UNIVERSITY OF CALIFORNIA SAN DIEGO

Estradiol Regulation of *Kiss1* Neurons

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

in

Biology

by

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The Thesis of Reanna Beverly Liaw is approved, and it is acceptable in quality and form for publication on microfilm and electronically:

Co-Chair

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LIST OF ABBREVIATIONS

3V	Third ventricle
ARC	Arcuate nucleus
AVPV	Anteroventral periventricular nucleus
BnST	Bed nucleus of the stria terminalis
CC	Corpus callosum
DT	Diphtheria toxin
DTR	Diphtheria toxin receptor
E_2	Estradiol
ERα	Estrogen receptor α
ERαKO	Estrogen receptor α knockout
ERβ	Estrogen receptor β
ERβKO	Estrogen receptor β knockout
FSH	Follicle-stimulating hormone
GnRH	Gonadotropin-releasing hormone
HPG	Hypothalamic-Pituitary-Gonadal
i.p.	Intraperitoneal
iDTR	Cre-inducible diphtheria toxin receptor mouse strain
ISH	*In situ* hybridization
Kiss1r (Gpr54)	Kisspeptin receptor (G-protein coupled receptor 54)
LH	Luteinizing hormone
LS	Lateral septum
LV	Lateral ventricle

MeA	Medial amygdala
PND	Postnatal day
T	Testosterone
VMH	Ventromedial hypothalamic nucleus
WT	Wildtype

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ABSTRACT OF THE THESIS

Estradiol Regulation of *Kiss1* Neurons

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Reanna Beverly Liaw

Master of Science in Biology

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Professor Alexander S. Kauffman, Chair
Professor Byungkook Lim, Co-Chair

Kisspeptin, encoded by the *Kiss1* gene, is required for reproductive function. There are several distinct *Kiss1* populations in the brain, and how these kisspeptin neurons are regulated is still not fully understood. The two largest hypothalamic *Kiss1* populations reside in the ARC and AVPV and respond to sex steroids in opposing manners, one being negatively regulated and the other being positively regulated, respectively. As a result, in research experiments it has been technically difficult to visualize *Kiss1* expression of both populations in the same animal. Here,

we sought to establish a dose of estradiol (E_2) for which both ARC and AVPV kisspeptin populations can be visualized with *in situ* hybridization. Using this E_2 treatment in conjunction with the inducible diphtheria toxin receptor mouse line, we next aimed to exploit the different developmental ontogenies of the *Kiss1* populations to selectively remove only the ARC *Kiss1* neurons, which have been implicated in the maintenance of the reproductive axis. We discovered that administration of diphtheria toxin (DT) during early juvenile stage to transgenic mice with selective sensitivity to DT led to specific ablation of ARC *Kiss1* neurons. Finally, to address lesser understood *Kiss1* populations outside the hypothalamus, we used two transgenic mouse models lacking either ERα or ERβ and found that E_2 positively regulates *Kiss1* cells in the lateral septum (LS) via ERα. Understanding the role of the ARC and LS *Kiss1* neurons may lead to a better understanding of how the reproductive axis is regulated and provide potential therapeutics for reproductive disorders.

INTRODUCTION

Neuroendocrine control of reproduction

Reproduction serves as the foundation of species survival. Despite its importance, much is still unknown about reproductive control and its initiation during pubertal development. A large milestone in understanding reproduction in vertebrates was the finding that the brain is the control center of reproduction via the secretion of reproductive hormones. A region of the brain known as the hypothalamus sends endocrine signals to the anterior pituitary and in turn, the pituitary signals to the gonads (1). This pathway is known as the hypothalamic-pituitary-gonadal (HPG) axis and serves to regulate fertility in vertebrates (Figure 0.1) (2).

The gonads, testes in males and ovaries in females, are sexually differentiated organs which serve to produce gametes, or spermatozoa and oocytes, respectively. In addition to gametogenesis, the gonads produce and secrete sex steroid hormones, such as testosterone (T) or estradiol (E_2). These sex steroids act to regulate reproductive physiology and behavior, thereby governing reproductive status.

The gonads themselves are regulated by hormones from the pituitary gland. The anterior pituitary produces and secretes two reproductive hormones, known as gonadotropins: luteinizing hormone (LH) and follicle-stimulating hormone (FSH). These gonadotropins act on their receptors in the gonads to signal varying effects (3,4). In females, LH stimulates steroidogenesis, primarily the synthesis of estrogens, which results in the initiation of the processes that trigger ovulation (5). In males, LH stimulates androgen, primarily testosterone, production, which results in spermatogenesis and secondary sexual characteristics (6,7). LH is released from the anterior pituitary in a pulsatile manner and in females, there is an additional preovulatory surge of LH

released midcycle which induces ovulation (8). Prior to the 1970s, a higher regulatory element of the gonadotropins was postulated, but not yet confirmed.

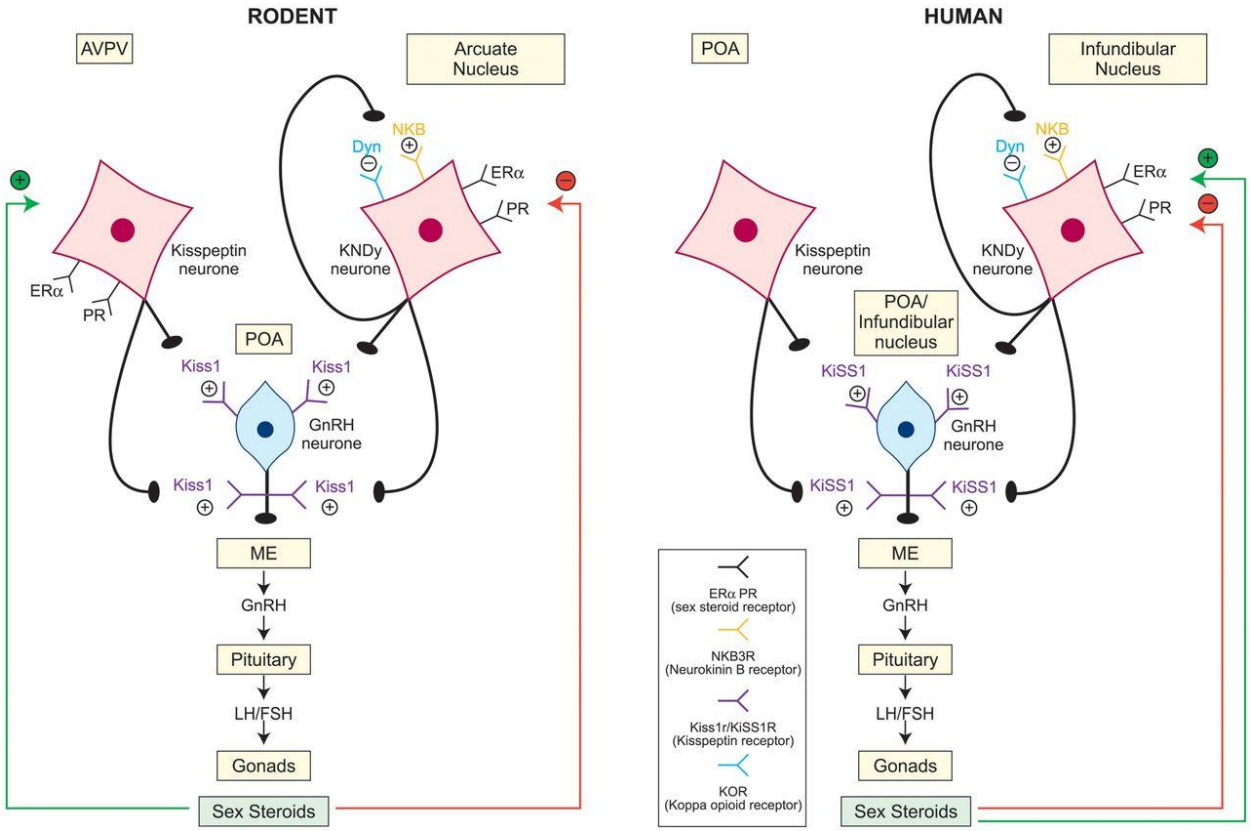

Figure 0.1 Cartoon schematic of the Hypothalamic-Pituitary-Gonadal Axis in the rodent and human. Adapted from Skorupskaite et al., 2014 (9)

Gonadotropin-Releasing Hormone

In the 1970s, the higher neuronal regulator of the reproductive axis, gonadotropin-releasing hormone (GnRH) was discovered (3,10). This peptide hormone has a sequence of ten amino acids which is highly conserved and has been identified in several animal classes including protochordates and vertebrates (11–13). GnRH is secreted by the hypothalamus into the median eminence region of the brain, where it travels through the hypophyseal portal vasculature to the anterior pituitary and signals to its receptor in the gonadotroph cells. Upon signaling, gonadotroph cells produce and secrete LH and FSH. The actions of the gonadotropins are varied, but importantly their signaling causes gonads to produce and secrete sex steroids such as testosterone and estradiol. These sex steroids ultimately feed-back to receptors in the hypothalamus to regulate their own production in a homeostatic feedback loop.

There are two modes by which GnRH is secreted. Under most circumstances, GnRH is released in small, well-timed pulses of approximately 60-90 minutes in frequency. In a second mode of secretion, exclusive to females at a specific stage of their reproductive cycle, GnRH is secreted in a large surge. GnRH pulses have been known to correlate nearly perfectly with downstream LH pulses secreted from the pituitary (14). When the hypothalamus was lesioned in female monkeys, LH pulses and menstrual cyclicity were eliminated (15,16). Interestingly, the menstrual cycles were recovered when exogenous pulsatile GnRH was administered to the monkeys at an appropriate frequency, yet LH secretion was suppressed if the exogenous GnRH pulse frequency was too high or low (17,18). A subsequent study in sheep found that LH pulses are initiated by GnRH pulses (19).

While it was initially believed that the LH surge was the summation of LH pulses, it was discovered that there was a unique GnRH surge secretion event that barely preceded the LH surge

in female sheep (20,21). This GnRH surge has been found in several other animal models to cause the LH surge, thus initiating ovulation (22,23). This LH surge is notably absent in males, which functionally correlates with their lack of ovulatory ability as they do not have ovaries.

