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Deciphering how kisspeptin neurons mediate effects of glucocorticoids on pubertal development in mice

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Thesis

DECIPHERING HOW KISSPEPTIN NEURONS MEDIATE EFFECTS OF GLUCOCORTICOIDS ON PUBERTAL DEVELOPMENT IN MICE

by

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DEDICATION

I would like to dedicate this work to my family; father, mother, sister, grandmother, dog, and to Dr. LB Kats who first introduced me to elegant science.

ACKNOWLEDGMENTS

I would like to thank Dr. Sidney Pereira. I would also like to acknowledge Lizzie Medve, Manar Zoghdan, and the Kaiser and Navarro Labs.

DECIPHERING HOW KISSPEPTIN NEURONS MEDIATE EFFECTS OF GLUCOCORTICOIDS ON PUBERTAL DEVELOPMENT IN MICE

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ABSTRACT

Background: Kisspeptin has recently been deemed the critical central regulatory factor for gonadotropin releasing hormone (GnRH) release. Through the control of GnRH release, kisspeptin is also responsible for the secretion of luteinizing hormone (LH) and follicle stimulating hormone (FSH), both downstream and regulated by GnRH. Kisspeptin is released from kisspeptin, neurokinin B, dynorphin A (KNDy) neurons within the hypothalamus' arcuate nucleus (ARC) and anteroventral periventricular nucleus (AVPV). As the most prominent regulator of GnRH release, KNDy neurons directly influence the hypothalamic pituitary gonadal (HPG) axis. The HPG axis primarily regulates reproduction and can be inhibited by the presence of stress hormones, glucocorticoids (GCs), and activation of the hypothalamic pituitary adrenal cortex (HPA) axis. It was found that exposure to stress before and during puberty can lead to short-term pubertal delay and long-term physiological and behavioral alterations. It is known that a large portion of kisspeptin neurons contain glucocorticoid receptors (GR) which bind GCs. However, the exact pathway that allows HPA axis activation to inhibit the HPG axis is still unknown.

Objective: The objective of this study is to understand how kisspeptin neurons mediate the relationship between the HPG and HPA axis and if the lack of GR from kisspeptin neurons alters pubertal development.

Methods: A mouse model with the targeted deletion of GR from Kiss1 expressing neurons $GR^{flox/flox}$; Kiss1-Cre ($Kiss1^{GRKO}$) was compared to control ($GR^{flox/flox}$) mice in phenotypic pubertal development studies. To determine if the removal of GR from Kiss1 expressing neurons affected pubertal development we began data recording of male and female mice phenotypes beginning on postnatal day (PND) twenty-one after weaning. We collected data assessing the body weight of the animal and age and body weight of preputial separation (PS), vaginal opening (VO), and first estrus, markers of pubertal development in mice.

Results: The removal of GR from Kiss1 expressing neurons did not cause a significant difference in pubertal development. Male and female $Kiss1^{GRKO}$ and $GR^{flox/flox}$ mice did not have significantly different ages or body weights at PS or VO, nor was age and body weight significantly altered at first estrus.

Conclusion: The removal of GR and therefore the loss of the ability for Kiss1 expressing neurons to bind GCs does not appear to significantly alter pubertal development in male and female $Kiss1^{GRKO}$ mice. Further research and studies are necessary to determine if GR in kisspeptin neurons mediate the effects of stress on pubertal development.

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LIST OF ABBREVIATIONS

ACTH ..... Adrenocorticotropic Hormone

ARC ..... Arcuate Nucleus

AVPV ..... Anteroventral Periventricular Nucleus

BNST ..... Bed Nucleus of the Stria Terminalis

BW ..... Body Weight

CRF ..... Corticotropin Releasing Factor

CRH ..... Corticotropin Releasing Hormone

FSH ..... Follicle Stimulating Hormone

GC ..... Glucocorticoid

GnRH ..... Gonadotropin Releasing Hormone

GR ..... Glucocorticoid Receptor

$GR^{flox/flox}$ ..... Control mice

HPA ..... Hypothalamic-Pituitary Adrenal Cortex

HPG ..... Hypothalamic-Pituitary Gonadal

H&E ..... Hematoxylin and Eosin

IACUC ..... Institutional Animal Care and Use Committee

KNDy ..... Kisspeptin, Neurokinin B, Dynorphin A

$Kiss1^{GRKO}$ ..... Knockout Mice

LH ..... Luteinizing Hormone

LoxP ..... Locus of x-over P1

LS..... Lateral Septum

LPS ..... Lipopolysaccharide

NKB..... Neurokinin B

PND ..... Post Natal Day

PVN ..... Paraventricular Nucleus

VO ..... Vaginal Opening

INTRODUCTION

The HPG Axis

The hypothalamus-pituitary-gonadal (HPG) axis (Figure 1) is known for its crucial role during puberty, reproductive life, and menopause. The HPG axis is a biological feedback loop that controls the secretion of sex steroids. The secretion of sex steroids from the gonads is controlled via the release and feedback loop mechanisms of other hormones and neuropeptides. Beginning in the hypothalamus, gonadotropin-releasing hormone (GnRH) is produced and in a pulsatile pattern, secreted. After GnRH is secreted, it moves in the blood through the hypophyseal portal system to the pituitary gland. GnRH then activates, stimulates the release, and therefore controls the production and release of gonadotropins, Luteinizing Hormone (LH) and Follicle Stimulating Hormone (FSH) from the anterior pituitary gland. These two hormones will consequently leave the pituitary gland and move through the bloodstream to mediate sex steroid and gamete production in the gonads. The hormones will bind their receptors which are present in the ovaries and testes (Amoss et al, 1971 and Matsuo et al, 1971). The sex steroids produced by the gonads will subsequently enter the bloodstream and complete the feedback loop by altering and adjusting the activation of GnRH production in the hypothalamus.

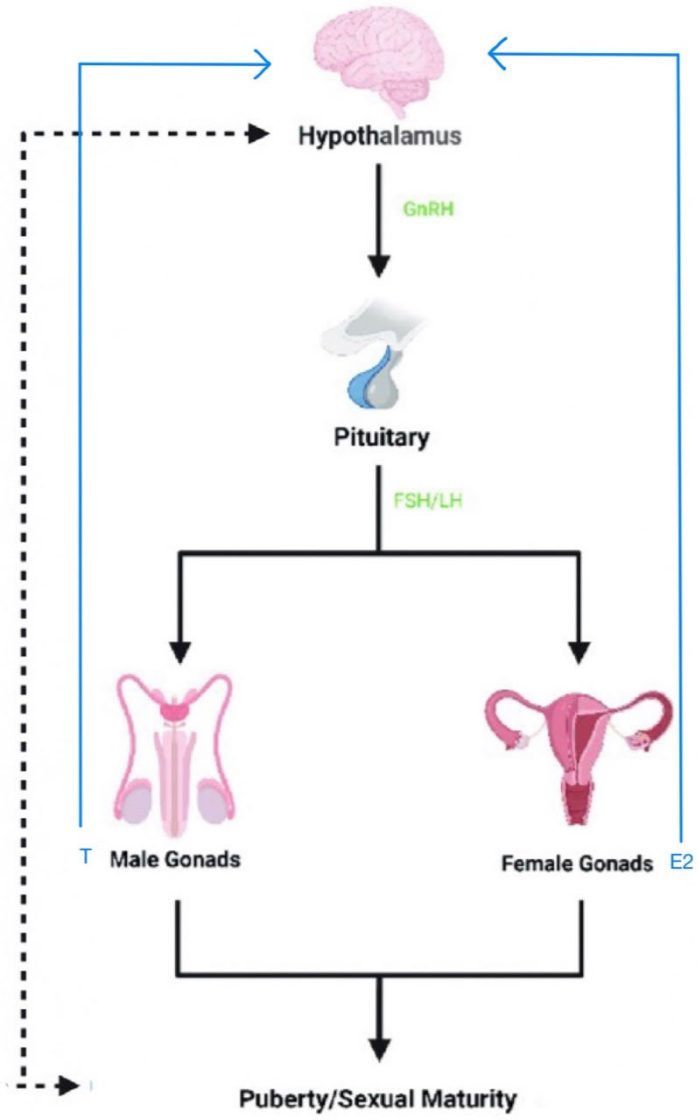

Figure 1. The hypothalamus-pituitary-gonadal (HPG) axis, beginning in the hypothalamus, gonadotropin-releasing hormone (GnRH) is produced and secreted in a pulsatile pattern. After GnRH is secreted, it moves in the blood through the hypophyseal portal system to the pituitary gland. GnRH then activates, stimulates, and therefore controls the production and release of gonadotropins, luteinizing Hormone (LH) and follicle Stimulating Hormone (FSH) in the pituitary gland. Figure adapted from Chung et al, 2021.

