DELETION OF ARCUATE NUCLEUS-SPECIFIC Kiss1 DISRUPTS ESTROUS CYCLICITY AND LH PULSATILITY IN FEMALE MICE

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ABSTRACT OF THE THESIS

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by NIMISHA NANDANKAR

Kisspeptin (encoded by Kiss1), a neuropeptide critically involved in neuroendocrine regulation of reproduction, is primarily synthesized in two discrete hypothalamic nuclei: the anteroventral periventricular nucleus (AVPV) and arcuate nucleus (ARC). Current data indicates that AVPV Kiss1 is important for the pre-ovulatory luteinizing hormone (LH) surge unique to females as well as estrogen-induced positive feedback control of GnRH and LH. In contrast, ARC Kiss1 neurons, which largely co-express the neuropeptides NKB and dynorphin (collectively known as KNDy neurons), are thought to be the major regulators of pulsatile release of GnRH and LH, and mediate estrogen-induced negative feedback control of both GnRH and LH. Previous studies have not definitively separated the specific roles of Kiss1 in the AVPV versus KNDy-ARC neurons in the downstream control of GnRH and LH release. Therefore, we generated a Pdyn-Cre/Kiss1^{0.0} (KO) mouse model to target Kiss1 in KNDy neurons to differentiate KNDy neuron-specific function from AVPV Kiss1 function in the maturation and maintenance of the reproductive axis. qRT-PCR data documented the loss of Kiss1 expression in the mediobasal hypothalamus (containing ARC) compared to controls, whereas Kiss1 in the preoptic area (containing AVPV) was similar in both KO and
controls. Immunofluorescent staining for kisspeptin confirmed the loss of Kiss1 specifically in the ARC of KO mice. Although no changes in pubertal body weight gain or pubertal onset were observed in KO animals, KO females exhibited disrupted estrous cyclicity in adulthood. Interestingly, KO female mice had disrupted estrous cycles presenting with persistent diestrus and a small vaginal opening. We tested the hypothesis that ARC KNDy neurons are necessary for generating and maintaining episodic LH pulsatile release by serial collection of whole blood and measuring LH. KO female mice exhibited significantly fewer LH pulses in a 3-hour timespan compared to controls, suggesting that KNDy neurons were functionally compromised. These observations indicate the central role of KNDy neurons in the regulation of GnRH/LH pulsatility and estrous cyclicity. The functional effects of disrupted estrous cyclicity and slowed LH pulsatility observed in KO females result in arrested folliculogenesis and infertility. Future experiments will determine whether ARC Kiss1 deletion disrupts the KNDy-driven negative feedback response of LH to gonadectomy, as well as address potential sex differences in ARC Kiss1-mediated negative feedback control of LH release.
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I dedicate this thesis to my family and loved ones, including my parents, brother, and soon-to-be sister-in-law.

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Introduction

Kisspeptin, encoded by *Kiss1*, is a 54 amino acid polypeptide that binds to and activates the kisspeptin receptor *Kiss1R* (encoded by *Kiss1r*). The critical role of kisspeptin in regulating normal pubertal onset and reproductive development came to light after the discovery of a family lacking the *Gpr54* gene (later confirmed to be the cognate kisspeptin receptor) and their clinical phenotype of idiopathic hypogonadotropic hypogonadism (5, 6). This finding was the first to suggest that kisspeptin has a major role in the hypothalamic-pituitary-gonadal (HPG) axis. Activation of the *Kiss1R* via kisspeptin administration in rats and monkeys elicits downstream effects on the HPG axis such as activation of the GnRH neuron and gonadotropin-releasing hormone (GnRH) secretion leading to the onset of puberty, providing further evidence for kisspeptin’s role in regulating the reproductive axis (8, 9, 10). The mechanism of kisspeptin action on GnRH secretion was demonstrated by several groups as a direct interaction between kisspeptin and *Kiss1R*, which is expressed in GnRH neurons (11, 12, 13). Thus, the downstream effects of GnRH on releasing the gonadotropins, luteinizing hormone (LH) and follicle-stimulating hormone (FSH) on gonadal function is, in a large part, regulated by hypothalamic kisspeptin neurons.

Hypothalamic kisspeptin is synthesized in two distinct nuclei and well-characterized – the arcuate nucleus (ARC) and the anteroventral periventricular nucleus (AVPV). AVPV kisspeptin neurons have been implicated in the onset of puberty and the pre-ovulatory LH surge unique to females (14, 15, 16). Whereas arcuate nucleus kisspeptin neurons are distinguished by their co-expression of neurokinin B and dynorphin, and due to the co-expression of these three neuropeptides, they are collectively referred to as KNDy neurons (17). Dynorphin and neurokinin B are thought
to act as inhibitory and stimulatory influences, respectively, on arcuate kisspeptin expression to generate and coordinate pulsatile GnRH release in both males and females (18-20). For this reason, arcuate kisspeptin neurons are believed to be the neuroanatomical site for GnRH pulse generation (21, 40-43). AVPV kisspeptin is thought to respond to estradiol-mediated positive feedback by ERα receptors, which allows for puberty onset, whereas ARC kisspeptin responds to negative feedback of estradiol for the homeostatic regulation of gonadotropin release (22). Altogether, hypothalamic kisspeptin plays an important role in the onset of puberty, ovulation, and the maintenance of GnRH pulse generation, as well as LH pulsatile secretion.