Despite understanding that GnRH activates the HPG axis, the mechanism by which the sex steroids feedback and negatively regulate GnRH neurons remained elusive, as it was discovered that GnRH neurons do not have estrogen receptor alpha (ERα) or androgen receptor, the principal receptors for sex-steroid regulation of the HPG axis (24,25). Without such receptors, it was not understood how estrogen (females) or testosterone (males) could feedback to the brain to inhibit GnRH release. It was theorized that there was another level of control upstream of GnRH. Indeed, in 2003, another protein was implicated in reproductive regulation with the discovery of reproductive disorders in humans and mice containing mutations in kisspeptin receptor (Kiss1r), formerly known as GPR54 (26,27).

Kisspeptin control of reproduction

Prior to its implication in the reproductive axis, kisspeptin (encoded by the *Kiss1* gene) was first known as a metastasis-suppressor gene (28). In 2003, separate research groups found that mutations in *Kiss1r* were associated with hypogonadotropic hypogonadism, a reproductive disease which causes infertility. These findings prompted interest in the role of kisspeptin in reproductive function.

In the following years, many experiments in multiple animal models confirmed the involvement of kisspeptin in reproduction, such as induction of an LH surge in rats following kisspeptin administration (29). To determine where kisspeptin might be acting in the brain, researchers looked for where *Kiss1r* was expressed. Fascinatingly, *Kiss1r* mRNA was shown to be expressed, via *in situ* hybridization (ISH), in GnRH neurons with nearly all GnRH neurons

expressing *Kiss1r* (30,31). Kisspeptin administration into the brain was shown to result in abundant in c-Fos expression (a transcription factor expressed upon neuronal activation) in GnRH neurons, suggesting that kisspeptin activates GnRH neurons (31). Later, it was confirmed that kisspeptin can directly stimulate GnRH neuron electrical activity (i.e., action potentials and neuron firing) (30,32). Additionally, kisspeptin administration ultimately leads to increased GnRH secretion, thereby stimulating downstream LH and FSH secretion (33). When kisspeptin was administered simultaneously with GnRH antagonists, the same results were not observed (33), indicating that kisspeptin's effects on LH and FSH are mediated via GnRH secretion.

In the brain, there are several discrete populations of *Kiss1*-expressing neurons which have been mapped via *Kiss1* mRNA expression or kisspeptin protein expression (33–35). In the hypothalamus, the two most prominent *Kiss1* populations are in the arcuate nucleus (ARC) and the anteroventral periventricular nucleus (AVPV). The ARC and AVPV *Kiss1* populations express sex steroid receptors, such as ERα, thus potentially bridging the divide between the gonadal sex steroids and GnRH neurons (36–39). Despite the arrival of a new player, kisspeptin, it was not understood how GnRH secretion was regulated to be either pulses or a large surge. The answer to this may lie in the several ways that the two primary *Kiss1* neuron populations differ, discussed further in depth below.

ARC Kiss1 population

In rodents, embryonic *Kiss1* neuron expression occurs solely in the ARC (40). In mice, the number of *Kiss1* neurons in the ARC is already fairly elevated in early neonatal and postnatal life, but increases to a moderate degree over the course of peri-pubertal development (postnatal day 24 - 30) (41).

In the ARC, kisspeptin neurons are present at high levels in both male and female mice in comparable numbers (i.e., no sex differences) (37,38). Compared to gonad-intact mice, mice that were castrated or ovariectomized (completely removing sex steroids) showed a large increase in kisspeptin expression in the ARC (37,38). Conversely, administration of T or E_2 in castrated male mice and administration of E_2 in ovariectomized female mice led to a significant reduction in kisspeptin expression in the ARC (37,38). Thus, sex steroids lower kisspeptin levels in the ARC. These data have led to a model in which ARC kisspeptin neurons are involved in the negative feedback mechanism of sex steroids which regulates GnRH pulses, which in turn regulate LH pulses.

AVPV Kiss1 population

Unlike ARC Kiss1 neurons, AVPV Kiss1 neurons are not expressed embryonically nor neonatally. The earliest documented onset of AVPV kisspeptin expression in mice occurs around postnatal day 10 to 12 (PND10 – PND12) (42). In female mice, there is a sharp and consistent increase in *Kiss1* expression in the AVPV from PND15 to PND28 (around the age of puberty onset), at which point adult levels of AVPV *Kiss1* expression are reached (41). This peri-pubertal increase in *Kiss1* neurons is more dramatic and of greater magnitude than the increase seen in the ARC.

In contrast to the ARC *Kiss1* neurons, the AVPV population is sexually dimorphic: female mice have ten-fold more kisspeptin neurons in the AVPV than males (34). Sex steroids also affect AVPV *Kiss1* levels. Ovariectomized mice have reduced *Kiss1* mRNA expression in the AVPV and estradiol administration causes a large increase in *Kiss1* expression (37). Though less dramatic, in males, castration also similarly leads to a reduction in *Kiss1* mRNA in the AVPV and testosterone replacement increased *Kiss1* expression (38). Thus, sex steroids stimulate *Kiss1*

expression in the AVPV. These observations were the antithesis of what was seen in the ARC, where sex steroids reduce *Kiss1* levels.

Whereas the ARC *Kiss1* neurons are implicated in regulating GnRH pulses, the AVPV *Kiss1* neurons are implicated in the preovulatory GnRH and LH surge that occurs only in females (36,43–47). AVPV kisspeptin neurons are currently understood to contribute to a positive feedback mechanism in which E_2 and progesterone increase GnRH secretion via activation of the AVPV kisspeptin neurons, resulting in the GnRH surge which helps trigger ovulation. The role of the AVPV *Kiss1* neurons in males is less understood, as males do not exhibit a similar sex steroid positive feedback response.

Extra-hypothalamic Kiss1 populations

There are several *Kiss1* neuron populations in the brain apart from the AVPV and ARC, including *Kiss1* cells in the bed nucleus of the stria terminalis (BnST), lateral septum (LS), and the medial amygdala (MeA) (33,35,48–50). Of these extra-hypothalamic populations, the MeA has been studied the most extensively.

MeA *Kiss1* cells are more prolific in gonad-intact male rodents than female rodents (51). Reminiscent of AVPV *Kiss1* regulation, gonadectomies lead to a large reduction in *Kiss1* levels in the MeA whereas T or E_2 treatment increased *Kiss1* levels. This sex steroid regulation likely occurs via estrogen receptors, as administration of DHT, a nonaromatizable androgen, had no effect on MeA *Kiss1* levels. Unlike the AVPV and ARC in which ERα is expressed in higher amounts than estrogen receptor beta (ERβ), both estrogen receptor subtypes are expressed highly in the MeA (37–39,52,53). In a separate study, it was found that of the two estrogen receptors, ERα is the main regulator of E_2-mediated *Kiss1* changes (54).

Similar to the hypothalamic kisspeptin populations, the LS region only expresses ERα, however the BnST expresses both ERα and ERβ (55). The functions of these LS and BnST kisspeptin populations and the mode of their endocrine regulation have yet to be elucidated.

Study Objectives

Experiment 1

As ARC and AVPV kisspeptin populations respond to sex steroids in opposing manners, it has been difficult experimentally to visualize both populations with adequate mRNA signal in the same animal after given a sex steroid treatment. High sex steroid levels reduce ARC *Kiss1* levels, making the neurons difficult to detect, whereas low sex steroid levels reduce AVPV *Kiss1* levels to low detectability. Here, we seek to establish an experimental "mid-range" dose of E_2 for which both ARC and AVPV *Kiss1* populations can be visualized in the same animal at sufficient levels with *in situ* hybridization.

Experiment 2

The ARC and AVPV kisspeptin populations are implicated in different GnRH-release patterns as well as in different aspects of reproductive events (puberty onset, pre-ovulatory LH surges, LH pulses, etc.). Presently, it is technically challenging to mechanistically disentangle the role of each of these two kisspeptin populations *in vivo* and to determine which is necessary or sufficient for a given reproductive event. One way to address this issue would be to remove just one of the two *Kiss1* populations but leave the other population functionally intact. For example, to determine if the ARC population is necessary and the AVPV population is sufficient for different aspects of reproductive function, it would be beneficial to have a method which could selectively ablate only the ARC *Kiss1* cells while leaving the AVPV *Kiss1* cells intact. In this

experiment, utilizing an inducible diphtheria toxin receptor transgenic mouse line, we will exploit the different developmental ontogenies of the hypothalamic kisspeptin populations to selectively remove only the ARC *Kiss1* neurons but leave the AVPV kisspeptin neurons fully intact. This will ultimately establish a new technical method to selectively ablate one population of kisspeptin neurons while leaving other populations intact and functional.

Experiment 3

Very little is understood about the regulation and role of extra-hypothalamic kisspeptin neurons, including the kisspeptin neurons recently discovered in the LS region. Here, we seek to understand if and how E_2 regulates *Kiss1* cells in the lateral septum (LS) by using two transgenic mouse models lacking ERα (ERαKO) or ERβ (ERβKO) and exploring the receptor pathway by which E_2 may directly regulate *Kiss1* expression in the LS.

METHODS

Animals

Mice were housed two to four females per cage or two to three males per cage on a 12L:12D cycle in a University of California, San Diego vivarium. Mice had access to food and water *ad libitum*. All experiments agreed with guidelines and were approved by the University of California San Diego Institutional Animal Care and Use Committee. Experiment 1 was conducted on wildtype (WT) C57BL6 female mice. Experiment 2 was conducted in female C57BL6 mice on a transgenic background. Experiment 3 was conducted in male C57BL6 mice on a transgenic background.

Experiment 1

At 7 weeks old, female C57BL6 mice were ovariectomized under 4% isoflurane anesthesia and subcutaneously implanted with an estradiol implant containing either 310 ng E_2 or 31 ng E_2. These doses were based on previous studies using a high physiological dose of 625 ng E_2. Estradiol implants are comprised of Silastic tubing of 12-mm length (1.98 mm inner diameter x 3.18 mm outer diameter) filled with 5-mm of 310 ng or 31 ng mixture of 17β-estradiol (Sigma) dissolved in sesame oil.