Kisspeptin Neurons

Within the hypothalamus, but upstream of the GnRH neurons are kisspeptin (Kiss1) releasing neurons. Kisspeptin, a neuropeptide, encoded by the *Kiss1* gene, was initially discovered by Lee et al, 1996 and although originally thought to be related to cancer growth, it is now known to be related to reproduction. These neurons can be found in the anteroventral periventricular (AVPV) and arcuate (ARC) nuclei of the hypothalamus, amygdala, the bed nucleus of the stria terminalis (BNST), and lateral septum (LS) (Gottsch et al, 2004). Both the AVPV and ARC Kiss1 neurons play an integral role in the feedback mechanisms of the HPG axis, mainly by controlling the production and secretion of GnRH. Specifically, the ARC's population of Kiss1 expressing neurons has been deemed the pulse generator of the GnRH (Terasawa et al, 2013 and Clarkson et al, 2017). This is triggered by the release of kisspeptin and its binding on the kisspeptin receptor (Kiss1r) in the axon terminals of GnRH neurons (Rance et al, 2010, Wakabayashi et al, 2010, Goodman & Inskeep 2015). Additionally, there is a sex steroid feedback mechanism regulating kisspeptin secretion that triggers GnRH (Herbison 2015). Within the HPG axis there are two populations of kisspeptin neurons of specific interest. Present in the AVPV and ARC, these kisspeptin neurons receive, respectively, positive and negative feedback from estradiol, Figure 2 (Navarro and Kaiser 2013). Due to estradiol's positive feedback to the AVPV, these kisspeptin neurons have been found to modulate the preovulatory LH surge (Oakley et al, 2009).

The kisspeptin secreting neurons found in the ARC are kisspeptin, neurokinin B (NKB), dynorphin A (KNDy) neurons (Lehman et al, 2010). KNDy neurons have

receptors for NKB, NK3R, and dynorphin A, KOR (Navarro et al., 2009; Lehman et al., 2010; Wakabayashi et al., 2010). The kisspeptin required for the relay of pulse generation is produced and secreted from these KNDy neurons (Terasawa et al. 2013). From this model, the KNDy neuron has now been termed the "GnRH pulse generator" according to a current study.

It has been proposed that the pulsatile release of kisspeptin is controlled by the stimulatory action of NKB and inhibitory action of dynorphin A while both acting on KNDy neurons (Cheng et al, 2010). NKB has been described to act on KNDy neurons to stimulate burst firing pulsatility while dynorphin A works as a "brake" to stop pulses. The generation and termination of GnRH pulses are correlated to the balance or ratio of stimulatory NKB and inhibitory dynorphin A (Kegami et al, 2022). These two neuropeptides control the kisspeptin output that will affect downstream GnRH neurons and therefore the rest of the HPG axis, Figure 2 and 3 (Qinying Xie et al, 2022).

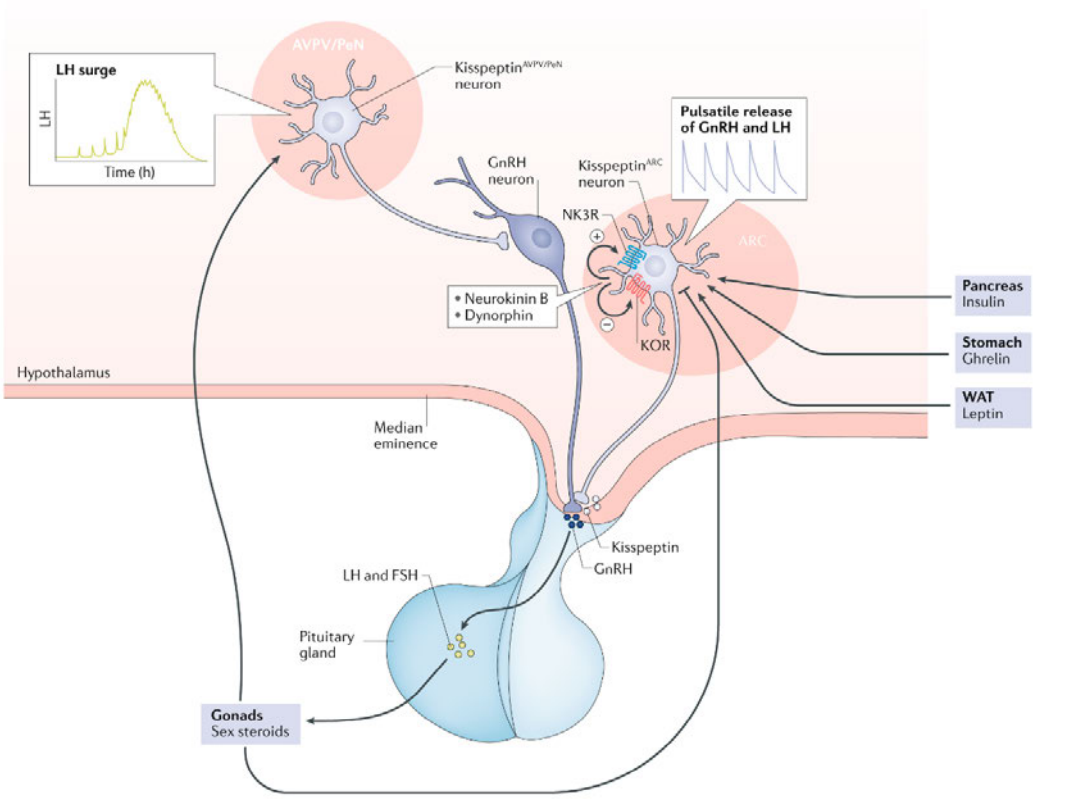

Figure 2. Kisspeptin secreting neurons found in the anteroventral periventricular (AVPV) and arcuate (ARC) (KNDy) neurons, express kisspeptin, neurokinin B (NKB), and dynorphin A. They are intertwined with the hypothalamus-pituitary-gonadal (HPG) axis. ARC kisspeptin releasing neurons help modulate the pulsatile release of gonadotropin-releasing hormone (GnRH) and LH. AVPV kisspeptin secreting neurons help modulate the luteinizing Hormone (LH) surge (Navarro, 2020).

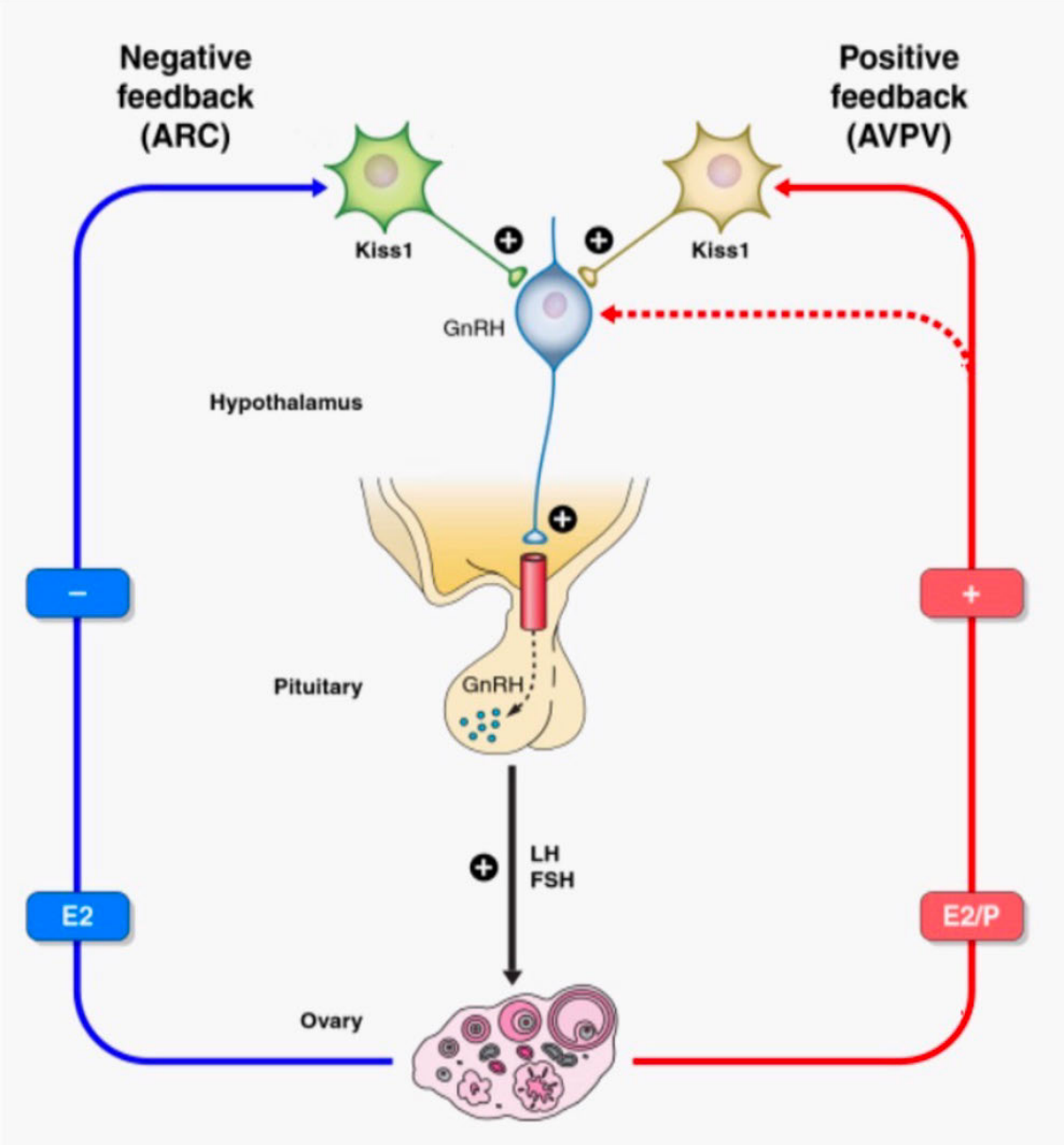

Figure 3. The two populations of kisspeptin secreting neurons and their feedback loops. This includes the feedback of gonadotropin-releasing hormone (GnRH) from the hypothalamus to the pituitary, which stimulates luteinizing Hormone (LH) and follicle Stimulating Hormone (FSH), which finally affects the gonads and sex steroid production. Sex steroid production is positive to the anteroventral periventricular (AVPV) as seen in red and negative to the arcuate (ARC) as seen in blue (Tena-Sempere, 2010).