To date, there has not been an in vivo model to dissect the functions of ARC and AVPV kisspeptin as having independent functions. Most animal models targeting ARC kisspeptin have used toxin-based ablation post-natally, Sox14 deletion based on embryonic RNA-seq data, or diphtheria toxin-based ablation done prenatally or post-natally (23, 24, 25, 35). However, kisspeptin communication with GnRH neurons begins in utero, which was not considered in previously published data in post-natal induced animal models with ARC Kiss1 deletion (26). In addition, the published toxin-based ablation or silencing approach may produce off-target effects as opposed to a genetically modified approach. In the case of the genetically-induced diphtheria toxin ablation, off-target effects are still a possibility since the toxin may not be completely sequestered in the targeted cells once translated into protein (23). Thus, we generated a conditional ARC Kiss1 knock-out mouse model using embryonic targeting with the Prodynorphin-IRES-Cre/Kiss1fl/fl mouse line (Pdyn-Cre/ Kiss1fl/fl KO). We chose prodynorphin due to dynorphin’s 95% co-expression with ARC Kiss1 neurons and no co-expression with
AVPV Kiss1 neurons, allowing for specific deletion of ARC Kiss1 neurons *in utero* (27).

With the development of this Pdyn-Cre/Kiss1^fl/fl^ KO mouse model, we are able to discern ARC Kiss1-specific effects on the HPG axis.
Materials and Methods

Animals

We crossed commercially available Prodynorphin-IRES-Cre mice, established by Dr. Bradford Lowell at Harvard University, from The Jackson Laboratory with a Kiss1 homozygous floxed (Kiss1^{fl/fl}) mouse line generated in our laboratory. We developed the Kiss1^{fl/fl} mouse with a BAC clone (Source Bioscience) which contains the entire coding sequence of all known Kiss1 splice variants and the coding and regulatory regions of Golt1a (Clone BMQ-203-m22). The coding sequence for kisspeptin is located in exons 2 and 3 for all transcript variants. Recombineering technology (60, 61) was used to flank LoxP sites around exon 2 of Kiss1, which includes the translation start site for Kiss1. Cre recombinase excised exon 2 resulting in a frameshift in exon 3 to produce a truncated protein product and complete absence of the Kiss1 protein. This targeted fragment was electroporated into embryonic stem cells of 129SV/J mice by the Johns Hopkins University ES Cell Targeting Core Laboratory to target Kiss1 using homologous recombination. Our knock-out (KO) mice are identified as heterozygous Cre^{+/-} and homozygous Kiss1^{fl/fl}. Mice are a mixed C57B6/J and 129SV/J strain. Animals were housed in the Child Health Institute of NJ (CHINJ) vivarium, maintained on a 12:12 light/dark cycle (lights ON at 6:00 am) and were fed ad libitum, with free access to water. All animal procedures were performed in accordance with the Institutional Animal Care and Use Committee (IACUC) at Rutgers University.
**q-RT-PCR**

We collected various mouse tissue for q-RT-PCR mRNA expression analysis and maintained the fresh-frozen tissue at -80°C until RNA extraction was performed. The mediobasal hypothalamus (MBH) and preoptic area (POA) were collected using a mouse brain matrix (Roboz) following a blocked-section collection method (58). RNA was homogenized with Qiagen’s TissueRuptor II handheld rotor-stator homogenizer and subsequently extracted from tissue using the TRIzol RNA isolation protocol by Thermo Fisher Scientific. Extracted mRNA was then reverse transcribed into cDNA using iScript cDNA Synthesis Kit by Bio-Rad per their protocol. cDNA was diluted 1:1 with Sigma Aldrich Molecular-Grade Water and used with Universal SYBR Green Master Mix in accordance with their protocol. The primers used to amplify Kiss1 were the sense primer 5’-AGCTGCTGCTTCTCCTCTGT-3’ and anti-sense primer 5’-GCATACCGCGATTCCCTTTTT-3’. The primers used for the housekeeping gene Rpl13a were sense primer 5’-GCTGCTCTCAAGGTTGTTCG-3’ and anti-sense primer 5’-CCTTTTCTTCCGTTTCTCC-3’.

**Perfusion and Brain Immunofluorescence**

Mice were anesthetized with a cocktail mix of ketamine/xylazine (100 and 10 mg/kg of body weight, respectively) and then underwent intracardiac perfusion with an in-house made 4% paraformaldehyde (PFA, pH 7.4) made in 0.1M PBS for 10-12 minutes. The brain was then carefully extracted, post-fixed in 4% PFA for 24 hours at 4°C, and then placed in 30% sucrose for 2 days, or until fully sunken in solution. Brains were then frozen on dry ice and stored at -80°C until sectioning. Brains were serially sectioned at 30 µm at -20°C in a cryostat starting from the rostral pre-optic area (Bregma 0.62 mm) to
the caudal extent of the arcuate nucleus (Bregma -2.80 mm) according to Franklin and Paxinos (59). Sections were collected in a polyvinylpyrrolidone-based cryoprotectant and stored at -20°C until staining.

Free-floating brain sections were washed 4 times in 1X PBS for 10 minutes each, then blocked for 1 hour at room temperature with in 5% normal goat serum and 0.3% Triton X-100 in 1x PBS. Sections were then incubated in a 1:10,000 dilution of primary antibody (AC#566, Rabbit Polyclonal anti-mouse kisspeptin-10), developed by Dr. Alain Caraty, in the same blocking solution for 1 hour at room temperature, and then 48 hours at 4°C. After primary antibody incubation, sections underwent 4 washes in 1X PBS for 10 minutes each. Sections were incubated in 1:500 Alexa 488 goat anti-rabbit secondary fluorescent antibody (Invitrogen/ThermoFisher), made in 0.3% Triton X-100 in 1X PBS, for 3 hours at room temperature and protected from light. Sections were washed 4 times in 1x PBS for 10 minutes each and mounted onto microscope slides. Finally, slides were coverslipped with DAPI Fluoromount-G® (Southern Biotech) and imaged using a confocal microscope.

**Microscopy and Imaging**

Images were acquired with a Zeiss 700 Laser Scanning Microscope (LSM700) using a Plan-Apochromat 10X objective and processed with Zen 2.3 software (Zeiss, Germany). Each channel visualized was scanned individually to prevent crosstalk between channels and fluorophores

**Pubertal Onset and Body Weight**

External markers of pubertal onset were assessed in female and male mice of both genotypes for comparison. In females, pubertal onset was determined as the presence of
vaginal opening (VO) and was assessed after daily clinical observations starting from the day of weaning (21 days of age). In males, pubertal onset was defined as the day that balanopreputial was fully achieved, and this was assessed daily starting at weaning age. Preputial separation was assessed by gentle manual retraction of the prepuce (1). We also measured anogenital distance in males, defined as the distance from the anus to the genital tubercle at 25 days of age as an external sign of androgen-sensitive growth (29-31). Body weight in females and males of both genotypes was measured on a tared laboratory scale every other day from weaning age, through puberty, until 49 days old to track weight gain and growth.