After one week of E_2 treatment, mice were anesthetized with 4% isoflurane and weighed. Blood was collected from each mouse via retro-orbital bleeding and mice were subsequently rapidly decapitated. Brains were immediately collected and flash-frozen on dry ice and stored at -80°C. Serum was isolated from the blood samples by centrifugation (5000 rpm x 15 min) and stored at -20°C until sent for hormone measurement.

Experiment 2

Female Kiss1 Cre mice (KissCre; provided by Dr. Carol Elias) were bred with male mice containing Cre-inducible diphtheria toxin receptor (iDTR; Jackson Labs). Wildtype mice do not have diphtheria toxin receptors (DTR). The iDTR mice have loxP sites which flank a transcriptional STOP cassette upstream of a gene encoding DTR. Thus, these mice do not normally express DTR due to the presence of the STOP cassette. However, in the presence of Cre recombinase, the STOP cassette is removed and DTR can be expressed.

The iDTR mice were first crossed with Kiss1 Cre mice, then the heterozygous offspring were backcrossed to iDTR mice to yield KissCre+ iDTR fl/fl and KissCre- iDTR fl/fl (control) mice. KissCre+ mice express Cre recombinase only in *Kiss1* cells, thus KissCre+ iDTR fl/fl mice express DTR specifically in *Kiss1* cells. When KissCre+ iDTR fl/fl mice are treated with diphtheria toxin (DT), these DTR-expressing cells undergo cell death (Figure 0.2).

Mice were injected intraperitoneally (i.p.) with 750 ng of DT (dissolved in saline) on PND12 and PND13. The timing of the DT treatment was chosen based on previous studies observing the development of ARC and AVPV *Kiss1* cells, which found that at PND12 and PND13, a majority of ARC *Kiss1* cells have developed while a majority of AVPV *Kiss1* cells have not developed (41,42). As a result, DT treatment in KissCre+ mice at PND12 and PND13 should result in the ablation of ARC *Kiss1* cells while AVPV *Kiss1* cells are left intact.

At 7 weeks old, mice were ovariectomized under 4% isoflurane anesthesia and subcutaneously implanted with an estradiol implant containing 31 ng E_2. Estradiol implants are comprised of Silastic tubing of 12-mm length (1.98 mm inner diameter x 3.18 mm outer diameter) filled with 5-mm of 31 ng of 17β-estradiol (Sigma) dissolved in sesame oil.

After one week of E_2 treatment, mice were anesthetized with 4% isoflurane and weighed. Blood was collected from each mouse via retro-orbital bleeding and mice were subsequently rapidly decapitated. Brains were immediately collected and flash-frozen on dry ice and stored at -80°C. Serum was isolated from the blood samples by centrifugation (5000 rpm x 15 min) and stored at -20°C until sent for hormone measurement.

Experiment 3

Heterozygous ERαKO mice (Chambon Lab) were bred to produce ERαKO and WT mice. To briefly explain the generation of these mice, the ERαKO mice were created using Cre-Lox technology (56). LoxP sites flank exon 3 of the ERα sequence, which encodes the first zinc finger of the DNA-binding domain of ERα. Male ERα mice with the targeted allele were crossed with CMV-Cre transgenic mice. CMV-Cre is expressed embryonically in all cells (57), thus the resulting mice of the cross have a ubiquitous deletion of ERα.

Heterozygous ERβKO mice (ERβKO, Jackson Labs) were bred to produce ERβKO and WT mice (Experiment 3). To generate these ERβKO mice, the Korach laboratory inserted a neomycin resistance gene in the reverse orientation into exon 3 of the ERβ gene, which introduces a stop codon in exon 3 resulting in premature termination of translation of ERβ mRNA (58).

At 7 weeks old, male ERαKO, ERβKO, and WT littermates were gonadectomized under 4% isoflurane anesthesia. One-week post-surgery, half of the mice received a subcutaneous implant of 8-mm length Silastic tubing (1.47 mm inner diameter x 1.96 mm outer diameter) containing 2-mm of a 1:25 mixture of β-estradiol in cholesterol (230 μg E_2). After 5 days of E_2 treatment, mice were anesthetized with 4% isoflurane and blood and brains were collected as in Experiments 1 and 2.

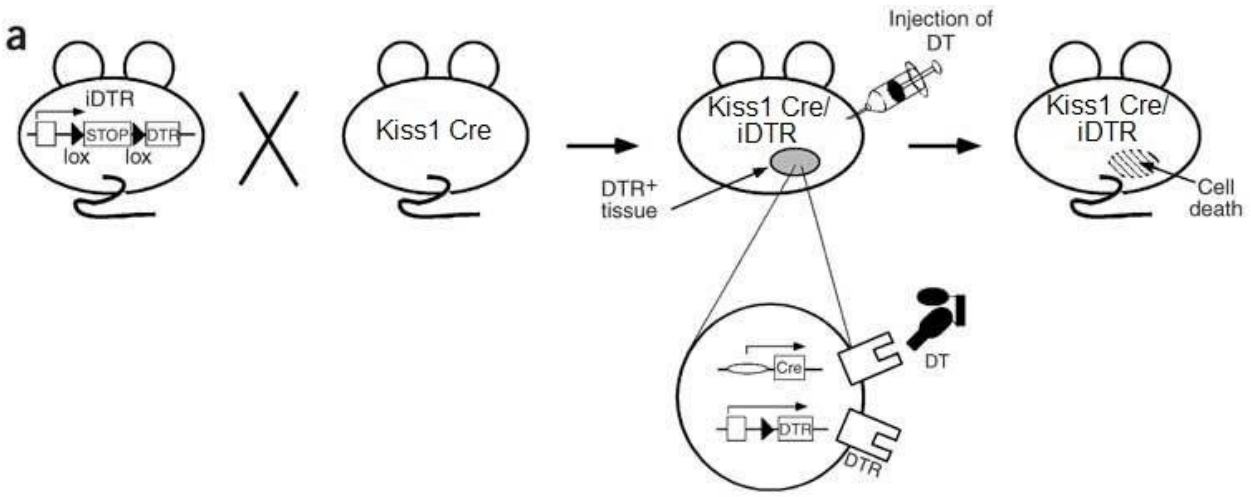

Figure 0.2 Cartoon schematic of the Cre-inducible diphtheria toxin mouse model. The STOP cassette upstream of the DTR gene is removed when the mouse is crossed with a Cre-expressing mouse. This results in the selective expression of DTR. Upon injection of DT, the Cre-expressing tissue are ablated. Adapted from Buch et al., 2005 (59).

Hormone measurements

All blood serum hormone levels were measured by the University of Virginia Ligand Assay Core (Charlottesville, VA). Serum LH was measured by a sensitive mouse radioimmunoassay (sensitivity: 0.04 ng/mL; average reportable range: 0.04 – 75 ng/mL). Serum E_2 was measured using a mouse enzyme-linked immunosorbent assay (sensitivity: 3 pg/mL; average reportable range: 3 – 300 pg/mL).

Single-label in situ hybridization

Frozen brains were cryosectioned into five sets of 20 μm sections, thaw-mounted onto Superfrost-plus slides, and stored at -80°C. One set of slides encompassing the entire AVPV and ARC (Experiments 1 and 2) or LS (Experiment 3) was used. Slides were fixed in 4% paraformaldehyde, pretreated with acetic anhydride, and rinsed in 2x sodium citrate/sodium chloride (SSC) solution. Subsequently, slides were delipidated in chloroform, dehydrated via ethanol washes (70%-100%), and allowed to air-dry for 90 minutes. Radiolabeled (P^{33}) antisense *Kiss1* riboprobe (0.04 pmol/mL) were added to yeast tRNA, heat-denatured, and combined with hybridization buffer to create a probe mixture. Following the application of 100 μL of probe mixture to each slide, the slides were coverslipped and allowed to hybridize in a humidity chamber at 55°C overnight. After hybridization, slides were washed in 4x SSC and placed into RNase A for 30 minutes at 37°C. Slides were washed in RNase buffer at 37°C for 30 minutes and in 2x SSC at room temperature for 30 minutes. Slides were then washed in 0.1x SSC at 62°C for one hour, dehydrated in room temperature ethanol washes, and air dried for 90 minutes. Slides were dipped in Kodak nitroblue tetrazolium salt emulsion, air dried for 90 minutes, and then stored at 4°C until development. Slides were developed 5-6 days later (depending on the assay) and coverslipped.

Quantification and analysis of ISH data

To quantify *Kiss1* expression for each slide, an automated custom grain-counting software (Dr. Don Clifton, University of Washington, Seattle, Washington) was used. The program counts the number of silver grain clusters, which represent cells expressing *Kiss1* mRNA, whilst considering the background staining. All slides were analyzed blind to treatment.

Statistical analysis

All data are expressed as the mean ± SEM for each group. For Experiments 1 and 2, differences among groups were assessed with a two-tailed unpaired t test. For Experiment 3, differences among groups were assessed with a two-way ANOVA and Bonferroni post-hoc test. Differences were considered significant when $p < 0.05$.

RESULTS

Experiment 1: A 31 ng E_2 dose allows for adequate visualization of both ARC and AVPV Kiss1 cells compared to a 310 ng E_2 dose

This experiment sought to determine a dosage of E_2 that would allow for ISH visualization of both ARC and AVPV *Kiss1* regions in the same animal. In adult female mice, as expected, circulating serum E_2 levels were significantly higher with the 310 ng E_2 dose compared to the 31 ng E_2 dose (Figure 1.1a). Serum LH levels were also significantly lower in mice treated with the higher dose of 310 ng E_2 compared to those treated with the lower E_2 dose, as predicted since LH levels are typically reduced by E_2-induced negative feedback (Figure 1.1b).

