Morphology. (A) The percent of motile sperms from the total sperms present in *Vax1*^{syn} WT and cKO. Data was analyzed by student's t-test. No significance was found. (B) Total sperm was counted in the control and cKO. Data was analyzed by student's t-test. No significance was found. (C) H&E staining of control and cKO testes.

Discussion

Fertility in humans and mice is orchestrated and maintained by circadian rhythms generated by the suprachiasmatic nucleus (SCN), uterus, and ovaries [36, 37]. Although we know that normal SCN function is necessary for fertility, the exact mechanism through which circadian rhythms control fertility remains unknown. Studies have shown that the ablation of the SCN in rats abolishes the preovulatory LH surge, leading to infertility [38, 39]. These studies have provided an experimental model to study the role

of the SCN in rodents. However, the precise lesion of this small hypothalamic structure is challenging, because the removal of the SCN might also cause collateral damage to nearby structures, especially those that have projections from the SCN, such as GnRH and kisspeptin neurons [5, 33, 40]. To provide insight into the complex regulation of fertility by the SCN, we sought to investigate the effects of a novel transcription factor, Ventral Anterior Homeobox (*Vax1*) in the SCN. We therefore generated the *Vax1*$^{fl/fl}$*:Synapsin*^{cre} (*Vax1*^{syn}) model that allows for the conditional knock down of VAX1 in the mature SCN. Our preliminary data showed that *Vax1* is not only required for SCN formation, but is also important for SCN function in adulthood. We also found that the absence of *Vax1* in the mature SCN significantly reduced the expression of vasoactive intestinal peptide (VIP), which is important for SCN signaling. Not surprisingly, the *Vax1*^{syn} cKO also had irregular wheel-running activity. The decreased VIP expression along with the wheel-running study is indicative of abnormal SCN output and therefore demonstrates the importance of VAX1 in regulating VIP expression and maintaining normal SCN function.

VIP expression in the SCN also plays a major role in fertility. SCN VIP can influence the hypothalamic-pituitary-gonadal (HPG) axis (Fig. 1) by either directly activating GnRH neurons [5] or by indirectly activating kisspeptin neurons via stimulation of SCN arginine vasopressin (AVP) neurons. [32, 33]. Illustrating the importance of VIP in fertility, studies have shown that *Vip* full body KO females have prolonged estrous cycles, less ovulation, and smaller litter size [29]. As expected, the *Vax1*^{syn} cKO females also had prolonged estrous cycles similar to the VIP-deficient model, but their ovulation frequency and litter size were unaffected. The reduced number

of litters of *Vax1*^{syn} cKO females suggests that they might either have problems with implantation or maintaining pregnancies. Another possibility is that the slightly desynchronized circadian rhythms could result in elevated stress levels and therefore result in cannibalism of the pups shortly after birth [41]. Overall, the *Vax1*^{syn} cKO females seem to have a less severe subfertility phenotype than the *Vip* full body KO females, because the *Vax1*^{syn} model specifically targets the SCN. The reduction of VIP expression only in the SCN is important for the characterization of the SCN's role in fertility, because VIP is also expressed in peripheral tissues, such as the intestines [42, 43]. In addition, as a transcription factor, VAX1 can have numerous targets in the SCN, including core clock genes. Indeed *Vax1* is able to regulate the expression of the clock genes, period (*Per1*) and brain muscle ARNT-like (*Bmal1*) *in vitro*. Similar to VIP, extensive research has characterized PER1 and BMAL1 as critical regulators in the maintenance of normal SCN output, rhythm generation, and fertility [44-47]. In our *Vax1*^{syn} model, we would expect that the deletion of VAX1 in the mature SCN would also alter the expression of PER1 and BMAL1 *in vivo*. Although we have not yet studied the expression of PER1 and BMAL1 in *Vax1*^{syn}, we have shown that *Vax1*^{syn} cKO have abnormal wheel running activity and reduced VIP expression. From these data, we can extrapolate that the *Vax1*^{syn} cKO might also have abnormal Per1 expression, because VIP has been identified as an inducer of PER1 [45]. Despite extensive research, the exact mechanism by which PER1 and BMAL1 control fertility remains unknown. However, we do know that *Bmal1* full body KO females have disrupted estrous cycles, no LH surge, but are somehow still able to ovulate [48]. The ability of BMAL-deficient females to ovulate in the absence of the LH surge is unconventional and is still not understood. Our

identification of VAX1 as a regulator of *Bmal1* and *Per1* can contribute to understanding of how fertility is intricately regulated by these core SCN peptides.