Assessment of Estrous Cyclicity

Starting on the day of VO, female mice underwent daily vaginal lavage with 0.9% saline solution in the afternoon for observation of vaginal cytology to determine the stages of the estrous cycle. This method was used to determine the day of first estrous to indicate their first ovulatory event. Estrous cyclicity was determined at two developmental ages: starting at 2 months of age, when reproductive maturity was reached, and from the day of VO until 2 months of age, in order to track possible irregularities in the estrous cycle during puberty. The stage of the estrous cycle was determined by observing vaginal epithelial cytology and assessing the proportion of leukocytes, nucleated epithelial cells, and cornified cells present in the collected saline samples under a brightfield microscope.

Gonad Histology

Ovaries were freshly dissected and fixed in 4% PFA. Testes were freshly dissected and fixed in Bouin’s solution. Fixed gonads were kept at 4°C prior to paraffin embedding by Rutgers University’s Pathology Core. Mouse ovaries and testes were sectioned cross-
sectionally at 5 \(\mu\)m thickness and stained using histological hematoxylin and eosin staining. To determine gonad weights, both sets of ovaries and both sets of testes, freshly dissected, were cleaned of gonadal white adipose tissue and weighed on a tared laboratory scale.

**Tail-tip Blood Collection and Ultrasensitive Mouse LH Assay**

Female and male mice (2–3 months old) underwent serial tail-tip blood collection between 10:00 am and 1:00 am. Female mice were assessed for estrous cycle and blood collection on the day of diestrus. Mice were acclimated by daily handling and placing in a mouse restrainer prior to the experiment. 6 \(\mu\)L of tail-tip blood was collected from mice, while inside a mouse restrainer, and directly pipetted into 54 \(\mu\)L of Ultra-Sensitive LH Assay Buffer (1:10 dilution) prepared according to UVA Ligand Assay Core’s protocol (2). Blood was collected every 6 minutes for 3 hours, kept on dry ice, and stored at -80°C prior to shipment and analysis by the University of Virginia Center for Research in Reproduction Ligand Assay and Analysis Core. The functional sensitivity, defined as the lowest concentration that demonstrates accuracy within 20% of expected values, is 0.016 ng/ml. Intra-assay co-efficient of variance (CV) is 2.2%. Inter-assay CVs are 7.3% (Low QC, 0.13 ng/ml), 5.0% (Medium QC, 0.8 ng/ml) and 6.5% (High QC, 2.3 ng/ml) (Steyn et al, 2013).

**LH Pulsatility Analysis**

LH pulses were determined based upon the following criteria: 1) peaks must have at least a 20% increase from the previous 1 or 2 LH values 2) peaks must be followed by a 10% decrease in the next 1 or 2 LH values, and 3) pulse amplitude (LH increase from nadir to peak) must be greater or equal to 0.32 ng/mL, which is the functional sensitivity of the
assay (3). LH pulsatility dynamics were assessed by calculating the following parameters:

1) Pulse frequency, calculated as the number of pulses in the 3-hour collection period
2) Pulse amplitude, calculated as the difference between the pulse peak and its preceding nadir (lowest of the 3 previous data points), and
3) Mean LH, calculated by averaging all LH values in the 3-hour collection period for each animal. Basal LH was calculated by averaging the lowest 10 data points for each.

**Male Testosterone Assay**

Serum from whole blood, collected during sacrifice, was collected using the UVA Center for Research in Reproduction Ligand Assay and Analysis Core’s protocol. Samples were allowed to clot for 90 minutes before centrifugation at 2000 X g for 15 minutes at room temperature. After centrifugation, serum was carefully pipetted into separate tubes and stored at 4°C prior to shipment for analysis. The mouse testosterone assay (Calbiotech Inc) was performed by the UVA Center for Research in Reproduction Ligand Assay and Analysis Core. Reportable range of the assay is 10.0 - 1600.0 ng/dL. Outside reportable range is classified as %CV > 20.

**Fertility Study**

Starting at 2-3 months of age, wild-type (WT) and knock-out (KO) virgin female mice were individually paired with a proven stud WT male for 90 days. Mice were checked daily for plugs, if present, and pups. The number of litters and number of pups per litter were recorded in addition to day-of-birth. After pups were born, they were euthanized immediately, and the dam was immediately placed with the stud male for the remainder of the 90-day duration.
**Statistical Analyses**

All statistical analyses were done using GraphPad Prism Versions 7 and 8. Statistical analysis comprised of the parametric, unpaired Student’s t-test to compare wild-type and knock-out groups. Statistical significance was defined as $p < 0.05$. 
Results

Validation of Kiss1 deletion within the ARC

The Pdyn-Cre/Kiss1<sup>1<sup>0.0</sup>/</sup> knock-out (KO) mice were generated by crossing Prodynorphin-IRES-Cre mice with homozygous Kiss1 floxed mice (Kiss1<sup>1<sup>0.0</sup>/</sup>), which resulted in excision of exon 2 of the Kiss1 gene, and thus a loss of kisspeptin expression in Cre-positive prodynorphin-expressing cells. Although there is no known co-expression of kisspeptin with prodynorphin in cell populations other than arcuate KNDy neurons, we validated the genetic specificity and accuracy of the knock-out model. In both sexes, q-RT-PCR (n = 2-6) analysis (Fig 1.A) conducted on a tissue panel demonstrates a significant reduction in knock-out (KO) mediobasal hypothalamic (MBH) Kiss1 mRNA expression, the location of arcuate Kiss1 neurons, when compared to wild-type controls (WT) (unpaired Student’s t-test, p < 0.005, n = 2-6). This provides evidence of successful deletion of arcuate Kiss1. Importantly, the continued presence of Kiss1 expression in the KO preoptic area (POA), where AVPV Kiss1 neurons reside, indicates that AVPV Kiss1 remains intact and is evidence of the specificity of the knock-out model. This information is corroborated by protein analysis using immunofluorescence staining for Kiss1 in ARC sections of wild-type and knock-out female mice, where Kiss1 signal is not seen (Fig 1.B). The decrease in Kiss1 expression in the KO MBH of the male and female tissue panels is validated by a complete loss of Kiss1 immunofluorescence in the ARC of KO males and females (Fig 1.B, Fig 1.C).
A. Females

![Bar chart showing mRNA fold change in females.]