HPA Axis

The hypothalamus-pituitary-adrenal (HPA) axis (Figure 4) is known for its ability to trigger a full body physiological response during stress. The HPA axis is a body wide feedback loop that is centered around corticotropin releasing factor (CRF). When activated, CRF triggers a cascade of responses that combine and trigger the release of glucocorticoids (GCs). GCs determine the severity, length, and type of response as well as provide feedback to the hypothalamus and complete yet another feedback loop. Beginning in the paraventricular nucleus (PVN) hypothalamus, corticotropin-releasing hormone (CRH) is secreted into the blood and moves through the hypophyseal portal system to the pituitary gland, Figure 4. Its effects on the pituitary gland stimulates the release of adrenocorticotropic hormone (ACTH). From the pituitary gland, ACTH acts on the adrenal cortex to stimulate cortisol, a glucocorticoid, and other secretions of corticosteroids. These stress hormones will then act on most of the tissues of the body to elicit a stress response (Smith and Vale, 2022). The effects of these stress hormones are widespread and can also affect the HPG axis and reproduction.

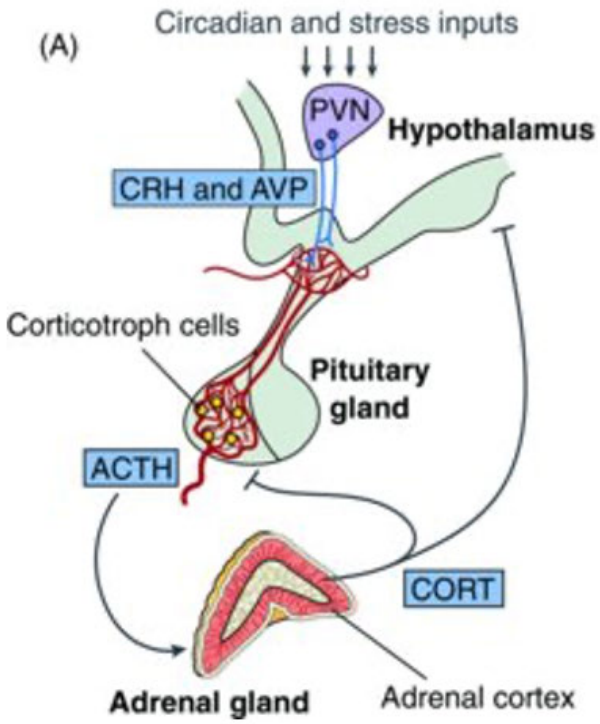

Figure 4. The hypothalamus-pituitary-adrenal (HPA) axis with external stimuli begins in the hypothalamus, corticotropin releasing hormone (CRH) secreted into the blood, moves through the hypophyseal portal system to the pituitary gland. Its effects on the pituitary gland stimulates the release of adrenocorticotropic hormone (ACTH). From the pituitary gland, ACTH acts on the adrenal cortex to stimulate cortisol and other secretions of corticosteroids. These stress hormones will then act on most of the tissues of the body to elicit a stress response (Spiga et al, 2014).

HPG and HPA Axis

The HPG axis is not only regulated by the pulsatile secretions of NKB and dynorphin A but also by nutritional load, environment, and most importantly, stress (Navarro and Kaiser 2013). It was shown in Rivier and Rivest, 1991; Tilbrook et al., 2002; and Williams et al., 2007 that metabolic, nutritional, psychological, and physical stress can reduce reproduction and negatively alter fertility in mammals, Figure 5 (Ralph

et al 2016). High levels of circulating GCs, from the HPA axis, can directly suppress GnRH production within the HPG axis (Chandran, et al., 1994; Whirledge and Cidlowski, 2010). Additionally, the HPA axis can also affect the HPG axis by suppressing Kiss1 expression in the AVPV and ARC. Kiss1 expression within KNDy neurons in the ARC maybe inhibited via KNDy neurons glucocorticoid receptors (GR). In summary, these studies as well as conclusions from an ongoing larger study in our group, GCs, binding via the GR, allow stress, from the HPA axis, to affect the HPG axis (Figure 6) (Kinsey-Jones et al., 2009; Takumi et al., 2012; Grachev et al., 2013; Luo et al., 2016; Yang et al., 2017). Much is unknown about the interaction of GC's within the different populations of Kiss1 expressing neurons and how this affects downstream neurons and larger bodily functions. The exact circuitry and upstream feedback loop of GRs, Kiss1 neurons, and KNDy neurons needs to be determined. Kiss1 and GnRH neurons both express GR but their interaction is unknown.

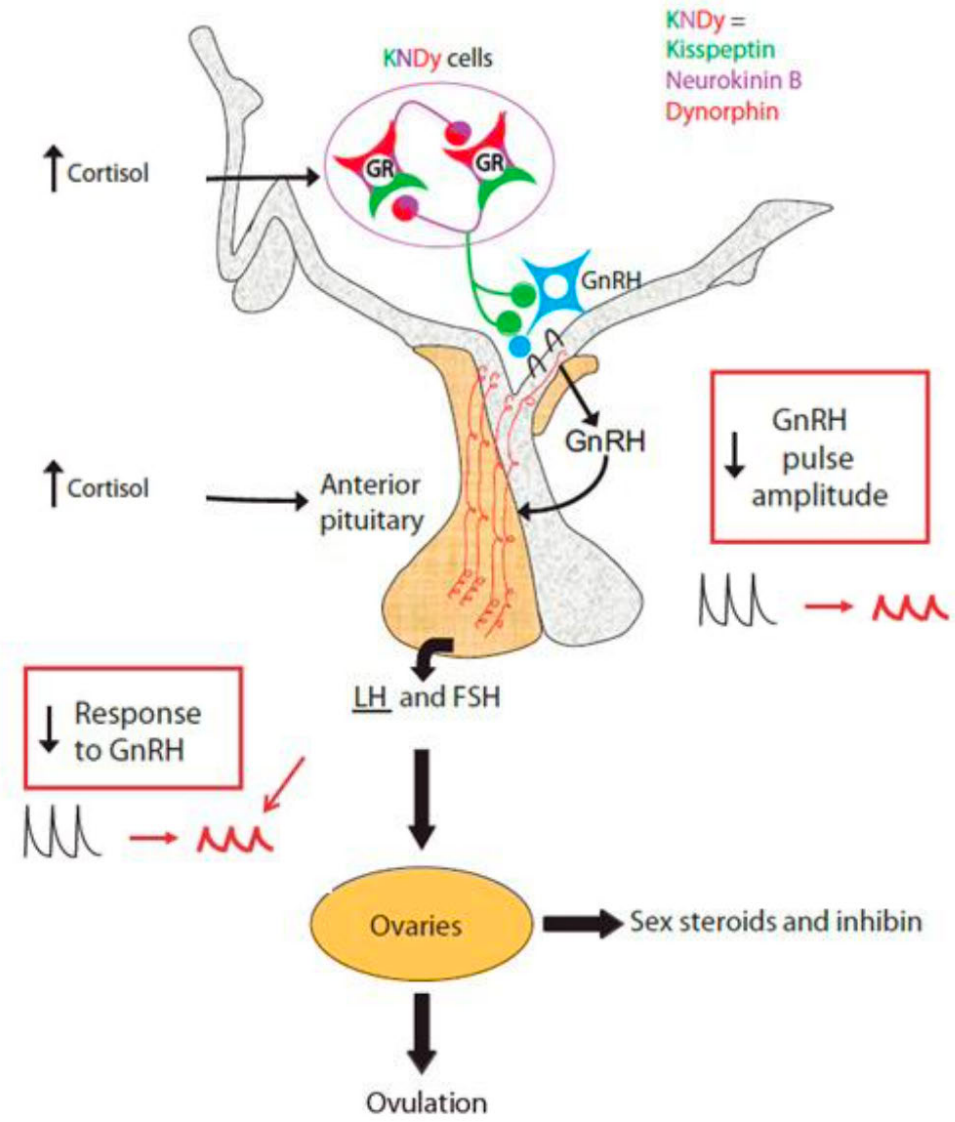

Figure 5. Feedback mechanism of how the hypothalamus-pituitary-gonadal (HPG) and hypothalamus-pituitary-adrenal (HPA) axes affect each other during high levels of glucocorticoids (GCs). Cortisol has effects on many of the tissues within the body acting through glucocorticoid receptor (GR) in many cells including kisspeptin, neurokinin B (NKB), dynorphin A (KNDy) neurons. When GCs levels are elevated, GCs bind GRs in many cells, including KNDy neurons in the arcuate (ARC) nucleus of the hypothalamus. This binding increases dynorphin A, while decreasing NKB and kisspeptin which inhibits gonadotropin-releasing hormone (GnRH) and the rest of the HPG axis. Diagram from Ralph et al. 2016.