The role of the SCN in fertility is more pronounced in females in both mice and humans [28], and as seen in the *Vax1*^{syn} cKO. In females, the synchronized timing of GnRH release in response to circadian input is essential for the LH surge and ovulation [38, 39]. We found that the deletion of VAX1 in the mature SCN disrupted circadian output, leading to subfertility only in the cKO females, while the male fertility seems to be unaffected. Although the *Vax1*^{syn} cKO males also had decreased VIP expression, their fertility starkly differed from VIP KO males, which had decreased testosterone and abnormal testes morphology [49]. This difference further illustrates that a small number of VIP expressing SCN neurons, as is the case for the *Vax1*^{syn} are sufficient to retain normal male fertility.

Until now, the effects of *Vax1* in the SCN have not been characterized. Our findings provide exciting insights into the possible mechanisms by which the SCN can regulate fertility, by demonstrating that *Vax1* is required in the mature SCN for normal SCN rhythm generation, which strongly regulates female fertility. We have also shown that the transcription factor *Vax1* in the SCN can regulate the expression of VIP, Bmal1-luciferase, and Per1-luciferase, which are key SCN components required for generation of circadian rhythms [31]. Our *Vax1*^{syn} model is also different from the other models, because it allows for the specific targeting of the SCN. This is important for studying the mechanism by which the SCN modulates fertility, because *Vax1,* along with the other SCN peptides, is also expressed in other peripheral tissues [42, 43].

Chapter II, in part, is currently being prepared for submission for publication of the material. I would like to thank the following co-authors for allowing me to share our data: Hanne M. Hoffmann, Brittainy Hereford, Michael R. Gorman, David K. Welsh, and Pamela L. Mellon. The thesis author is the second author of this material.

General Discussion

Fertility is tightly regulated and maintained through a complex coordination of hormone release in the hypothalamic-pituitary-gonadal (HPG) axis. Circadian rhythms produced by the hypothalamic suprachiasmatic nucleus (SCN) synchronize the rhythmic release of gonadotropin-releasing hormone (GnRH) from GnRH neurons. GnRH acts on the pituitary to stimulate the secretion of luteinizing hormone (LH) and follicle-stimulating hormone (FSH), which then regulate the release of gonadal testosterone, estrogen, and progesterone from the gonads. Although this general schematic of how fertility is regulated has been well established, there are other key regulators that have not been characterized. We have here identified Ventral anterior homeobox 1 (VAX1) as a transcription factor that is necessary for normal GnRH neuron maturation and SCN function. We have characterized the role of VAX1 in two distinct stages of fertility: development and adulthood. The deletion of *Vax1* in GnRH neurons during early development completely eliminates GnRH expression leading to delayed puberty, hypogonadism, and infertility [8]. The effects of *Vax1* on GnRH neurons are direct as VAX1 can bind to and regulate the GnRH promoter and deletion of *Vax1* specifically within the GnRH neurons results in absence of GnRH expression [8]. Meanwhile, the deletion of *Vax1* in the mature SCN weakens circadian output by decreasing VIP expression, which leads to female subfertility. VAX1 can also regulate two vital clock genes, period 1 (*Per1*) and brain muscle ARNT-like (*Bmal1*). Collectively, these data highlights the importance of VAX1 in the development and maintenance of fertility.

As infertility conditions are becoming increasingly widespread in our society due to more people working shift-work, the use of artificial light to stay up late into the night, and disease conditions and pharmaceuticals that are associated with poor sleep behaviors, it is important that we increase our understanding of the interplay between disrupted circadian rhythms and hormone release. Our characterization of *Vax1* in relation to development of GnRH neurons and the SCN, as well as its role in the function of the post-developmental SCN, brings us one step closer to deciphering the complex interplay between circadian rhythms and reproduction. Our study provides *Vax1* as a novel candidate gene to be studied for infertility diseases that are not completely understood, such as idiopathic hypogonadotropic hypogonadism (IHH). There is a wide range of infertility issues that affect humans, generally resulting in subfertility. Most couples that are experiencing delayed conception are not completely infertile and some will be able to conceive spontaneously [50]. The subfertility of the $Vax1^{syn}$ cKO also reflects our current understanding that the female reproduction is more vulnerable to disruptions in circadian rhythms. In conclusion, I have shown that the novel transcription factor VAX1 is required for the normal development and function of GnRH neurons and the SCN.

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