### AVPV Female

KO

WT

KO – Left 3V

KO – Right 3V

B. Males

![Bar chart showing mRNA fold change in males.]

### AVPV Female

KO

WT

KO – Left 3V

KO – Right 3V

### DAPI

KO

WT

KO – Left 3V

KO – Right 3V

### Kiss1

KO

WT

KO – Left 3V

KO – Right 3V

### Merge

KO

WT

KO – Left 3V

KO – Right 3V
Figure 1. Generation and Validation of Pdyn-Cre/Kiss1<sup>−/−</sup> Knockout Mouse. (A)

Relative mRNA expression of Kiss1 was determined in the mediobasal hypothalamus (MBH), preoptic area (POA), neocortex (CTX), liver, and testes in KO animals compared to WT controls. (Female n = 2-6, Male n = 2-7) (****p < 0.0001, **p < 0.005 KO vs.
WT). (B) Representative photomicrographs revealing kisspeptin peptide immunofluorescence in brain sections from the AVPV and ARC of WT and KO females; representative photomicrographs revealing kisspeptin immunofluorescence in brain sections of the ARC of WT and KO male mice. Expression of kisspeptin by immunofluorescence is similar in the AVPV of KO mice and controls. In the ARC of both sexes, a decrease in Kiss1 immunoreactivity in neurons in the KO mice is seen. 3V, Third Ventricle.

*Arcuate Kiss1 is not necessary for pubertal onset in mice*

We evaluated clinical parameters indicating the onset of puberty in mice in males and females of both genotypes (Figure 2). For females (n = 7-11), the presence of a vaginal opening indicates the onset of puberty, whereas in males (n = 8-12) it is the presence of full preputial separation (Fig. 2.A, 2.B). In both sexes, there was no significant difference between WT and KO groups in these indicators of pubertal onset (unpaired Student’s t-test, Females: p = 0.6237; Males: p = 0.6548). In addition, vaginal cytology was assessed in females from the day of vaginal opening to monitor for cornified cells signifying the day of first estrus, which is an indicator of their first ovulatory event (Fig. 2.A). Day of first estrus was not significantly different in KO females compared to WT females (unpaired Student’s t-test, p = 0.9693). In pubertal males, anogenital distance (AGD) was used as an indicator of testosterone responsiveness; AGD in KO males was not significantly different from that of the WT group (unpaired Student’s t-test, p = 0.4514) (Fig. 2.B). Additionally, we measured body weight every other day from the weaning age of 21 days, until 49 days of age in both sexes and found no differences in weight gain.
between WT and KO groups, other than the first time-point in females where female KO mice were significantly lighter (unpaired Student’s t-test, p < 0.005, Fig. 2.C).

Figure 2. Pubertal Assessment and Growth. (A) Pubertal onset for female mice was assessed by daily clinical observation after weaning (age 21 days) to the day of vaginal opening. (WT n = 11, KO n = 7). After vaginal opening, vaginal cytology was assessed by daily saline vaginal lavage to determine the date of first estrous (WT n = 6, KO n = 8). (B) Pubertal onset for male mice was assessed by daily observation after weaning (age 21 days) until the day of full balanopreputial separation (WT n = 8, KO n = 12). Anogenital distance was measured at 25 days of age for each cohort and then compared. (WT n = 10, KO n = 12). (C) Body weight gain throughout the duration of pubertal onset. Mice were weighed every other day from weaning until 49 days of age (***p < 0.005 KO vs. WT, Females n = 3-6, Males n = 9-12).
**Adult Pdyn-Cre/Kiss1\(^{+/\beta} \)** KO females exhibit abnormally long diestrus periods

Estrous cyclicity was assessed by daily examination of vaginal cytology starting at reproductive maturity (2 months old). As expected, WT females demonstrated the expected normal 4- to 5-day estrous cycles, exhibiting the 3 stages of the cycle – proestrous, estrous, and metestrous/diestrous. However, age-matched KO females demonstrated persistent metestrus/diestrus with occasional proestrus and estrus events (Fig 3.A). Our summarized findings between the age-matched, reproductively mature WT and KO estrous cycles demonstrate that KO mice spend significantly less time in estrus (unpaired Student’s t-test, \(p = 0.000002\)) and proestrus (unpaired Student’s t-test, \(p = 0.000273\)), while spending more time in diestrus/metestrus (\(p = 0.000001\)) (Fig 3.C).

**A.**

**WT – 2 Months Old**

**KO – 2 Months Old**

**B.**

**Estrous Cyclicity**

<table>
<thead>
<tr>
<th>Stage</th>
<th>WT</th>
<th>KO</th>
</tr>
</thead>
<tbody>
<tr>
<td>E</td>
<td>0.5</td>
<td>1.5</td>
</tr>
<tr>
<td>P</td>
<td>1</td>
<td>1.5</td>
</tr>
<tr>
<td>D/M</td>
<td>1.5</td>
<td>1</td>
</tr>
</tbody>
</table>
**Figure 3. Estrous Cyclicity in PdynCre/Kiss1\textsuperscript{fl/fl} KO Mice.** (A) Vaginal cytology was collected and assessed daily for 15 consecutive days after reproductive maturity was reached. KO mice demonstrated persistent diestrus (n = 7) vs. regular cycles observed in WT mice (n = 8). (B) After reproductive maturity, KO females spent significantly less time in estrous and proestrus and more time in diestrus/metestrus (**** p < 0.0001, *** p < 0.0005 KO vs. WT). D/M, Diestrus/Metestrus. P, Proestrus. E, Estrus. (WT n = 8, KO n = 7).