Brains were analyzed for *Kiss1* mRNA levels after mice were treated for one week with one of two E_2 doses. *Kiss1* expression was observed in both the ARC and AVPV with either the 310 ng E_2 or 31 ng E_2 dose (Figure 1.2), but the degree of *Kiss1* expression varied based on dose. In the ARC, few ARC *Kiss1* cells were detected with the higher dose (310 ng) of E_2, resulting in low visualization of this kisspeptin population. In contrast, the number of detectable *Kiss1* cells and the amount of *Kiss1* mRNA/cell was elevated following 31 ng E_2 treatment in comparison to the 310 ng E_2 treatment ($p < 0.05$) (Figure 1.3). In the AVPV, the reverse pattern occurred, with a significant decrease in the number of *Kiss1* cells and mRNA/cell following 31 ng E_2 treatment compared to the 310 ng E_2 treatment (Figure 1.3). Even so, the AVPV *Kiss1* expression with 31 ng E_2 was still elevated, ensuring proper visualization of the cells in this region. Thus, the 31 ng E_2 dose resulted in adequate numbers of detectable *Kiss1* cells in both the ARC and AVPV, unlike the 310 ng E_2 dose which only permitted detection in the AVPV.

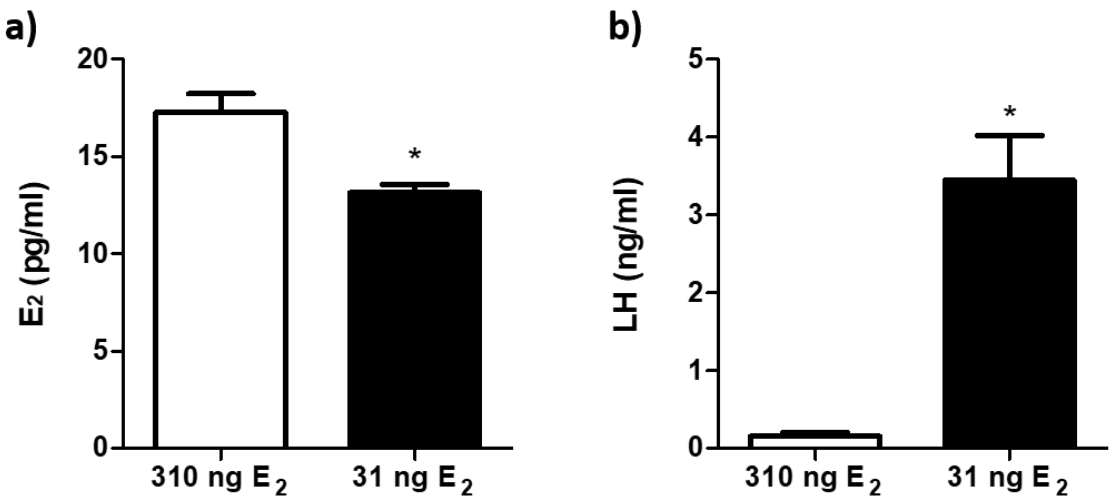

Figure 1.1 The mean and SEM serum (a) E_2 and (b) LH levels for female mice treated with 310 ng E_2 or 31 ng E_2. n = 4 mice per group. Asterisk denotes significant differences between the two groups treated with two different E_2 doses ($P < 0.05$)

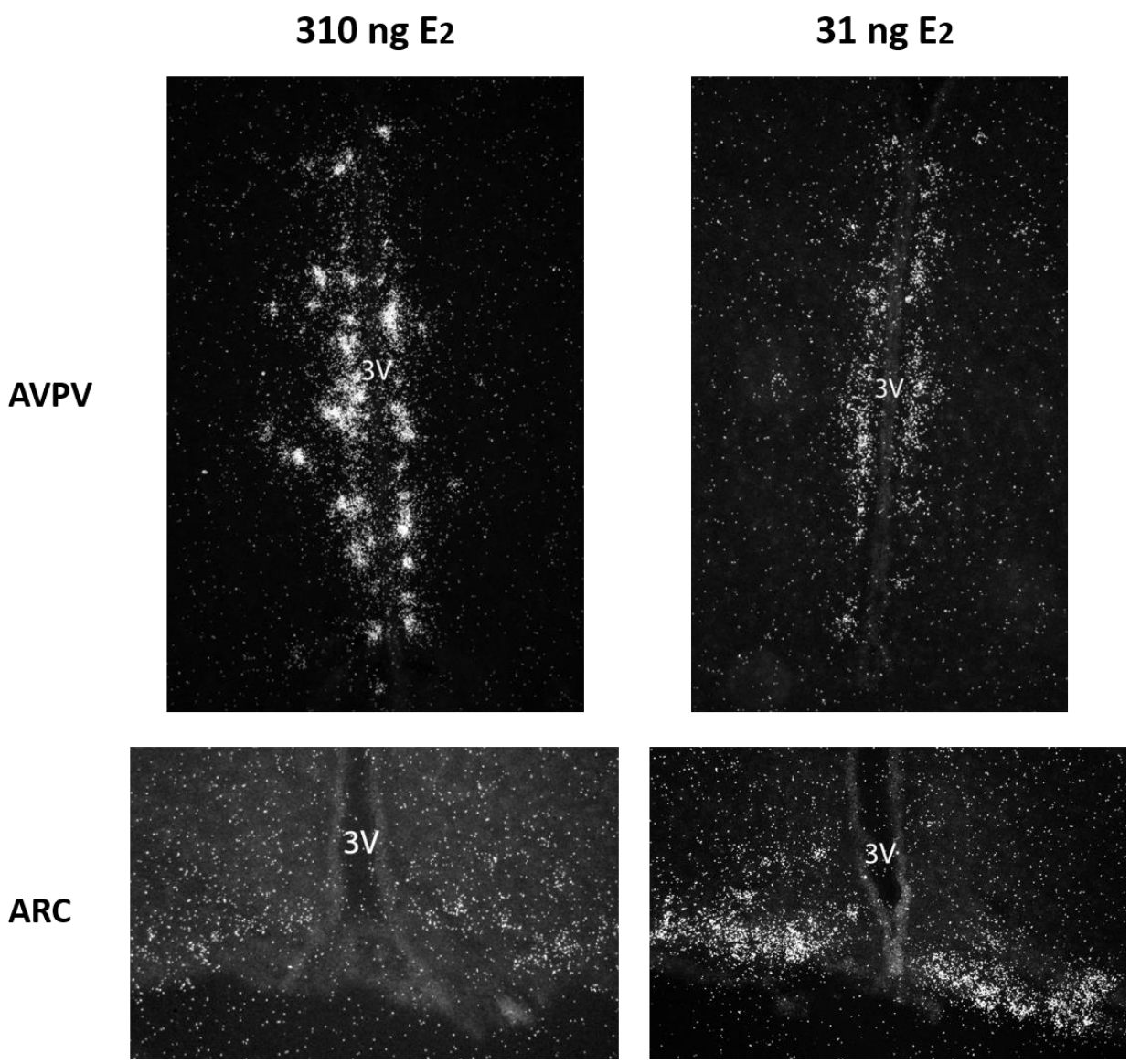

Figure 1.2 Representative photomicrographs of *in situ* hybridization for *Kiss1* mRNA in the ARC and AVPV of mice given a dose of 310 ng E_2 or 31 ng E_2. 3V = third ventricle

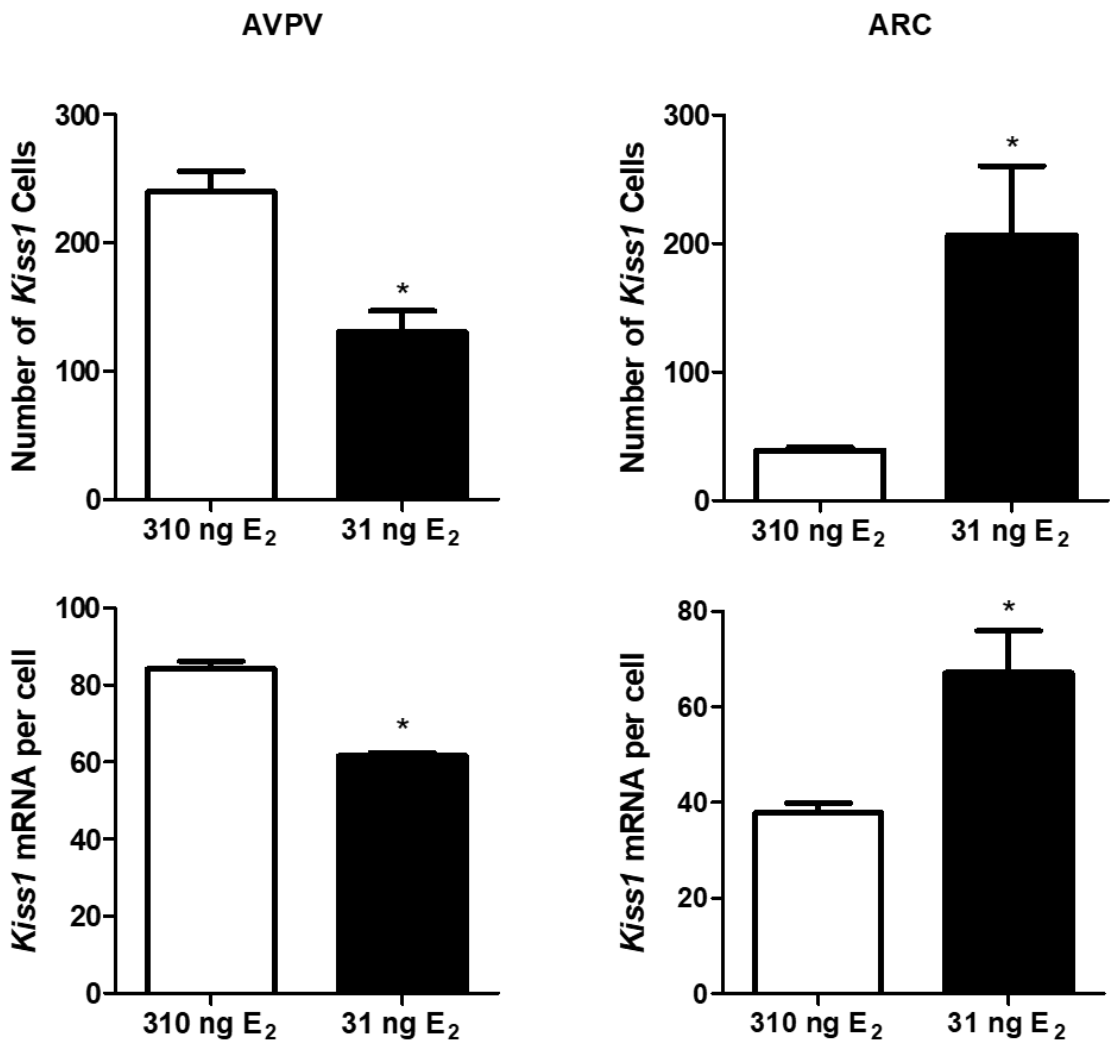

Figure 1.3 The mean and SEM number of Kiss1 mRNA-positive cells and grains per cell in the AVPV and ARC of mice given a dose of 310 ng E_2 or 31 ng E_2. n = 4 mice per group. Asterisk denotes significant differences between the two groups treated with different E_2 doses ($P < 0.05$)