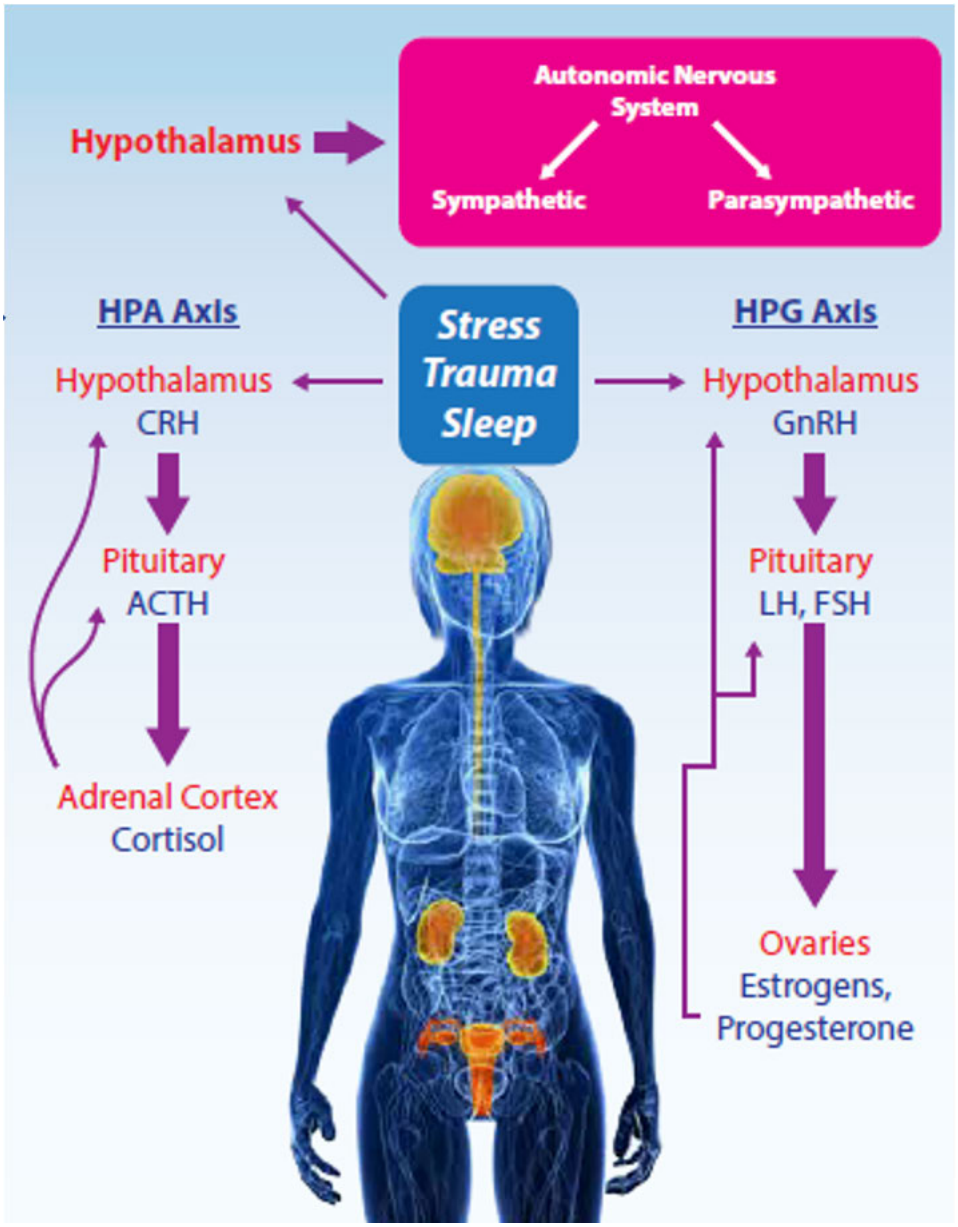

Figure 6. Shows the intermingling and parallel nature of both the hypothalamus-pituitary-gonadal (HPG) and hypothalamus-pituitary-adrenal (HPA) axes within the human body. Stress, trauma, and sleep all affect the HPG and HPA axis as well as the hypothalamus as a whole. Within the HPG axis there is feedback from sex steroids to the pituitary and to the hypothalamus. The HPA axis' cortisol production affects both the pituitary and hypothalamus as well. The hypothalamus is intertwined with the autonomic nervous system that modulates parasympathetic and sympathetic nervous system activity (Us, 2021).

Stress and Pubertal Development

Puberty is characterized by the initiation of sexual maturation of the gonads to allow the transition into adulthood. Puberty is not only known for the development of secondary sex characteristics but also for its collection of physiological, behavioral, and anatomical changes that occur in order for the organism to reach full fertile development. It was shown in Colledge, 2004 that the G protein coupled receptor, GPR54 (or Kiss1 receptor), in coordination with its ligand metastin, define as kisspeptin for the reproduction field is vital for the initiation of puberty in mice. Mice without Kiss1r did not experience puberty, harbored immature reproductive organs, had low levels of sex steroids and gonadotrophin hormones, but maintained normal levels of GnRH in the hypothalamus.

Puberty is the last stage of maturation for the HPG axis. The maturation of this feedback loop is characterized by increased sex steroids and changing in the levels of gonadotropins (Pinter et al, 2007). Puberty onset is crucial for population growth and many external and internal factors such as nutrition, genetics, and stress can alter its progression. Phenotypically, in female mice, the development of sexual maturity during puberty is often characterized by vaginal opening (VO) and first estrus. Female mice that do not experience puberty fail to conceive when paired with fertile male mice. Male mice maturation is characterized by the growth of testis, spermatozoa production, increasing testosterone levels, and the development of the preputial glands. Male mice that did not undergo puberty and therefore sexual development did not experience the growth of the testis, severely impaired spermatozoa production, low testosterone levels, and

phenotypically, the lack of development of the preputial glands of the penis as seen in figure 7 (Colledge, 2004). Animals exposed to caloric and restraint stress early in pubertal development were shown to weigh significantly less. Pubertal female mice were also found have delayed onset of vaginal opening, a phenotypic physical sign of puberty as well as having “irregular” estrus cycles (Nieves et al 2019).

The re-emergence of the pulsatile action of GnRH secretion at the onset of puberty allows these phenotypic, behavioral, and physiological changes to begin (Herbison, 2016). When GnRH production is suppressed, it affects not only HPG axis but also impacts pubertal development, fertility, and ovulation. Wagenmaker et al, 2009 found that GC’s can inhibit the pulsatile release of GnRH into the portal blood to the pituitary. Much about this process is still unelucidated including where within or above the feedback loop GC’s make contact to inhibit GnRH.

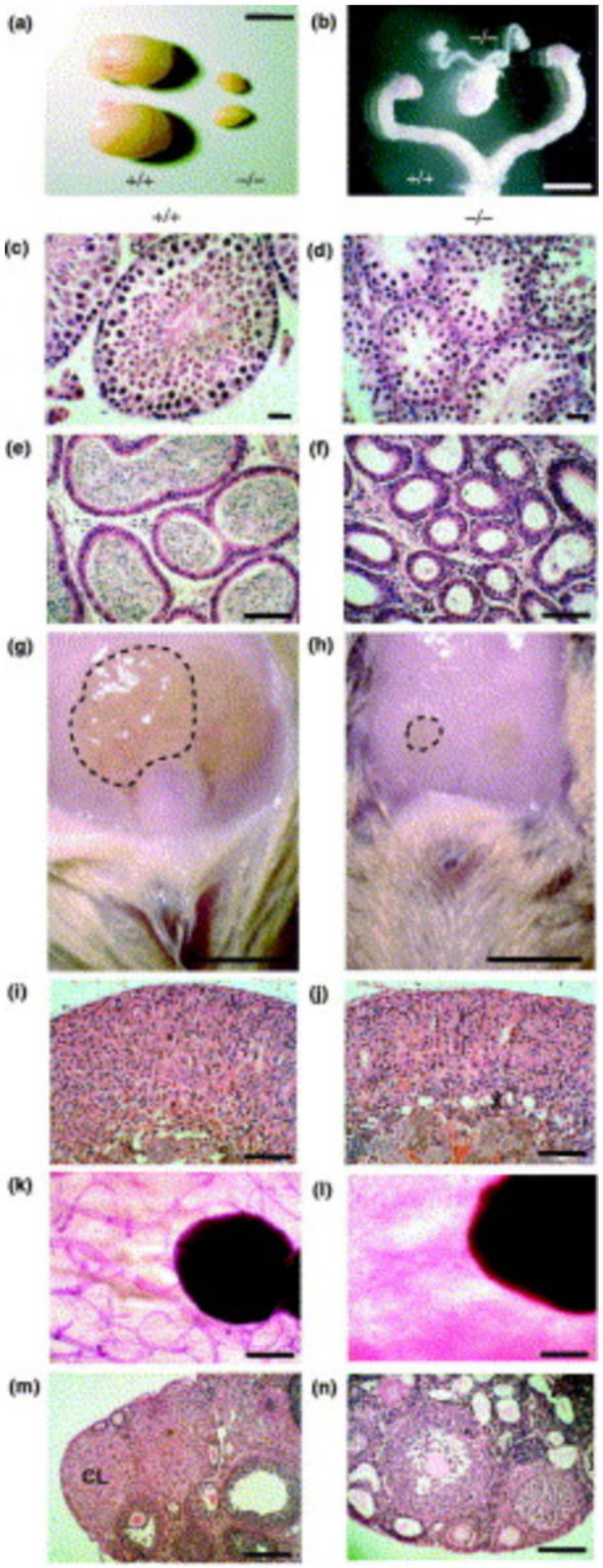
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Figure 7. From Seminara et al, 2003, mice (Kiss1r, knockout) that do not experience puberty, have immature gonads and less pronounced secondary sex characteristics. A: Reduction in size of testis. B: Smaller ovaries and uterus indicating the lack of ovulation. C,D: Severely reduced number of spermatozoa within the seminiferous tubules indicating failure to produce sperm. E,F: Absence of sperm within the epididymis. G,H: Low testosterone levels that indicates reduced development of preputial glands. I, J: A marker of prepubescents, retention of zone X in the adrenal gland. K, L: Lack of development of the mammary ducts. M, N: Absence of corpora lutea of large antral follicles indicating a lack of ovulation.