**Adult Pdyn-Cre/ Kiss1\textsuperscript{fl/fl} KO females have decreased LH pulse frequency**

KNDy neurons in the ARC are thought to be the putative site of GnRH pulse generation (21, 40-43), hence we sought to investigate how the loss of ARC kisspeptin (the major secretagogue for GnRH) affected the dynamic secretion of LH, as a proxy for GnRH secretion. We used an ultrasensitive assay to measure and follow LH pulses. Serial tail-tip blood collection every 6 minutes for 3 hours on the morning of diestrous from representative KO females (n = 4) demonstrated a decrease in pulse frequency during the 3-hour testing interval compared to that of WT females (n = 5) (Fig 4.A). This is statistically significant (Fig 4.B, unpaired Student’s t-test, p = 0.0047) as KO females had an average of 2 pulses during the 3-hour testing interval while WT females had 4.25 pulses in the same interval frame. Pulse amplitude (unpaired Student’s t-test p = 0.6413), mean LH (unpaired Student’s t-test, p = 0.9460), and basal LH (unpaired Student’s t-test, p = 0.8718 were not significantly different between WT and KO groups (Fig 4.B).
Figure 4. LH Pulsatility in Females. (A) Representative profiles of LH pulsatile release in ovary-intact females. LH for pulse analysis was collected using serial tail-tip blood collections every 6 minutes for 3 hours beginning between 10 and 11 AM on the day of diestrus. Asterisks denote identified pulses. (B) Parameters of LH pulsatility included pulse frequency, pulse amplitude, mean LH, and basal LH. Pulses are significantly less frequent in KO mice (n = 4) compared to WT mice (n = 5) (** p < 0.005, KO vs. WT), while peak amplitude, mean LH, and basal LH remained similar between genotypes.
**Adult Pdyn-Cre/ Kiss1^{0/0} KO males exhibit normal LH pulsatility**

We observed that KO males had underdeveloped testes, specifically lower weight compared to WT testes. In order to address whether this was due to irregular gonadotropin release, we also assessed LH pulsatility in KO males. In contrast to our findings in KO females, LH pulse frequency in KO males was similar to WT males (unpaired Student’s t-test, p = 0.6578) (Fig 5.A). Additionally, mean LH (p = 0.7558), basal LH (p = 0.8964), and pulse amplitude (p = 0.1458) remained similar between genotypes (unpaired Student’s t-test) (Fig 5.B). In addition, serum testosterone levels were also not significantly different between WT and KO males (unpaired Student’s t-test, p = 0.6374). One WT and one KO mouse were detected as significant outliers and were therefore excluded from serum testosterone statistical analysis (Grubb’s test, two-sided, p < 0.05).

A.  

![Graph A](image1.png)

B.  

![Graph B](image2.png)
Figure 5. LH Pulsatility in Males. (A) Representative profiles of LH pulsatile release in testes-intact males. LH for pulse analysis was collected using serial tail-tip blood collections every 6 minutes for 3 hours starting between 10 and 11 AM. Asterisks denote identified pulses. (B) Parameters of LH pulsatility were pulse frequency, pulse amplitude, mean LH, and basal LH. Pulses have similar frequency for both the KO mice (n = 4) and WT mice (n = 2). (p = 0.6578, KO vs. WT). LH pulse amplitude, mean LH, and basal LH are similar between genotypes. (C) Testosterone levels are not significantly different between WT (n = 3) and KO (n = 6) males (p = 0.6374 KO vs. WT).

Adult Pdyn-Cre/Kiss1<sup>0/0</sup> KO females exhibit signs of arrested folliculogenesis

In order to address the end-organ result of irregular estrous cyclicity in KO females, we examined ovarian morphology. H&E staining of the innermost sections of the WT ovary revealed evidence of each stage of progressive folliculogenesis including primary/secondary follicles, antral follicles and corpora lutea. Qualitative analysis of KO ovaries, however, revealed a larger proportion of early-stage follicles with few antral follicles and no corpora lutea (Fig 6.A). In addition, the uteri of KO females presented with thinner endometrium, myometrium, and circular myometrium layers in comparison to WT mice (Fig 6.C). For males, testes from both genotypes were processed in a similar fashion and H&E staining revealed spermatogenesis in both WT and KO males (Fig 6.B). Prior to fixation and histological processing, gonads from males and females, as well as
uteri, were weighed. Ovarian weight in KO females was significantly lower compared to WT ovaries (unpaired Student’s t-test, \( p = 0.0007 \)). Uterine weight in KO females was also significantly lower compared to WT uteri (unpaired Student’s t-test, \( p = 0.0016 \)).

Further, KO males showed significantly lower wet testicular weight compared to WT testes (unpaired Student’s t-test, \( p < 0.0001 \)) (Fig 6.D).
Figure 6. Gonadal and Uterine Histology & Weight. (A) 5 μm sections from the ovary were obtained starting from the innermost portion of the ovary and stained with hematoxylin and eosin (H&E). KO ovaries have more early-stage follicles, fewer antral follicles and no corpora lutea. CL, corpus luteum. (B) 5 μm sections from the innermost portion of the testis were obtained and stained with H&E. KO testes had no morphological differences in spermatogenesis compared to WT testes. (C) 5 μm sections from the innermost portion of the uterine horn were obtained and stained with H&E. KO uteri were thinner than WT uteri. (D) Gonads/uteri were freshly dissected from mice and wet organ weights measured immediately after. Adult mice were age-matched and ranged from 2-6 months old. For ovarian and testicular weights, both gonads were weighed together. Ovaries, uteri and testes weighed significantly less in KO mice. Ovaries n = 2-6, Uterus n = 2-6, Testes n = 3-7.; ** p < 0.005, **** p < 0.0001 KO vs. WT).