Experiment 2: Diphtheria toxin administration in PND12 and PND13 female mice causes a large reduction in adult ARC Kiss1 cells, but not adult AVPV Kiss1 cells

This experiment sought to determine a time and dosage of DT administration which would allow ARC *Kiss1* cells to be ablated but leave AVPV *Kiss1* cells intact. Brains of adult animals that had been treated with DT in juvenile life (PND12 – PND13) were examined for *Kiss1* expression levels in both hypothalamic regions using ISH. At PND12 and PND13, a majority of ARC *Kiss1* cells have developed while a majority of AVPV *Kiss1* cells have not yet developed (41,42). Based on the developmental ontogenies of the two hypothalamic *Kiss1* populations, DT treatment in KissCre+ mice at PND12 and PND13 should result in the ablation of only ARC *Kiss1* cells while AVPV *Kiss1* cells are left intact. In Kiss1 Cre- animals (controls, no DTR expressed), *Kiss1* expression was observed at detectable levels in both the AVPV and ARC regions (Figure 2.1). In contrast, Kiss1 Cre+ mice (which express DTR in *Kiss1* cells) had dramatically reduced *Kiss1* expression in the ARC. Quantification showed that there was a significant difference in the number of ARC *Kiss1* cells of Cre+ DT-treated animals in comparison to Cre- DT-treated animals. However, in contrast, AVPV *Kiss1* expression did not show a significant reduction. Indeed, in the AVPV, there was no significant difference in number of *Kiss1* cells nor amount of *Kiss1* mRNA/cell between the Cre- and Cre+ animals (Figure 2.2). There was no significant difference in *Kiss1* mRNA/cell in the ARC of Cre- and Cre+ cells treated with DT, indicating that any non-ablated cells in the Cre+ mice were expressing *Kiss1* at normal levels.

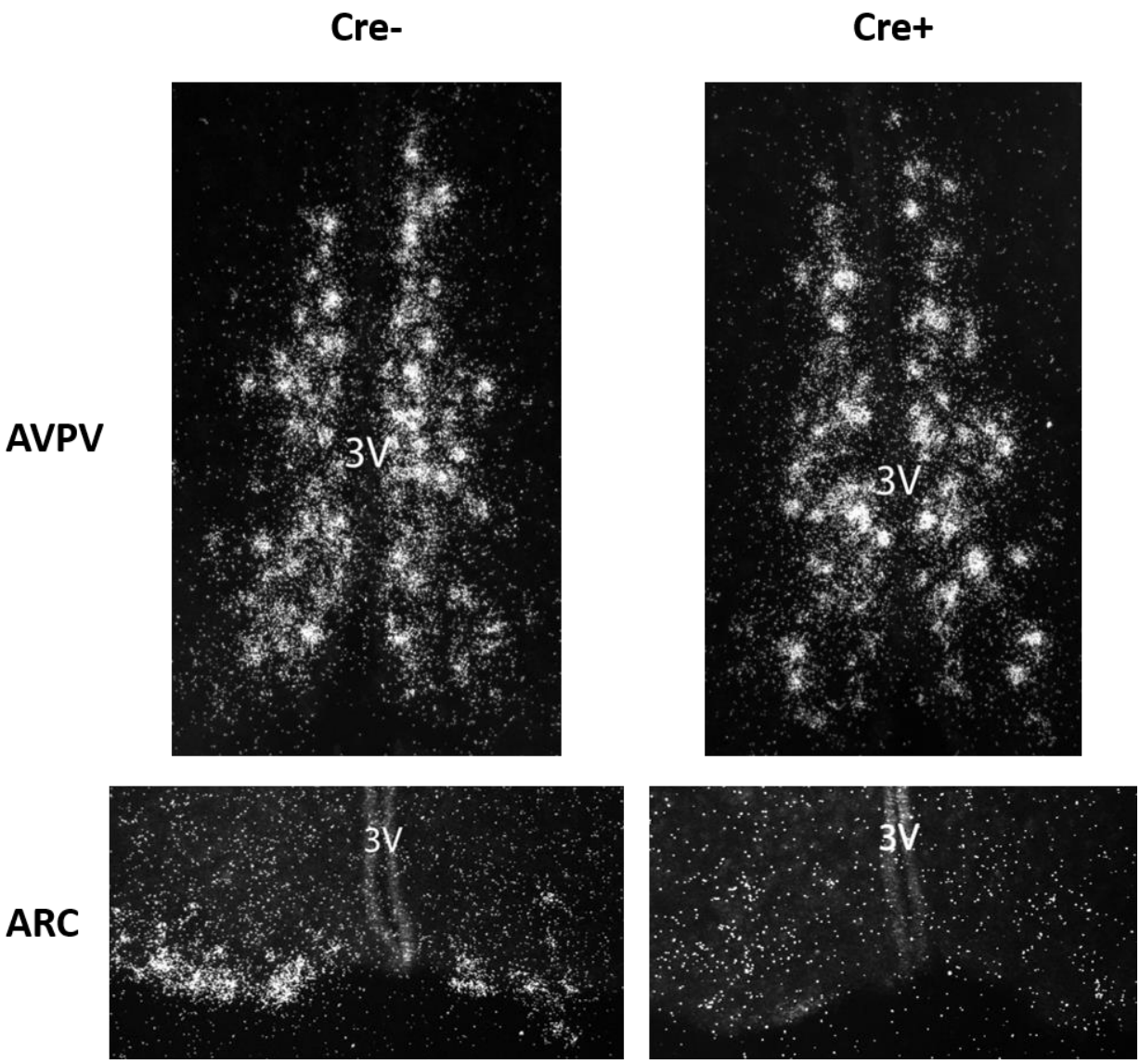

Figure 2.1 Representative photomicrographs of *in situ* hybridization for *Kiss1* mRNA in the AVPV and ARC of adult KissCre- and Cre+ mice treated with DT at PND12 and PND13. 3V = third ventricle

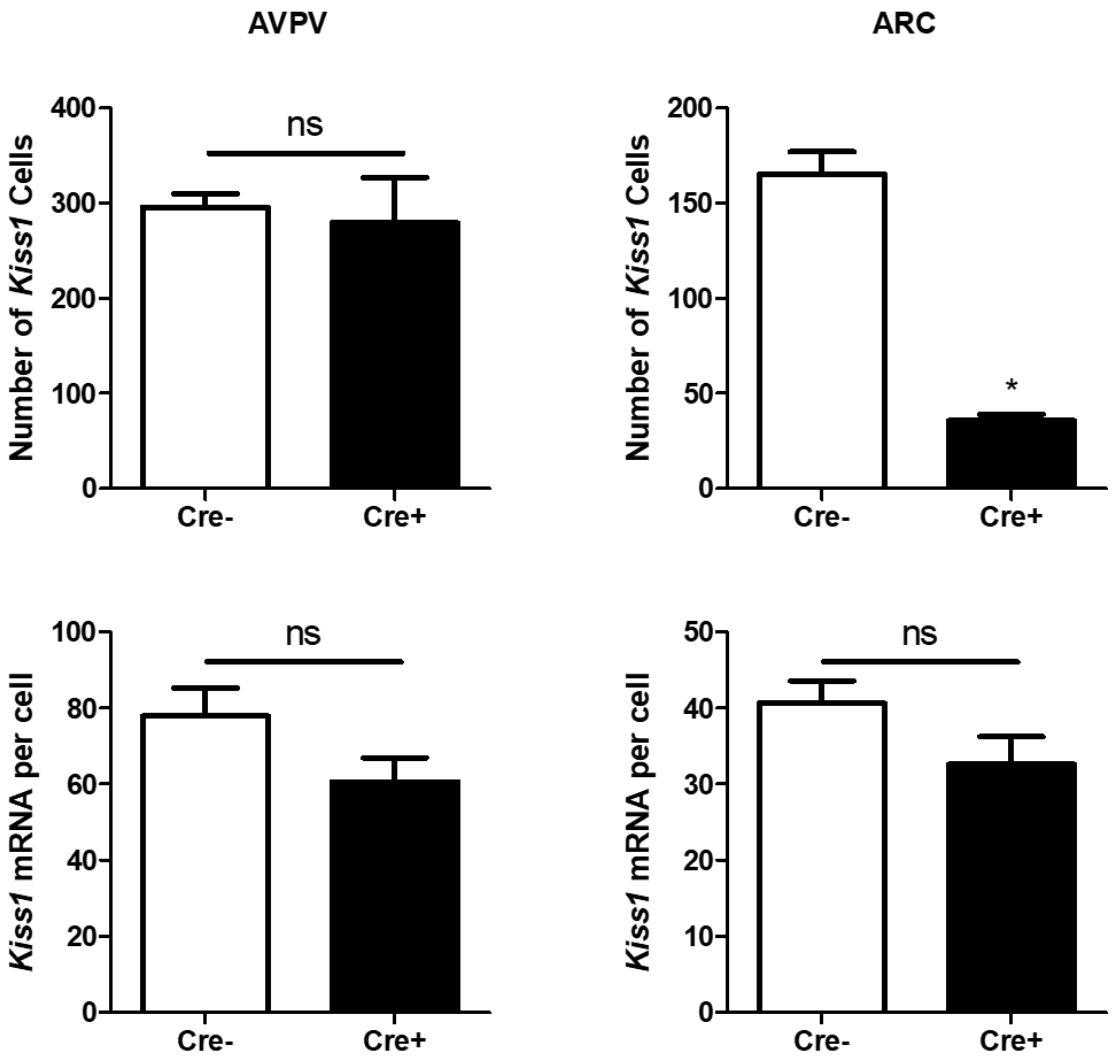

Figure 2.2 The mean and SEM number of Kiss1 mRNA-positive cells and grains per cell in the AVPV and ARC of adult KissCre- and Cre+ mice treated with DT at PND12 and PND13. n = 2 mice per group. Asterisk denotes significant differences between the two groups ($P < 0.05$)

Experiment 3: E_2 increases Kiss1 expression in the LS via ERα, not ERβ

It is unknown if E_2 regulates *Kiss1* levels in the LS, and if so, if this effect is stimulatory or inhibitory. This experiment sought to determine the effects of E_2 on *Kiss1* expression in the LS and if an effect was observed, through which estrogen receptor was the change mediated. E_2 treatment led to a significant increase in number of *Kiss1* cells in the LS in gonadectomized WT and ERβKO mice (Figure 3), indicating that E_2 has stimulatory effects on LS *Kiss1* expression. In contrast, there was no increase in the number of *Kiss1* cells in the LS in gonadectomized ERαKO mice following E_2 treatment. This indicates that ERα is necessary for E_2 to stimulate *Kiss1* in the LS.