SPECIFIC AIMS

It has been shown that physical, metabolic, psychological, and nutritional types of stress negatively impact mammal reproduction. Stress in mammals has been exhibited to activate the hypothalamic pituitary adrenal cortex (HPA) axis and interrupt the hypothalamic pituitary gonadal (HPG) axis (Tilbrook et al, 2022 & Williams et al, 2007). Not only does stress affect these body system arching feedback loops but ultimately disrupts puberal development and ovarian cyclicity in females (Magiakou et al, 1997). After HPA axis activation the levels of adrenal stress hormones, glucocorticoids (GCs), increase (Rivier et al, 1986). It has been shown, in Dubey et al, 1985 Saketos et al, 1993, Kinsey-Jones et al, 2009, Lou et al, 2016, that systemic presence of GCs inhibit the activity of the HPG axis in mice, rats, monkeys, and humans.

Within the hypothalamus, kisspeptin neurons play an integral role in regulating gonadotropin releasing hormone (GnRH) pulsatile secretion, affecting, and regulating the HPG axis and puberty. In the arcuate (ARC) nucleus, kisspeptin neurons are known as KNDy neurons releasing kisspeptin, neurokinin B, and dynorphin A. It should also be noted that a large portion of kisspeptin neurons contain glucocorticoid receptors (GRs) (Takumi et al, 2012). During stress, (physical restraint, metabolic hypoglycemia, and inflammatory lipopolysaccharides) Kiss1 expression was decreased within the hypothalamic ARC (Kinsey-Jones et al, 2009). As Kiss1 expression decreases, GnRH release as well as LH pulse frequency decreases (Pinilla et al, 2012).

We hypothesize that the deletion of GR in kisspeptin expressing neurons would alter pubertal development in mice, due to their “inability” to experience GCs signaling. .

Our specific aim is to determine if GR in Kiss1 neurons are necessary for normal development and pubertal maturation. Through the use of a mouse animal model with the selective deletion of GR from Kiss1 neurons, we hope to understand how kisspeptin neurons mediate the HPG and HPA axis relationship during puberty and if these mechanisms are mediated by kisspeptin neurons directly.

METHODS

Study Approval and Animal Care

These studies were approved by the Brigham and Women's Hospital Institutional Animal Care and Use Committee (IACUC) in the Center for Comparative Medicine. The mice were group housed according to sex and bred under constant conditions including temperature (22–24°C), light [12 hr light (06:00)/dark (18:00) cycle], fed with standard mouse chow (Teklad F6 Rodent Diet 8664), and were given open access to tap water.

Mouse Model

In this project we aimed to understand the role of GR on Kiss1 neurons. In order to execute this, we generated a knockout mouse model where GR was specifically removed from Kiss1 neurons (Kiss1^{GRKO} mice). Mice were obtained from crossing breeding GR$^{flox/flox}$ mice with Kiss1-Cre mice using the Cre LoxP System (Kim et al, 2018). Kiss1-Cre mice, created by Dr Carol Elias, (STOCK Tg(Kiss1-cre)J2-4Cfe/J, Jackson Labs, stock# 023426) were crossed with GR$^{flox/flox}$ (B6.Cg-Nr3c1<tm1.1Jda>/J, Jackson Labs, stock# 021021) mice in which the loxP sites flank exon 3 of the *Nr3c1* gene. All the mice were genotyped by PCR analyses on genomic DNA isolated from ear biopsies, by Transnetyx (transnetyx.com) using the primers shown in table 1.

	Forward
Cre (+/-)	TTAATCCATATTGGCAGAACGAAAACG
Kiss1-1 WT	TCCCTGGAAGGAGACTGTAGAC
Nr3c1-10 FL	TGCTATACGAAGTTATGCGATCGG
Nr3c1-10 WT	TGTTGTTTCCCATTTCCTATACCATGT
	Reverse
Cre (+/-)	CAGGCTAAGTGCCTTCTCTACA
Kiss1-1 WT	GCGACACAGAGGAGAAGCA
Nr3c1-10 FL	TCCCCCTTCTCATTCCATGTCA
Nr3c1-10 WT	CTGTCTCCTAATAAGGTGTAGTGTAATCCT

Table 1. Primers used for PCR analyses testing from DNA isolated from ear biopsies from Transnetyx (transnetyx.com)

Cre Lox P System

A preferred means of studying human disease is using genetically engineered mouse models. The Cre-loxP system is integral to investigating tissue or cell specific genes. Cre recombinase is a 38 kDa flipase, D6 specific recombinase, and tyrosine site specific recombinase. Cre recognizes locus of x-over P1 (loxP) which is 34 base pairs, specific DNA fragment sequences and mediates this site-specific deletion or excision of the DNA between in the two loxP sites as seen in figure 8 (Kim et al, 2018). In the mouse model used in this project, Cre recombinase is only active in tissues expressing Kiss1

with a *Kiss1* promoter while the loxP sites flank the gene encoding for GR in these cells. This allows for mice with Kiss1^{GRKO} and GR$^{flox/flox}$.

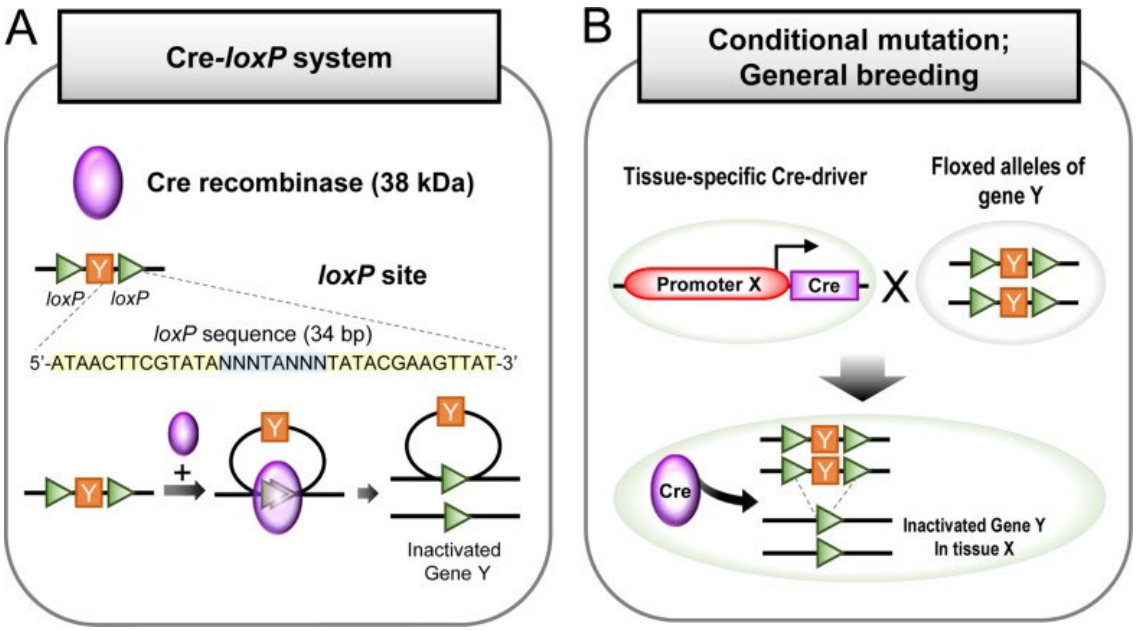

Figure 8. The Cre loxP system. A: overview of Cre recognizing loxP within the DNA sequences flanking the gene "Y". B: In creating a mouse model with the loxP and Cre genes, it is required to have one mouse with the cre gene and another mouse with the loxP DNA sequences. When combined in offspring, the cre mediates excision of gene "Y" between loxP. Figure from Kim et al, 2018

Validation of the Mouse Model

To verify the selective knockout of GR from mice Kiss1 neurons, a dual IHC of hypothalamic sections of experimental, Kiss1^{GRKO} mice and their littermate controls, GR$^{flox/flox}$, were compared. The brains sections were stained with both GR (rabbit anti-GR #PA1-511A, 1:500) and kisspeptin (sheep anti-kisspeptin AC #053,1:1000) in the hypothalamic ARC, an area of the brain known to express both proteins.