**Pdyn-Cre/Kiss1<sup>fl/fl</sup> KO females are infertile**

After observing significant deficiencies in ovarian development, cyclicity and LH pulsatility, we investigated the impact of these reproductive impairments on female fertility. We paired either a WT or KO virgin female with a proven, stud WT male, and monitored for parity events within 90 days of continuous mating, only separating the females from stud males during pregnancy and after birth of a litter. One WT mouse gave birth 3 times in a 90-day period, producing a total of 25 pups with an average of 8.67
pups per litter. The second WT mouse gave birth 4 times, producing a total of 28 pups with an average of 7 pups per litter. The 3 KO mice, however, did not produce any pups in the entire 3-month period, making the number of pups per litter significantly decreased in KO females (unpaired Student’s t-test, p = 0.0011) (Figure 7).

Figure 7. Female Fertility Assessment. (A) WT or KO females were paired with one proven, WT stud male each over a span of 90 days, beginning at female reproductive maturity. Breeding pairs were observed daily for pregnancy and birth of litters during this 90-day testing period. Yellow circles indicate litters, with numbers inside indicating the number of live pups in that litter. The sum of live pups from all litters of each individual dam are indicated on the right under ‘Total Pups’. WT n = 2, KO n = 3. (B) Number of pups, date of birth, and number of litters were recorded prior to sacrificing pups and re-pairing test females with stud male for the duration of the study. KO females demonstrate infertility having 0 litters in 90 days (** p < 0.005, WT vs. KO).
Discussion

In summary, our novel Pdyn-Cre/Kiss1\textsuperscript{fl/fl} (KO) mouse model has demonstrated a specific and significant decrease (p < 0.0001, p < 0.005) in Kiss1 mRNA expression in the MBH of KO female and male mice (Fig 1.A). The precision of the gene deletion was demonstrated by expression of Kiss1 in specific peripheral and CNS tissues, while the MBH lacked significant expression. Since KNDy neurons in the arcuate nucleus (ARC) could not be isolated, q-RT-PCR results for Kiss1 in the MBH reflects a large number of cells that do not express kisspeptin, although no kisspeptin has been localized outside of the KNDy neurons in the ARC by current methods. Unfortunately, some tissues in the male and female tissue panels exhibit considerable variability. In order to address this, we are breeding additional mice to increase the number assessed.

Kiss1 immunofluorescence in female mice clearly shows the presence of AVPV Kiss1 neurons, corroborating our mRNA data (Fig 1.B). The arcuate (ARC) sections of the male and female KO mice demonstrate a complete loss of green fluorescent Kiss1 signal in the arcuate nucleus, flanking the third ventricle, in the KO mouse. Presently, we are conducting AVPV immunofluorescence staining on male WT and KO mice.

Since the targeting paradigm was to perform an ARC-specific knock-out of kisspeptin neurons, and our initial experimental assessment using q-RT-PCR and immunofluorescence were consistent, we did not expect to see any phenotypic changes related to AVPV kisspeptin function related to GnRH secretion and pubertal onset (28). In fact, we did not see any differences in onset of vaginal opening in KO females or full balanopreputial separation in KO males (Fig 2.A, 2.B), indicating the functional preservation of AVPV kisspeptin neurons and hence pubertal onset and progression. The downstream observations of lower ovarian and uterine mass alongside disrupted...
folliculogenesis (Fig 6) implies that there are impaired estradiol levels in the circulating blood. This implication alongside the conserved pubertal onset would indicate a possible connection or cross-talk between ARC kisspeptin neurons and AVPV kisspeptin neurons. Specifically, the ARC kisspeptin neuronal knock-out could hinder full AVPV kisspeptin neuronal activation at the time of puberty, thus hindering the magnitude of GnRH stimulation on gonadotropins. This could allow for enough gonadotropin signaling for pubertal onset (Fig 2), but not for complete pubertal maturation of ovarian follicles or mass, resulting in smaller ovaries and arrested folliculogenesis (Fig 6). This reduction in ovarian mass could cause a decrease in circulating estradiol levels, negatively affecting uterine size (Fig 6) and reducing the capability of estradiol inducing its positive feedback onto AVPV kisspeptin for onset of ovulation – causing fewer incidences of proestrous and estrous (Fig 3).

Previous publications have highlighted the synaptic connections of arcuate kisspeptin neurons with agouti-related peptide (AgRP), neuropeptide Y (NPY), and proopiomelanocortin (POMC) expressing neurons, all implicated in energy homeostasis due to their shared lineage with ARC kisspeptin neurons (32, 33, 34). An ARC Kiss1 loss-of-function mouse model, induced by AAV1-DIO-GFP:tetanus toxin (TeTx) viral stereotaxic injection into adult Kiss1-Cre female mice, demonstrated increased body weight and obesity due to dysregulated feeding behavior (24). To examine whether this was a function specific to kisspeptin in KNDy neurons, we weighed WT and KO mice every other day from 21 to 49 days of age; finding no differences between genotypes or the sexes (Fig 2.C). Off-target effects from the toxin-based silencing in AAV1-DIO-GFP:TeTx mice were likely a determining factor of abnormal weight gain, which was
not seen in our PdynCre/Kiss1\textsuperscript{fl/fl} KO mice (24). Another explanation, although less likely, may be that an embryonic onset of kisspeptin deficiency in our model may allow for developmental compensation of energy homeostasis in utero.