Experiment 3 has been accepted for publication in *Endocrinology* (August 2018). The thesis author contributed to quantification and analysis of ISH data. Shannon Stephens and Noelia Di Giorgio were the primary investigators and authors of the manuscript. Ruby Parra, Jennifer Yang, and Navdeep Chahal contributed to quantification and analysis of ISH data. Victoria Lux-Lantos and Alexander Kauffman oversaw the project and assisted with statistical analysis and manuscript preparation.

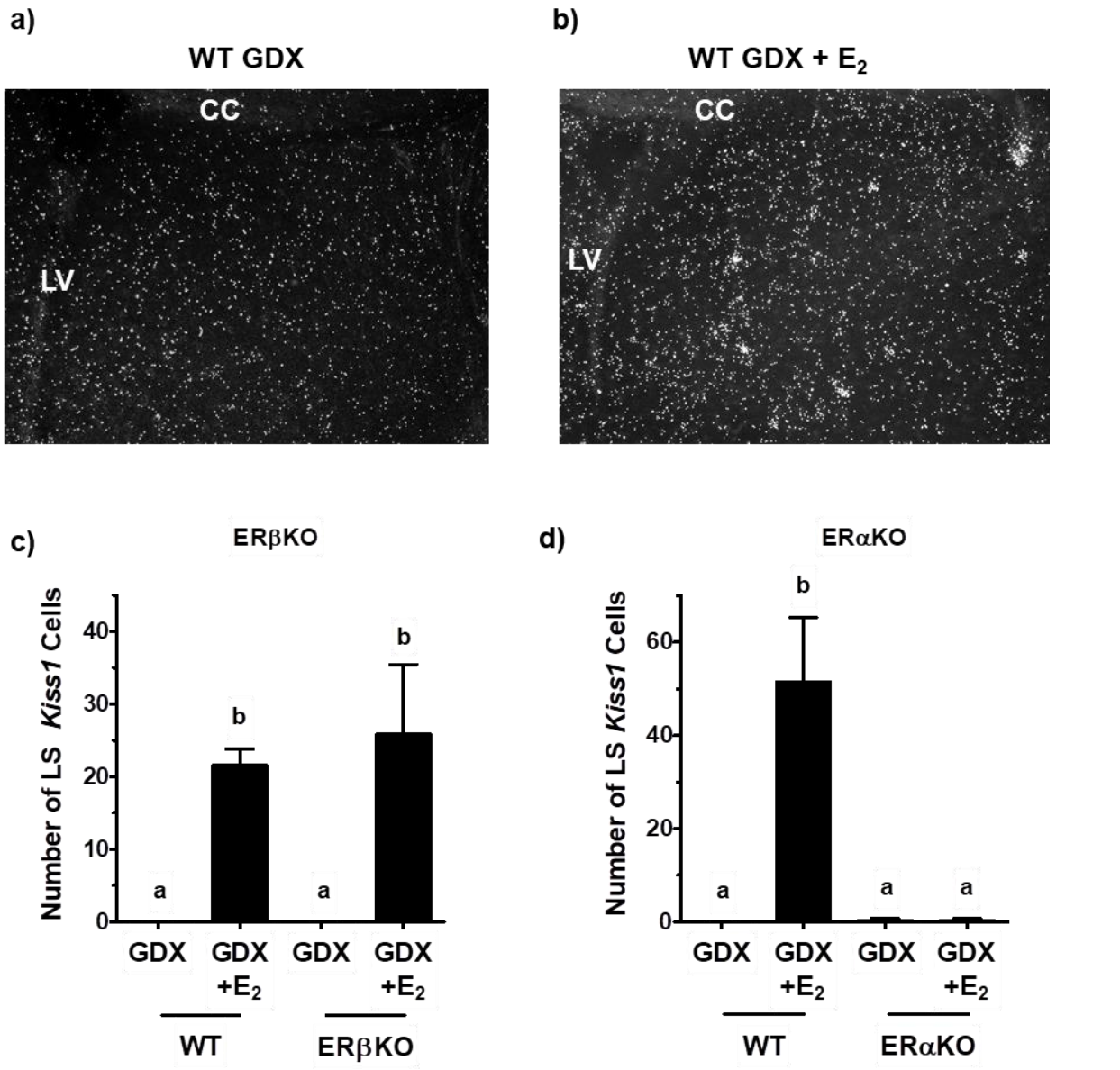

Figure 3 *Kiss1* mRNA expression in the lateral septum (LS) of ERβKO and ERαKO mice. (a) and (b) are representative images of LS *Kiss1* expression in a gonadectomized WT male and WT E2-treated male, respectively. The mean and SEM number of *Kiss1* mRNA-positive cells in the LS of gonadectomized WT and ERβKO mice without and with E2 (c) and of gonadectomized WT and ERαKO mice without and with E2 (d). n=6-9 mice per group. Different letters denote significant group differences ($P < 0.05$). CC = corpus callosum; LV = lateral ventricle

DISCUSSION

Kisspeptin has been shown to be critical for regulating reproduction, initiating puberty, and influencing the secretion patterns of GnRH. Despite knowing that kisspeptin plays a role in these processes, there is still much that is not known about how the kisspeptin neurons themselves are regulated. The *Kiss1* neurons are located in several regions of the brain, with the two largest populations being in the ARC and AVPV nuclei of the hypothalamus. These two *Kiss1* neuron populations differ in several ways, including in their response to sex steroids. This difference in their response to sex steroids and several other functional differences may provide a clue for their different roles in reproduction. The ARC is negatively regulated by sex steroids while the AVPV is positively regulated by sex steroids. As a result, it has been difficult to adequately visualize *Kiss1* expression of both populations in the same animal as the absence of sex steroids leads to strong expression of *Kiss1* in the ARC but little to no expression in the AVPV while a high dose of sex steroids leads to strong expression in the AVPV but little to none in the ARC. Here, we demonstrate that a dose of 31 ng E_2 may be an optimal dose to allow for visualization of both ARC and AVPV *Kiss1* populations via ISH. In addition, using this E_2 dose in conjunction with the KissCre-iDTR mouse model, we found a timing and dosage of DT which would selectively ablate just ARC *Kiss1* neurons when administered during the early juvenile stage. Finally, to address the lesser understood extrahypothalamic *Kiss1* populations, we used two transgenic mouse models lacking either ERα or ERβ and found that estradiol positively regulates *Kiss1* cells in the LS via ERα

Experiment 1

Mice that were treated with the higher 310 ng E_2 dose had increased visualization of AVPV *Kiss1* cells but had fewer visible ARC *Kiss1* cells in comparison to mice treated with the lower 31

ng E_2 dose. The 31 ng E_2 dose resulted in good visualization in both the ARC and AVPV *Kiss1* populations.

As a result, this dose can be used to validate models in which one of the hypothalamic populations is expected to be present while another is not. For example, in the case of Experiment 2, as we know that the 31 ng E_2 dose allows for visible ARC and AVPV *Kiss1* cells, we can determine if the number of ARC *Kiss1* cells are decreased due to successful ablation rather than due to the negative feedback of E_2 on the ARC population.

Experiment 2

DT administration at PND12 and PND13 in KissCre+ female mice resulted in decreased *Kiss1* expression in the adult ARC but did not significantly change AVPV *Kiss1* expression compared to DT-treated KissCre- control animals. This confirms this timing and dosage paradigm as a method for selectively ablating a majority of ARC *Kiss1* cells.

A prior study done by the O'Byrne Lab in 2015 attempted to knockdown the expression of kisspeptin in the ARC or AVPV regions in female rats by using bilateral stereotaxic injections of recombinant adeno-associated virus encoding kisspeptin antisense into either region. They found that a 32% knockdown of kisspeptin in the ARC did not alter pubertal onset but decreased both LH pulse frequency and LH surge amplitude (60). The lack of effect on puberty may be because too many kisspeptin cells were remaining (68%) and potentially able to confer full functionality for pubertal activation. Our new iDTR technique allows for a much greater knockdown (78%) than the 32% used in the rat study.

Our i.p. injections are also less intrusive than stereotaxic injections and are successful in permanently ablating the ARC *Kiss1* population, an effect that is conserved into adulthood. This

more robust ARC *Kiss1* knockdown model would allow us to study the necessity of ARC *Kiss1* cells in the different secretion patterns of GnRH, such as the pulse and surge patterns. Our technique is also less invasive, as it only requires two i.p. injections versus bilateral stereotaxic brain injections used in the rat study. This is critical when studying young, prepubertal rodents of small size.

This new model could potentially allow us to study the onset of puberty as well as reproductive phenotypes in mice with ablated ARC *Kiss1* cells. Although the O'Byrne study did not find an affect of ARC *Kiss1* knockdown on pubertal onset, perhaps a knockdown of greater magnitude would lead to a difference in puberty onset. However, there are limitations to this as our study also found that Cre recombinase is expressed in *Kiss1* cells of the ovaries at PND10 and potentially earlier (not shown).