Immunohistochemistry

For GR and kisspeptin immunohistochemistry, brains were removed, stored in 4% paraformaldehyde diluted in PBS overnight and then transferred into sucrose solution (Thermo Fisher Scientific; 20% sucrose in PBS) at 4˚C. Following sucrose infiltration the tissue was frozen in Tissue-Tek® O.C.T. Compound (Sakura® Finetek-4583) and cut on a freezing stage microtome (Leica® CM3050S Cryostat) into 30 µm coronal sections. The brain sections were subjected to heat-mediated antigen retrieval in 10 mM sodium citrate buffer (pH 6.0) for 20 minutes at 90 ºC. The sections were blocked prior to addition of primary antibody with PBS-1X containing 0.3%Triton™ X-100 9 (Sigma Aldrich) and 2% normal donkey serum (NDS) in a humidified chamber at RT for 2 hours. Post blocking, sections were incubated with both primary antibodies, GR (rabbit anti-GR #PA1-511A, 1:500) and kisspeptin (sheep anti-kisspeptin AC #053,1:1000) diluted PBS-1X-0.3%Triton™ X-100 -2% normal donkey serum NDS. The 30 µm sections were then exposed and incubated for 1 hour with the secondary antibodies, anti-rabbit (1:500) and anti-sheep (1:500) against GR and kisspeptin primary antibodies, respectively. Slides were mounted with Vectashield mounting medium containing DAPI (Vector lab). The images were captured by using a fluorescent/light microscope (Nikon Eclipse 90i).

Body Weight

The body weights (BW) of male and female $Kiss1^{GRKO}$ mice (n=19) and (n=20) and their littermate controls $GR^{flox/flox}$ mice (n=13) and mice (n=24), respectively, were recorded every seven days from postnatal day (PND) 21 to PND 126. Additionally, BW

was recorded when preputial separation (PS), vaginal opening (VO) and first estrus occurred.

Male Pubertal Phenotyping – Preputial Separation

Male Kiss1^{GRKO} mice (n=19) and GR$^{flox/flox}$ mice (n=13) were monitored daily for complete preputial separation, a marker of pubertal development in male mice, from weaning PND-21. Preputial separation was confirmed by pushing the abdomen around the mouse's prepuce. A confirmed preputial separation was considered when the glans penis was a visible and dark red throughout as seen in Figure 9.

Figure 9. Penis of a mouse before preputial separation (PS) (left) and after PS (right). Male mouse on the right no longer has prepuce covering the penis and retracted to show the glans penis, image from Korenbrot et al, 1977.

Female Pubertal Phenotyping – Vaginal Opening (VO) and First Estrus

Female Kiss1^{GRKO} mice (n=20) and GR$^{flox/flox}$ mice (n=24) were monitored daily for VO (Figure 10) and first estrus, markers of pubertal development in female mice, from weaning PND-21. Vaginal opening was confirmed by the ability to use a pipette to lavage the vaginal canal as seen in Figure 10.

Female Kiss1^{GRKO} mice (n=20) and GR$^{flox/flox}$ mice (n=24) were monitored daily, around the same time of day, for first estrus from the first day of vaginal opening. The vaginal canal was lavaged with 15 µL water and the vaginal cells smeared on a glass slide as seen in figure 11. The slide was subsequently stained with hematoxylin and eosin (H&E) and examined by light microscopy. The first estrus was categorized by the presence of cornified cells on the vaginal mucosa. The cells present on the vaginal smear were categorized in one of four stages, pro-estrus, estrus, metestrus, and diestrus (Figure 12).

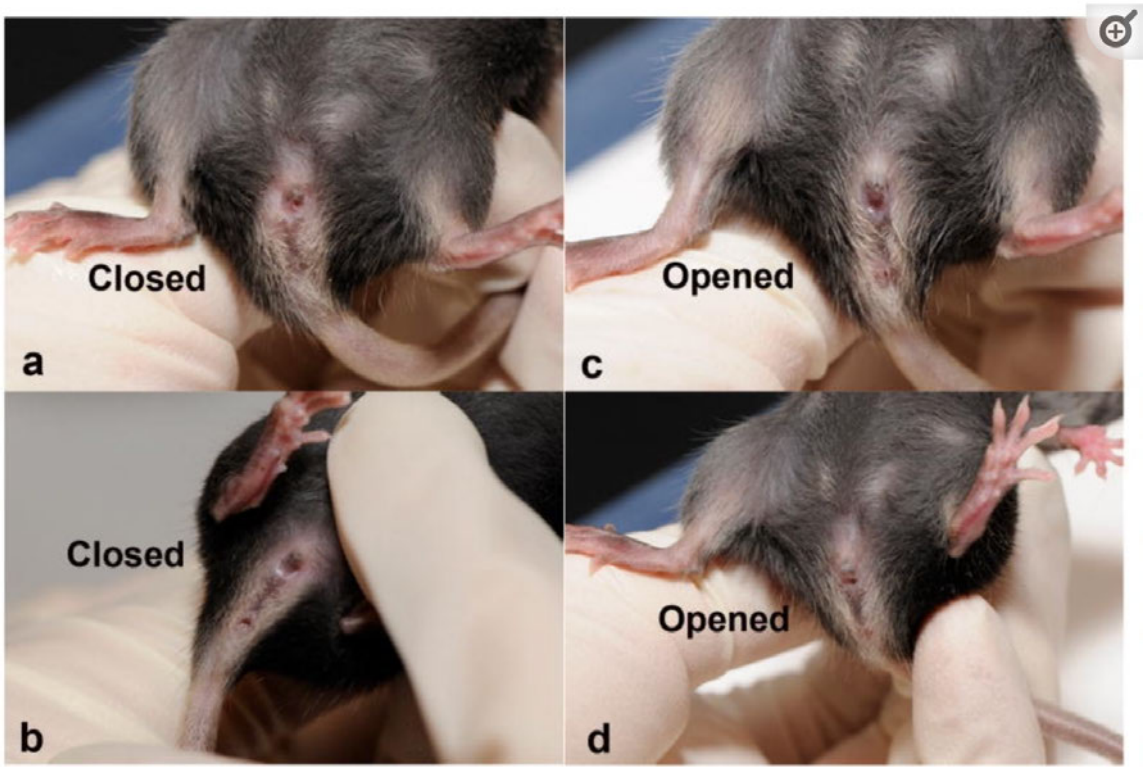

Figure 10. Vaginal opening in female mice at approximately 26 days old, showing no vaginal opening (A, B) and vaginal opening (C, D), image from Caligoni, 2009.

Figure 11. Collection of vaginal cells in female mice. After the female mouse has been restrained, the tip of a plastic pipette (~15 µL) filled with water is introduced in the vaginal opening of the mouse (a, detail in b) and the liquid is lavaged to collect a sample. Image from Caligoni, 2009.

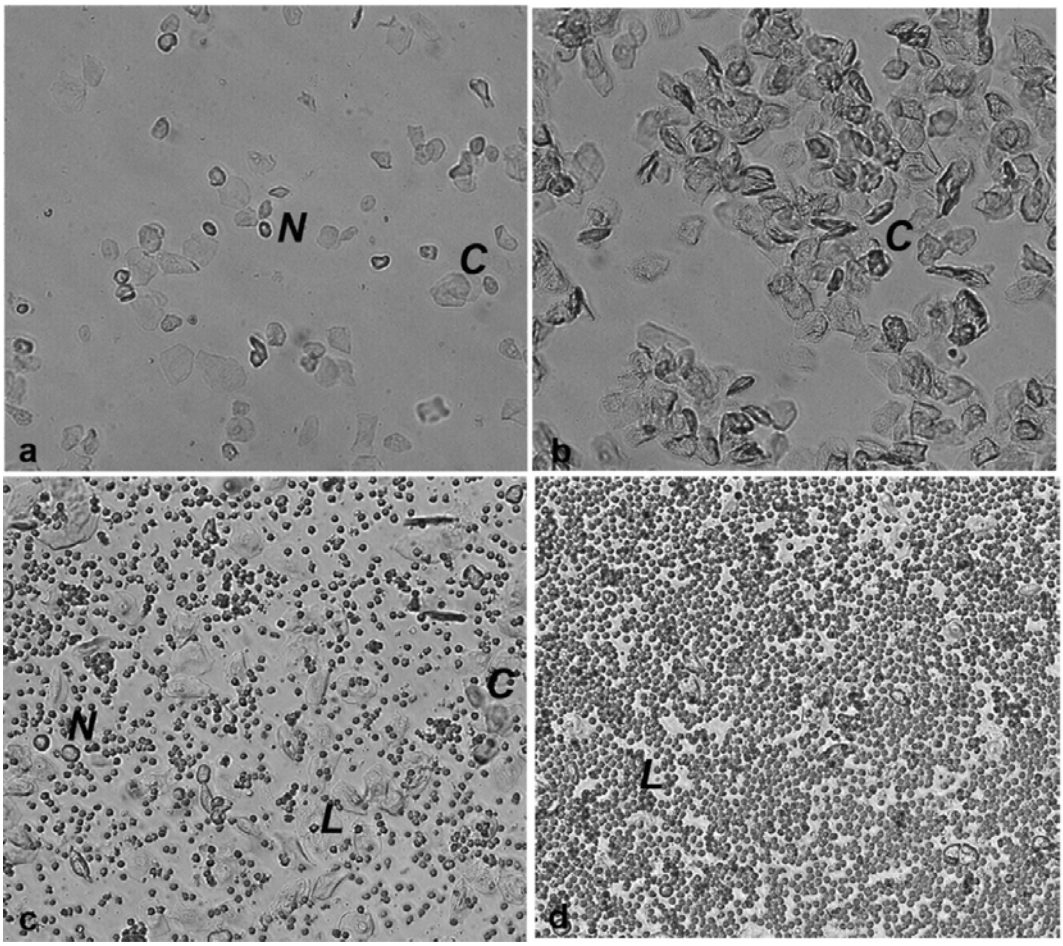

Figure 12. Photos of vaginal smears of the four types of vaginal cells. A: pro-estrus, nucleated epithelial cells. B: estrus, anucleated cornified cells. C: metestrus, cornified and anucleated epithelial cells and leukocytes. D: diestrus: leukocytes. N: nucleated epithelial cells, L: leukocytes, C: cornified cells. Figure adapted from Caligioni, 2009.