Previous studies of toxin-ablated or gene targeted silencing of ARC Kiss1 in rodent models have shown that experimental female mice present with persistent diestrous, which is the quiescent stage of the estrous cycle (24, 25, 35). Estrus and proestrus, indicated by cornified cells and nucleated epithelial cells, signify the ovulatory and pre-ovulatory stages of the estrous cycle, respectively (54, 55). The aforementioned AAV1-DIO-GFP:TeTx mouse model, which silenced ARC Kiss1 neurons in adulthood, reported persistent diestrous in female mice (24). Another study conducted stereotaxic injections of a rAAV kisspeptin antisense (rAAV-Kisspeptin-AS) virus into the ARC of adult rats. They noted a tendency of rAAV-Kisspeptin-AS female rats to undergo abnormal estrous cycles (35). However, they did not observe differences in percentage of time spent in each estrous cycle stage, unlike in AAV1-DIO-GFP:TeTx mice, which could be due to mechanistic differences between species, type of virus used, or differing off-target effects of the viruses. In contrast, diphtheria-toxin genetic ablation of kisspeptin-expressing cells demonstrated normal estrous cyclicity, with slightly prolonged estrous periods – however, in mice expressing diphtheria-receptor in kisspeptin neurons that were injected with diphtheria toxin, affected mice experienced prolonged diestrous periods (23). These conflicting results could be due to the non-specificity of kisspeptin neuronal ablation, since the diphtheria toxin targeting system was not targeted solely on arcuate kisspeptin neurons. Our Pdyn-Cre/Kiss1\textsuperscript{fl/fl} KO females, generated without the use of a virus, spend significantly more time in
diestrus/metestrus than in proestrus or estrus, which corroborates the findings in AAV1-DIO-GFP:TeTx mice (Figure 3). Although Pdyn-Cre/Kiss1\(^{fl/fl}\) KO mice spend most of their time in diestrus/metestrus, they exhibited occasional estrous and proestrous events, indicating that AVPV Kiss1 may produce rare ovulatory events. These findings provide strong evidence that ARC Kiss1 is responsible for the maintenance of regular estrous cyclicity (36, 37). The discrepancies between estrous cyclicity studies in viral ARC Kiss1 knock-down in adult mice and Pdyn-Cre/Kiss1\(^{fl/fl}\) KO mice may be due to the timing of the onset of ARC Kiss1 impairment. In addition, off-target effects from viral injections may be another contributing factor to this variable phenotype.

The ideal method to measure GnRH secretion would be using hypothalamic-pituitary portal samples, however this is not feasible in small mammals (44, 45). Since GnRH pulses and LH pulses have been well-documented to be synchronized, we used LH pulsatile measurements as a proxy for the GnRH pulse (46,62). Earlier studies in rats and monkeys with a lesioned ARC nucleus to target Kiss1 neurons, have demonstrated the necessity of this neuronal population in maintaining normal LH pulsatility (38, 39). With specific deletion of Kiss1 expression in our genetically modified mouse model, we observed a significant decrease in LH pulse frequency in female KO mice, as we hypothesized (Figure 4). Pulse amplitude was also lower in KO females, although this was not statistically significant (Figure 4.B). With an increased number of mice in each cohort, pulse amplitude may become statistically significant. In contrast, male KO mice exhibit no significant differences in LH pulsatility. Current studies are underway to increase the number of male mice studied (Figure 5). This will aid in clarifying whether there is a sex difference in LH pulsatility. However, since pulsatility is not absolutely
required for male fertility, we presume that kisspeptin does not have a major role in determining LH pulsatility in males. Interestingly, in both sexes, mean and basal LH were similar between WT and KO groups (Fig 4.B, 5.B). AAV1-DIO-GFP:TeTx female mice with silenced ARC Kiss1 neurons via TeTx viral injections in adulthood exhibited a 40% decrease in LH (24), which contrasts our findings that show no difference in mean and basal LH of KO mice. This differences could be attributed to off-target effects of viral delivery or yet unidentified factors responsible for basal LH secretion. Our findings confirm that timed regulation of GnRH in females, and subsequently pulsatile gonadotropin release, is mediated by kisspeptin in KNDy neurons, rather than kisspeptin regulating the total amount of gonadotropin released (57). Further evidence is forthcoming from serum drawn at sacrifice to determine serum LH and FSH levels.

Due to the persistent diestrous observed in KO females, we explored the ability of these mice to ovulate as they had impaired LH pulse generation. Firstly, we analyzed ovarian histology using H&E staining of fixed tissue. We observed distinct and varied stages of folliculogenesis in WT ovaries including early-stage follicles, antral follicles, and corpora lutea in relatively even proportions representing the sequential stages of folliculogenesis. In contrast, KO ovaries exhibited mostly early-stage follicles, with few antral follicles, and no corpora lutea (Fig 6.A). This suggests that the deletion of ARC Kiss1 results in arrested folliculogenesis at the antral follicle stage. Primordial, primary, and secondary follicles formed prior to the antral stage, referred to as pre-antral follicles, develop independent of gonadotropins. After the antral follicle stage, there is a developmental switch that allows folliculogenesis to become gonadotropin-dependent (47-50). Since we observe the presence of antral follicles in KO mice, we posit that the
temporal lag in gonadotropin secretion causes folliculogenesis to progress only to the point of antral follicle production. However, it is likely not to be sufficient to maintain the antral follicles, as seen by fewer antral follicles in KO mice than in WT mice. We are currently performing additional corpora lutea counts in the whole ovary in order to adequately confirm the finding, as well as to measure ovulation rate (4, 7). To further determine if arrested folliculogenesis is due to impaired gonadotropin release or timed gonadotropin pulsatile release, we plan on administering exogenous gonadotropins to rescue folliculogenesis.