Kisspeptin in the ovaries have been implicated in the control of follicular and oocyte development, steroidogenesis, and ovulation (61). Injecting female mice at PND12 and PND13 i.p. with DT may also result in the removal of *Kiss1* cells in the ovary, though this is not confirmed. As a result, if impaired fertility were observed, there would be a confounding variable of whether the phenotype is due to ablated ARC or ovarian *Kiss1* cells. To circumvent this, DT may be administered directly into the brain via intracerebroventricular injection, though this method is more invasive in nature.

Experiment 3

It was previously unknown if E_2 has any effect on *Kiss1* in the LS region. We found that E_2 increases expression of *Kiss1* in the LS of male mice and ERα is necessary for this regulatory effect. While the population of *Kiss1* cells in the LS have only recently been discovered, the LS has been closely studied since the 1970s. In a 1997 study, it was found that there are projections from the LS to the medial hypothalamus (62), and some specifically to the ventromedial hypothalamus (VMH), which has been implicated in feeding and sexual activity (63).

Lesions to the VMH in female rats lead to the absence of lordosis, a reflex for sexual receptivity in female rodents (64). Estradiol treatment in castrated males coupled with lesions in the LS of male rats also led to lordosis behavior, which is atypical of male rodent behavior (65). As septal lesions also facilitated lordosis in female and androgen-sterilized female rats, it appears that the LS is responsible for inhibiting lordosis (66). Of interest, the same study found no effect of septal lesions on gonadotropin release, thus the neurons in this region are not likely to contribute to the regulating the HPG axis.

Direct implantation of E_2 into the LS stimulated lordosis behavior in female rats, but did not alter activity in male rats given the same implant (67). This suggests that there is a sex difference in the septal inhibition of sexual receptivity. It is still not understood how estradiol regulates the LS inhibition of lordosis and why there is a resulting sex difference (68), or if kisspeptin neurons in the LS are involved in these sex behaviors.

A study in kisspeptin neurons found that kisspeptin increases mating and sexual behavior in female mice (69). This coupled with our understanding of LS as an inhibitor of sexual behavior suggests that the LS *Kiss1* population may be responsible for regulating the LS inhibition of

lordosis, perhaps by inhibiting the VMH. We hypothesize that E_2 increases *Kiss1* expression, which may increase female-like sexual behavior.

Our study only looked at the LS of males. Future experiments should study female *Kiss1* expression in the LS to determine if *Kiss1* is the missing link that is responsible for the difference in sexual response to E_2 in male and female mice. Understanding how the LS *Kiss1* population may be involved with sexual behavior could lead to a potential therapeutic for treating women with hypoactive sexual desire disorder or similar dysfunctions, especially as humans also have *Kiss1* expression in the LS with kisspeptin fibers leading to the VMH (70,71).

REFERENCES

1. Hohlweg W, Junkmann K. Die Hormonal-Nervöse Regulierung der Funktion des Hypophysenvorderlappens. *Klin Wochenschr*. 1932.

2. Sower SA, Freamat M, Kavanaugh SI. The origins of the vertebrate hypothalamic-pituitary-gonadal (HPG) and hypothalamic-pituitary-thyroid (HPT) endocrine systems: New insights from lampreys. General and Comparative Endocrinology. 2009.

3. Schally A V, Arimura A, Kastin AJ, Matsuo H, Baba Y, Redding TW, Nair RM, Debeljuk L, White WF. Gonadotropin-releasing hormone: one polypeptide regulates secretion of luteinizing and follicle-stimulating hormones. *Science* [Internet]. 1971 Sep 10 [cited 2018 Jul 3];173(4001):1036–8. Available from: http://www.ncbi.nlm.nih.gov/pubmed/4938639

4. Schally A V., Arimura A, Baba Y, Nair RMG, Matsuo H, Redding TW, Debeljuk L, White WF. Isolation and properties of the FSH and LH-releasing hormone. *Biochem Biophys Res Commun*. 1971.

5. Young JM, McNeilly AS. Theca: The forgotten cell of the ovarian follicle. Reproduction. 2010.

6. Walker WH. Testosterone signaling and the regulation of spermatogenesis. *Spermatogenesis*. 2011.

7. Nef S, Parada L. Hormones in male sexual development. *Genes Dev*. 2000.

8. Savoy-Moore RT, Schwartz NB. Differential control of FSH and LH secretion. *Int Rev Physiol* [Internet]. 1980 [cited 2018 Aug 7];22:203–48. Available from: http://www.ncbi.nlm.nih.gov/pubmed/6104650

9. Skorupskaite K, George JT, Anderson RA. The kisspeptin-GnRH pathway in human reproductive health and disease. Human Reproduction Update. 2014.

10. Baba Y, Matsuo H, Schally A V. Structure of the porcine LH- and FSH-releasing hormone. II. Confirmation of the proposed structure by conventional sequential analyses. *Biochem Biophys Res Commun*. 1971.

11. Gorbman A, Sower SA. Evolution of the role of GnRH in animal (Metazoan) biology. General and Comparative Endocrinology. 2003.

12. Okubo K, Nagahama Y. Structural and functional evolution of gonadotropin-releasing hormone in vertebrates. Acta Physiologica. 2008.

13. Morgan K, Millar RP. Evolution of GnRH ligand precursors and GnRH receptors in protochordate and vertebrate species. General and Comparative Endocrinology. 2004.

14. Krsmanovic LZ, Martinez-Fuentes AJ, Arora KK, Mores N, Tomić M, Stojilkovic SS, Catt KJ. Local regulation of gonadotroph function by pituitary gonadotropin-releasing hormone. *Endocrinology*. 2000.

15. Knobil E, Plant TM, Wildt L, Belchetz PE, Marshall G. Control of the rhesus monkey menstrual cycle: Permissive role of hypothalamic gonadotropin-releasing hormone. *Science (80-).* 1980.

16. Dierschke DJ, Bhattacharya AN, Atkinson LE, Knobil E. Circhoral oscillations of plasma LH levels in the ovariectomized rhesus monkey. *Endocrinology*. 1970.

17. Wildt L, Häusler A, Marshall G, Hutchison JS, Plant TM, Belchetz PE, Knobil E. Frequency and amplitude of gonadotropin-releasing hormone stimulation and gonadotropin secretion in the rhesus monkey. *Endocrinology*. 1981.

18. Pohl CR, Richardson DW, Hutchison JS, Germak JA, Knobil E. Hypophysiotropic signal frequency and the functioning of the pituitary-ovarian system in the rhesus monkey. *Endocrinology*. 1983.

19. Moenter SM, Brand RM, Rees Midgley A, Karsch FJ. Dynamics of gonadotropin-releasing hormone release during a pulse. *Endocrinology*. 1992.

20. Moenter SM, Brand RC, Karsch FJ. Dynamics of gonadotropin-releasing hormone (Gnrh) secretion during the gnrh surge: Insights into the mechanism of gnrh surge induction. *Endocrinology*. 1992.

21. Moenter SM, Caraty A, Karsch FJ. The estradiol-induced surge of gonadotropin-releasing hormone in the Ewe. *Endocrinology*. 1990.

22. Freeman ME. Neuroendocrine control of the ovarian cycle of the rat. In: Knobil and Neill's Physiology of Reproduction. 2006.

23. Goodman RL, Inskeep EK. Chapter 44 - Neuroendocrine Control of the Ovarian Cycle of the Sheep. In: Knobil and Neill's Physiology of Reproduction (Third Edition). 2006.

24. Hrabovszky E, Steinhauser A, Barabas K, Shughrue PJ, Petersen SL, Merchenthaler I, Liposits Z. Estrogen receptor-beta immunoreactivity in luteinizing hormone-releasing hormone neurons of the rat brain. *Endocrinology*. 2001.

25. Herbison AE, Theodosis DT. Localization of oestrogen receptors in preoptic neurons containing neurotensin but not tyrosine hydroxylase, cholecystokinin or luteinizing hormone-releasing hormone in the male and female rat. *Neuroscience*. 1992.

26. de Roux N, Genin E, Carel J-C, Matsuda F, Chaussain J-L, Milgrom E. Hypogonadotropic hypogonadism due to loss of function of the KiSS1-derived peptide receptor GPR54. *Proc Natl Acad Sci*. 2003.

27. Seminara SB, Messager S, Chatzidaki EE, Thresher RR, Acierno JS, Shagoury JK, Bo-Abbas Y, Kuohung W, Schwinof KM, Hendrick AG, Zahn D, Dixon J, Kaiser UB, Slaugenhaupt SA, Gusella JF, O'Rahilly S, Carlton MBL, Crowley WF, Aparicio SAJR, Colledge WH. The *GPR54* Gene as a Regulator of Puberty. *N Engl J Med*. 2003.

28. Lee JH, Miele ME, Hicks DJ, Phillips KK, Trent JM, Weissman BE, Welch DR. KiSS-1, a novel human malignant melanoma metastasis-suppressor gene. *J Natl Cancer Inst*. 1996.

29. Navarro VM, Castellano JM, Fernández-Fernández R, Barreiro ML, Roa J, Sanchez-Criado JE, Aguilar E, Dieguez C, Pinilla L, Tena-Sempere M. Developmental and hormonally regulated messenger ribonucleic acid expression of KiSS-1 and its putative receptor, GPR54, in rat hypothalamus and potent luteinizing hormone-releasing activity of KiSS-1 peptide. *Endocrinology*. 2004.

30. Han S-K. Activation of Gonadotropin-Releasing Hormone Neurons by Kisspeptin as a Neuroendocrine Switch for the Onset of Puberty. *J Neurosci*. 2005.

31. Irwig MS, Fraley GS, Smith JT, Acohido B V., Popa SM, Cunningham MJ, Gottsch ML, Clifton DK, Steiner RA. Kisspeptin activation of gonadotropin releasing hormone neurons and regulation of KiSS-1 mRNA in the male rat. *Neuroendocrinology*. 2004.