Statistical Analysis

Data were analyzed using the GraphPad Prism 9.3 statistical package. All data sets were presented as mean ± SEM. $P \leq 0.05$ was considered statistically significant. Analysis performed include 2 way ANOVA/ Sidaks or Unpaired Student's t test.

RESULTS

Mouse Model Validation

The frozen sections of hypothalamic ARC were treated with rabbit anti-GR and sheep anti-kisspeptin antibodies. Kisspeptin immunoreactivity was detected in both $Kiss1^{GRKO}$ mice and their littermate controls, $GR^{flox/flox}$ mice (Figure 13A and Figure 13B, green). GR immunoreactivity was detected in both $Kiss1^{GRKO}$ mice and their littermate controls, $GR^{flox/flox}$ mice (Figure 13C and Figure 13D, red). GR staining was observed in the $GR^{flox/flox}$ mice Kiss1 neurons (Figure 13F, yellow – green/red merged) but not in the same cells of the $Kiss1^{GRKO}$ mice (Figure 13E, green and red separated).

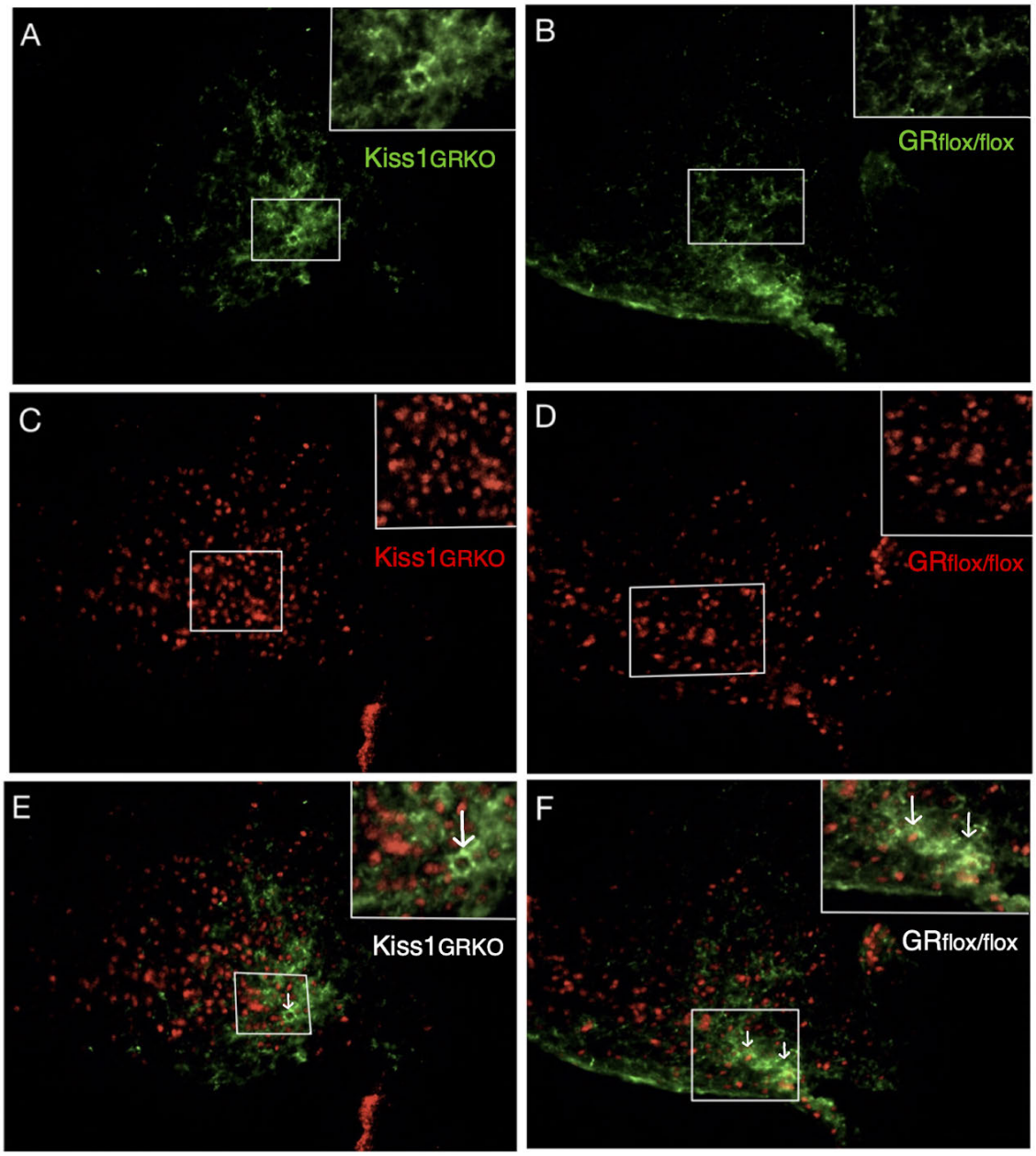

Figure 13. Immunoreactivity in the arcuate nucleus (ARC) of Kiss1^{GRKO} mice and GR$^{flox/flox}$ mice. A: Kisspeptin staining in Kiss1^{GRKO} mice, green. B: Kisspeptin staining in GR$^{flox/flox}$ mice, green. C: GR staining in Kiss1^{GRKO} mice, red. D: GR staining in GR$^{flox/flox}$ mice, red. E: Merged kisspeptin and GR staining in Kiss1^{GRKO} mice, green kisspeptin and red GR not colocalized in the same neurons, arrowheads. F: Merged kisspeptin and GR staining in GR$^{flox/flox}$ mice, green kisspeptin and red GR colocalized in the same neurons, yellow and arrowheads.

Pubertal Phenotyping

Body Weight

There was no significant difference in female mice body weight during week three to week fourteen of life between the two groups, Kiss1^{GRKO} mice (n=20), and GR$^{flox/flox}$ control mice (n=24) Figure 14A. There was also no significant difference in male mice body weight during week three to week fourteen of life between the two groups, Kiss1^{GRKO} mice (n=19) and GR$^{flox/flox}$ mice (n=13), Figure 14B.

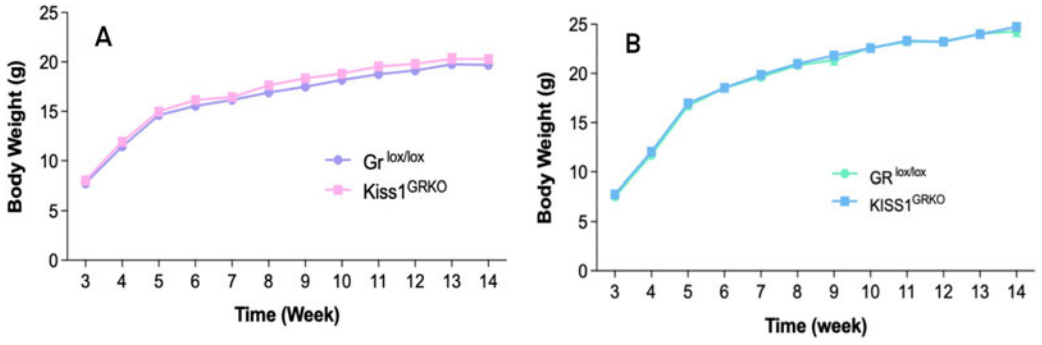

Figure 14. Body weight of Kiss1^{GRKO} mice compared to GR$^{flox/flox}$ control mice. (A) Female body weight from age three weeks to fourteen of life, n= 20-24 (B) Males body weight from age three weeks to fourteen of life, n= 13-19. Data represent mean +/- SEM by 2 way ANOVA/ Sidaks.

Preputial Separation

There was no significant difference between the two groups, Kiss1^{GRKO} mice (n=19) and GR$^{flox/flox}$ control mice (n=13) in preputial separation percentage over time, Figure 15A. There was no significant difference between the two groups for age at preputial separation, Figure 15B (Kiss1^{GRKO}: 32.68 +/- 0.50 days vs. GR$^{flox/flox}$: 32.67 +/-

0.68 days, p: 0.98) and weight at preputial separation, Figure 15C (Kiss1^{GRKO}: 15.61 +/- 0.25, grams vs. GR$^{flox/flox}$: 15.52 +/- 0.32, grams, p: 0.84).