Normal cyclic alterations in uterine weight occur during the estrous cycle. During diestrus the uterine horns are thinner, and during estrus/proestrus the uterine horns become engorged due to cellular proliferation required for implantation (51, 52). The decreased uterine weight observed in KO mice is likely due to the predominant time spent in diestrus compared to their WT counterparts. Presently, all layers of the uterus are present in the KO, however they appear thinner compared to WT uterine layers (Fig 6.C). Additional collection and measurement of WT and KO uterine layers will clarify the morphological changes in our KO females. The KO females’ persistent diestrus suggests that there is a disruption in normal estradiol levels that accompany estrous cyclic changes, which will be clarified with serum estradiol measurements. The thin KO uterus is likely due to a lack of the normal dynamic changes in estradiol secretion. To test this, we will ovariectomize (OVX) mice and observe uterine morphology post-estradiol administration to test uterine responsivity to estradiol. The decreased size and weight of KO ovaries compared to WT ovaries (Fig 6.D) is likely due to some degree to the disrupted LH pulsatility in the KO females, which unlike in males, is critically important
for ovarian development and folliculogenesis. However, since FSH also contributes to ovarian development and folliculogenesis, it will be important to evaluate FSH levels as a contributing factor for diminished ovarian weight and arrested folliculogenesis. Our laboratory has previously reported inhibition of kisspeptin signaling to GnRH neurons, in GKIRKO mice resulting in hypogonadotropic hypogonadism (31), the observation of smaller gonads in male and female Pdyn-Cre/Kiss1\textsuperscript{fl/fl} KO mice provides evidence for an additional etiology of central hypogonadism.

Although we observed arrested folliculogenesis in females, we have not observed any difference to date in spermatogenesis between WT and KO males (Fig 6.B). Although we note some variability in our H&E staining, we believe this may be due to our fixation methods and after a literature search for alternative methods, we recently switched from 4% paraformaldehyde to Bouin’s fixative (53). In males, we measured anogenital distance (AGD) as an indirect correlate of circulating androgen levels at the time of puberty (29, 30) which was unaffected in KO males (Fig 2.B), suggesting that circulating androgen levels between WT and KO groups are similar. In parallel, we measured serum testosterone levels in WT and KO adult males, which proved to be similar between genotypes (although variable) and supported the AGD data (Fig 5.C). Despite our observations that KO males have normal mean and basal LH, testosterone levels, anogenital distance and spermatogenesis, we observed decreased testicular weight in KO males (Fig 6). These observations of smaller ovarian size are like that of the diphtheria toxin-based ablation of kisspeptin neurons, however those KissIC/R26-DTA mice exhibit normal folliculogenesis as well as corpora lutea, likely due to the global targeting of kisspeptin neurons embryonically, leading to compensatory mechanisms that
would conserve folliculogenesis (23). To determine whether the source of this phenotype is due to abnormalities at the central level of the reproductive axis, we plan to evaluate several parameters. We will measure FSH levels to address whether the impaired folliculogenesis and low testicular weight are due to impaired FSH. In addition, we will be conducting GnRH and kisspeptin stimulation tests to evaluate the responsivity of KO mice to gonadotropins (LH and FSH). Any loss in gonadotropin responsivity will suggest that ARC kisspeptin is necessary for pituitary gonadotropin response to GnRH, GnRH neuronal response to kisspeptin, or both. If gonadotropin responsivity is hindered at the level of GnRH neurons, we will proceed to evaluate Kiss1r and Gnrh expression in the hypothalamus, and GnRH protein expression using immunofluorescence staining in the brain. In summary, FSH levels and gonadotropin responsivity must be explored in order to clarify whether kisspeptin signaling via the GnRH neuron is affected in our ARC Kiss1 KO model.

Due to arrested folliculogenesis, disrupted estrous cyclicity, decreased LH pulsatility, and decreased ovarian weight, we sought to assess fertility in female KO mice which we hypothesized would be abnormal. Indeed, none of the KO females have given birth in continuous paired-housing with proven, stud WT males, while WT females produced pups at least 3 times during the 90-day study (Fig 7.A). This provides strong evidence that Pdyn-Cre/Kiss1^fl/fl KO female mice are infertile. Their infertility is most likely due to their arrested folliculogenesis, hindering their ability to produce fully mature ovarian follicles and ovulate. Additionally, we are beginning to conduct a male fertility study to assess the reproductive ability of Pdyn-Cre/Kiss1^fl/fl KO males. Due to
preliminary data indicating intact spermatogenesis, we currently do not expect fertility issues among KO males.

It is important to realize that with an embryonic, conditional knock-out mouse model like the Pdyn-Cre/Kiss1^{0/1} KO mice, compensatory effects are quite a possibility in assessing their collective phenotype. In addition to refining some methodology and increasing our n-value to clarify the sexual dimorphism in ARC Kiss1 regulation, future directions include assessing sex steroid negative feedback in Pdyn-Cre/Kiss1^{0/1} KO mice, since ARC Kiss1 responds to estradiol negative feedback (56). To do so, we will perform gonadectomy (GDX) on male and female mice at reproductive maturity at 2 months of age, allow for at least 1 week of recovery, and analyze negative feedback using LH pulsatility as a read-out. Sex steroid administration post-GDX, we will provide insight into whether post-GDX changes can be rescued through sex steroid administration.
Conclusion

The Pdyn-Cre/Kiss1^fl/fl^ KO mouse line, derived using Cre-LoxP technology, resulted in a conditional arcuate-nucleus specific deletion of kisspeptin and for the first time discriminates the role of ARC Kiss1 neurons in the hypothalamic-pituitary-gonadal axis independent of AVPV Kiss1. Our data, demonstrating the normal onset of puberty in the mice bearing a deletion of kisspeptin in the arcuate nucleus is strong evidence that AVPV kisspeptin is the mediator of pubertal onset. Additionally, ARC Kiss1 neurons in female mice are shown to be primarily responsible for normal estrous cyclicity and GnRH pulse generation. The disruption in temporal GnRH/LH pulsatility arrests folliculogenesis in KO females and results in female infertility. Males, however, do not appear to have pulsatile abnormalities and maintain normal spermatogenesis. Thus, this genetically modified mouse model provides evidence that ARC Kiss1 neurons are responsible for GnRH/LH pulsatility, estrous cyclicity, and ultimately, fertility in female mice.
References/Bibliography


Acknowledgement of Previous Publications

Although the data in this manuscript has not been in a previous publication, we acknowledge that the data in this manuscript will be submitted for publication in a scientific research journal.