32. Pielecka-Fortuna J, Chu Z, Moenter SM. Kisspeptin acts directly and indirectly to increase gonadotropin-releasing hormone neuron activity and its effects are modulated by estradiol. *Endocrinology*. 2008.

33. Gottsch ML, Cunningham MJ, Smith JT, Popa SM, Acohido B V., Crowley WF, Seminara S, Clifton DK, Steiner RA. A role for kisspeptins in the regulation of gonadotropin secretion in the mouse. *Endocrinology*. 2004.

34. Clarkson J, Herbison AE. Postnatal development of kisspeptin neurons in mouse hypothalamus; sexual dimorphism and projections to gonadotropin-releasing hormone neurons. *Endocrinology*. 2006.

35. Clarkson J, d'Anglemont de Tassigny X, Colledge WH, Caraty A, Herbison AE. Distribution of kisspeptin neurones in the adult female mouse brain. *J Neuroendocrinol*. 2009.

36. Smith JT, Popa SM, Clifton DK, Hoffman GE, Steiner RA. Kiss1 Neurons in the Forebrain as Central Processors for Generating the Preovulatory Luteinizing Hormone Surge. *J Neurosci*. 2006.

37. Smith JT, Cunningham MJ, Rissman EF, Clifton DK, Steiner RA. Regulation of Kiss1 gene expression in the brain of the female mouse. *Endocrinology*. 2005.

38. Smith JT, Dungan HM, Stoll EA, Gottsch ML, Braun RE, Eacker SM, Clifton DK, Steiner RA. Differential regulation of KiSS-1 mRNA expression by sex steroids in the brain of the male mouse. *Endocrinology*. 2005.

39. Roa J, Vigo E, Castellano JM, Gaytan F, Navarro VM, Aguilar E, Dijcks FA, Ederveen AGH, Pinilla L, Van Noort PI, Tena-Sempere M. Opposite roles of estrogen receptor (ER)-α and ERβ in the modulation of luteinizing hormone responses to kisspeptin in the female rat: Implications for the generation of the preovulatory surge. *Endocrinology*. 2008.

40. Desroziers E, Droguerre M, Bentsen AH, Robert V, Mikkelsen JD, Caraty A, Tillet Y, Duittoz A, Franceschini I. Embryonic Development of Kisspeptin Neurones in Rat. *J Neuroendocrinol*. 2012.

41. Semaan SJ, Kauffman AS. Daily successive changes in reproductive gene expression and neuronal activation in the brains of pubertal female mice. *Mol Cell Endocrinol*. 2015.

42. Semaan SJ, Murray EK, Poling MC, Dhamija S, Forger NG, Kauffman AS. BAX-dependent and BAX-independent regulation of Kiss1 neuron development in mice. *Endocrinology*. 2010.

43. Herbison AE. Estrogen positive feedback to gonadotropin-releasing hormone (GnRH) neurons in the rodent: The case for the rostral periventricular area of the third ventricle

(RP3V). Brain Research Reviews. 2008.

44. Pineda R, Garcia-Galiano D, Roseweir A, Romero M, Sanchez-Garrido MA, Ruiz-Pino F, Morgan K, Pinilla L, Millar RP, Tena-Sempere M. Critical roles of kisspeptins in female puberty and preovulatory gonadotropin surges as revealed by a novel antagonist. *Endocrinology*. 2010.

45. Clarkson J, d'Anglemont de Tassigny X, Moreno AS, Colledge WH, Herbison AE. Kisspeptin-GPR54 Signaling Is Essential for Preovulatory Gonadotropin-Releasing Hormone Neuron Activation and the Luteinizing Hormone Surge. *J Neurosci*. 2008.

46. Dror T, Franks J, Kauffman AS. Analysis of Multiple Positive Feedback Paradigms Demonstrates a Complete Absence of LH Surges and GnRH Activation in Mice Lacking Kisspeptin Signaling. *Biol Reprod*. 2013.

47. Robertson JL, Clifton DK, De La Iglesia HO, Steiner RA, Kauffman AS. Circadian regulation of Kiss1 neurons: Implications for timing the preovulatory gonadotropin-releasing hormone/luteinizing hormone surge. *Endocrinology*. 2009.

48. Yeo SH, Kyle V, Morris PG, Jackman S, Sinnett-Smith LC, Schacker M, Chen C, Colledge WH. Visualisation of Kiss1 Neurone Distribution Using a Kiss1-CRE Transgenic Mouse. *J Neuroendocrinol*. 2016.

49. Xu Z, Kaga S, Mochiduki A, Tsubomizu J, Adachi S, Sakai T, Inoue K, Adachi AA. Immunocytochemical localization of kisspeptin neurons in the rat forebrain with special reference to sexual dimorphism and interaction with GnRH neurons. *Endocr J*. 2012.

50. Cravo RM, Margatho LO, Osborne-Lawrence S, Donato J, Atkin S, Bookout AL, Rovinsky S, Frazão R, Lee CE, Gautron L, Zigman JM, Elias CF. Characterization of Kiss1 neurons using transgenic mouse models. *Neuroscience*. 2011.

51. Kim J, Semaan SJ, Clifton DK, Steiner RA, Dhamija S, Kauffman AS. Regulation of Kiss1 expression by sex steroids in the amygdala of the rat and mouse. *Endocrinology*. 2011.

52. Shughrue PJ, Scrimo PJ, Merchenthaler I. Evidence for the colocalization of estrogen receptor-beta mRNA and estrogen receptor-alpha immunoreactivity in neurons of rat forebrain. *Endocrinology*. 1998.

53. Osterlund M, Kuiper GGJM, Gustafsson J-Å, Hurd YL. Differential distribution and regulation of estrogen receptor-alpha and -beta mRNA within the female rat brain. *Brain Res Mol Brain Res*. 1998.

54. Stephens SBZ, Chahal N, Munaganuru N, Parra RA, Kauffman AS. Estrogen stimulation of Kiss1 expression in the medial amygdala involves estrogen receptor-α but not estrogen receptor-β. *Endocrinology*. 2016.

55. Mitra SW, Hoskin E, Yudkovitz J, Pear L, Wilkinson HA, Hayashi S, Pfaff DW, Ogawa S, Rohrer SP, Schaeffer JM, McEwen BS, Alves SE. Immunolocalization of estrogen receptor beta in the mouse brain: comparison with estrogen receptor alpha. *Endocrinology*. 2003.

56. Dupont S, Krust A, Gansmuller A, Dierich A, Chambon P, Mark M. Effect of single and compound knockouts of estrogen receptors alpha (ERalpha) and beta (ERbeta) on mouse

reproductive phenotypes. *Development*. 2000.

57. Schwenk F, Baron U, Rajewsky K. A cre-transgenic mouse strain for the ubiquitous deletion of loxP-flanked gene segments including deletion in germ cells. *Nucleic Acids Res*. 1995.

58. Krege JH, Hodgin JB, Couse JF, Enmark E, Warner M, Mahler JF, Sar M, Korach KS, Gustafsson J a, Smithies O. Generation and reproductive phenotypes of mice lacking estrogen receptor beta. *Proc Natl Acad Sci U S A*. 1998.

59. Buch T, Heppner FL, Tertilt C, Heinen TJAJ, Kremer M, Wunderlich FT, Jung S, Waisman A. A Cre-inducible diphtheria toxin receptor mediates cell lineage ablation after toxin administration. *Nat Methods*. 2005.

60. Hu MH, Li XF, McCausland B, Li SY, Gresham R, Kinsey-Jones JS, Gardiner J V., Sam AH, Bloom SR, Poston L, Lightman SL, Murphy KG, O'Byrne KT. Relative importance of the arcuate and anteroventral periventricular kisspeptin neurons in control of puberty and reproductive function in female rats. *Endocrinology*. 2015.

61. Hu KL, Zhao H, Chang HM, Yu Y, Qiao J. Kisspeptin/kisspeptin receptor system in the ovary. Frontiers in Endocrinology. 2018.

62. Risold PY, Swanson LW. Connections of the rat lateral septal complex. Brain Research Reviews. 1997.

63. Kurrasch DM, Cheung CC, Lee FY, Tran P V., Hata K, Ingraham HA. The Neonatal Ventromedial Hypothalamus Transcriptome Reveals Novel Markers with Spatially Distinct Patterning. *J Neurosci*. 2007.

64. Pfaff DW, Sakuma Y. Deficit in the lordosis reflex of female rats caused by lesions in the ventromedial nucleus of the hypothalamus. *J Physiol*. 1979.

65. Nance DM, Shryne J, Gorski RA. Facilitation of female sexual behavior in male rats by septal lesions: An interaction with estrogen. *Horm Behav* [Internet]. 1975 Sep 1 [cited 2018 Aug 19];6(3):289–99. Available from: https://www.sciencedirect.com/science/article/pii/0018506X7590015X?via%3Dihub

66. Nance DM, Shryne J, Gorski RA. Septal lesions: Effects on lordosis behavior and pattern of gonadotropin release. *Horm Behav*. 1974.

67. Satou M, Yamanouchi K. Effect of direct application of estrogen aimed at lateral septum or dorsal raphe nucleus on lordosis behavior: Regional and sexual differences in rats. *Neuroendocrinology*. 1999.

68. Tsukahara S, Kanaya M, Yamanouchi K. Neuroanatomy and sex differences of the lordosis-inhibiting system in the lateral septum. Frontiers in Neuroscience. 2014.

69. Hellier V, Brock O, Candlish M, Desroziers E, Aoki M, Mayer C, Piet R, Herbison A, Colledge WH, Prévot V, Boehm U, Bakker J. Female sexual behavior in mice is controlled by kisspeptin neurons. *Nat Commun*. 2018.

70. Hrabovszky E, Ciofi P, Vida B, Horvath MC, Keller E, Caraty A, Bloom SR, Ghatei MA, Dhillo WS, Liposits Z, Kallo I. The kisspeptin system of the human hypothalamus: Sexual

dimorphism and relationship with gonadotropin-releasing hormone and neurokinin B neurons. *Eur J Neurosci*. 2010.

71. Lehman MN, Merkley CM, Coolen LM, Goodman RL. Anatomy of the kisspeptin neural network in mammals. Brain Research. 2010.