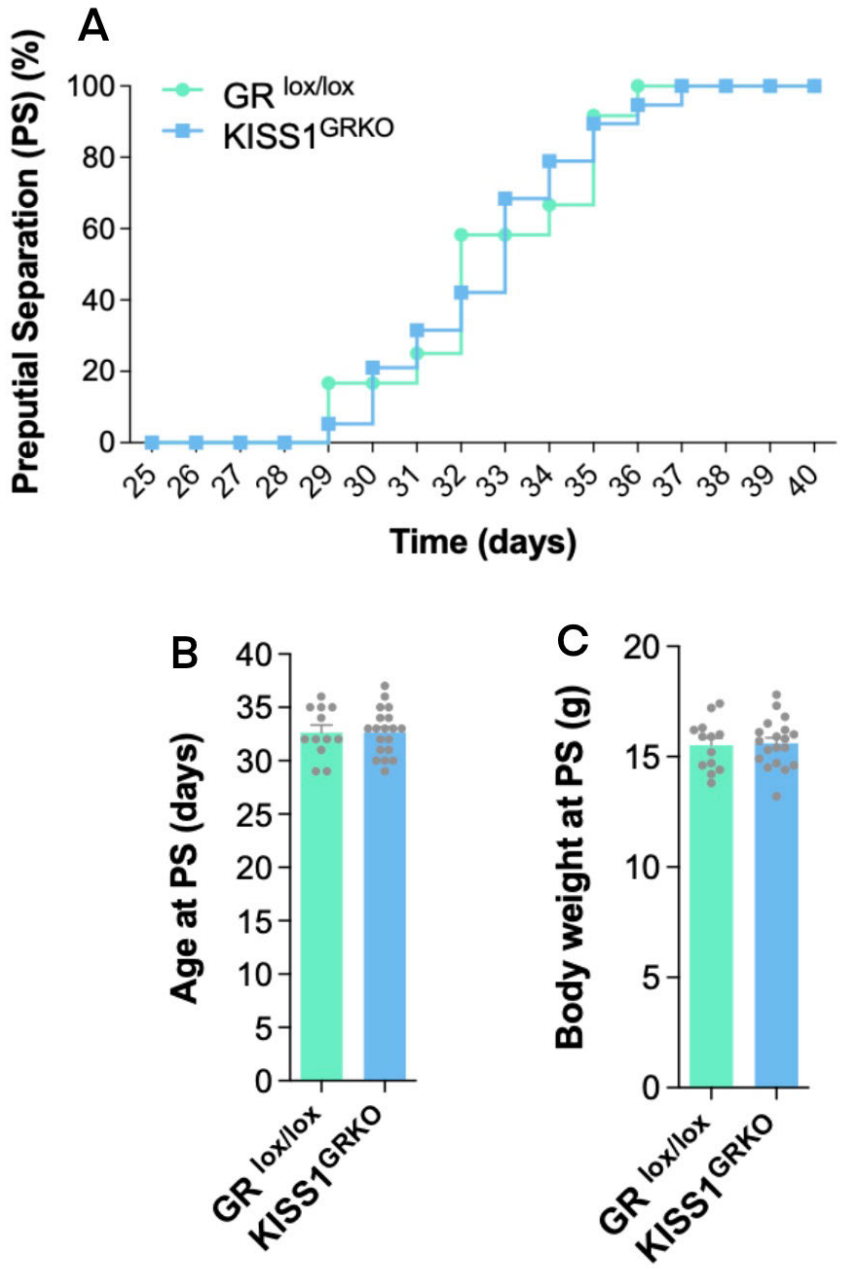

Figure 15. Preputial separation of Kiss1^{GRKO} mice compared to GR$^{flox/flox}$ control mice. (A) Percentage of males at postnatal day (PND) age of preputial separation from PND-25 to PND-40, n= 13-19. (B) PND age when preputial separation occurred Body weight when preputial separation occurred, n= 13-19. Data represent mean +/- SEM by unpaired t test.

Vaginal Opening

There was no significant difference between the two groups, Kiss1^{GRKO} mice (n=20) and GR$^{flox/flox}$ control mice (n=24) in vaginal opening percentage over time, Figure 16A. There was no significant difference between the two groups for age at vaginal opening, Figure 16B (Kiss1^{GRKO}: 29.32 +/- 0.47 days vs. GR$^{flox/flox}$: 29.65 +/- 0.45 days, p: 0.61) and body weight at vaginal opening, Figure 16C (Kiss1^{GRKO}: 12.65 +/- 0.20 grams vs. GR$^{flox/flox}$: 12.39 +/- 0.23 grams, p: 0.39).

, n= 12-19. (C)

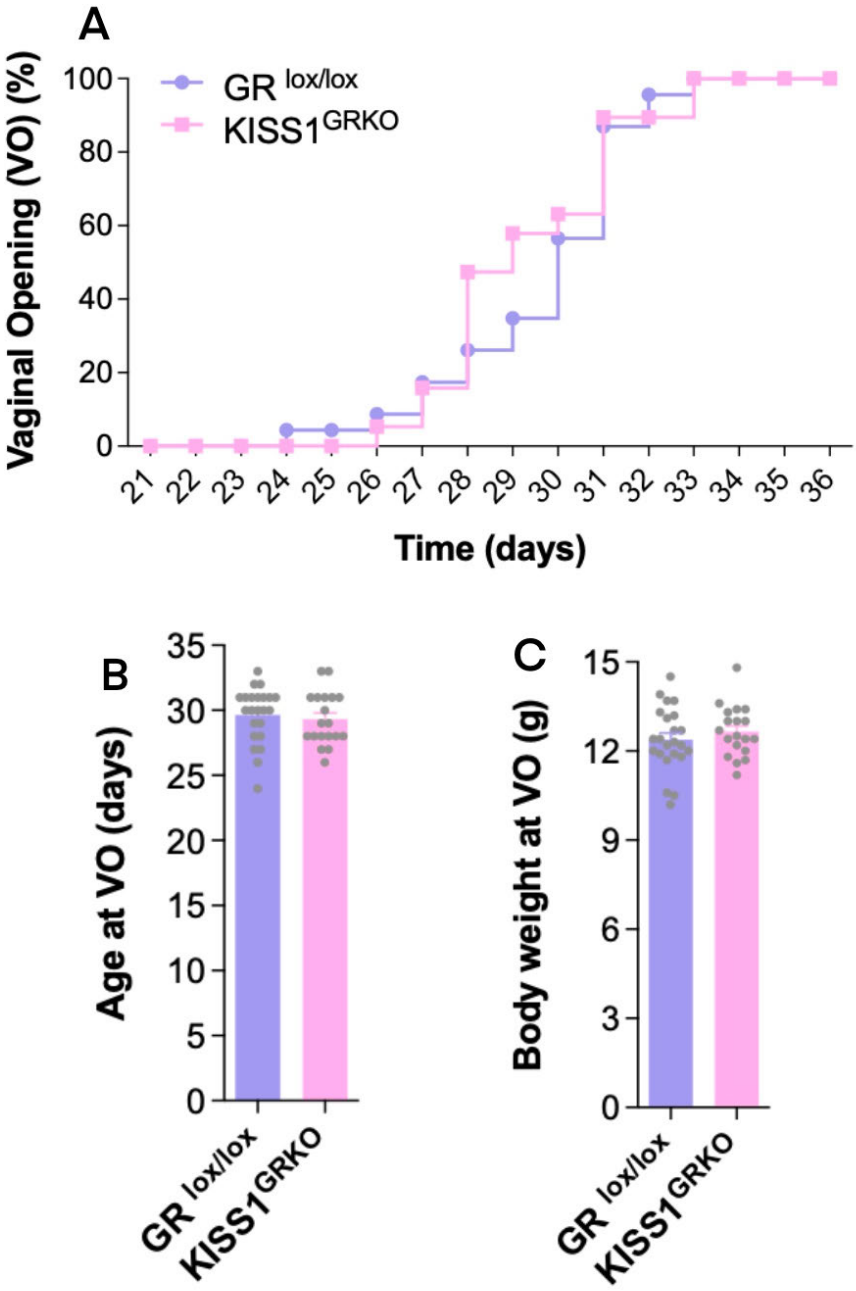

Figure 16. Vaginal opening of Kiss1^{GRKO} mice compared to GR$^{flox/flox}$ control mice. (A) Percentage of females at PND age of vaginal opening from PND-21 to PND-36. (B) PND age when vaginal opening occurred, n= 19-23. (C) Body weight when vaginal opening occurred, n= 19-23. Data represent mean +/- SEM by unpaired t test.

First Estrus

There was no significant difference between the two groups, $Kiss1^{GRKO}$ mice (n=20) and $GR^{flox/flox}$ control mice (n=24) in first estrus percentage over time, Figure 17A. There was no significant difference between the two groups for age at first estrus, Figure 17B ($Kiss1^{GRKO}$: 44.47 +/- 1.60 days vs. $GR^{flox/flox}$: 46.10 +/- 1.25 days, p: 0.42) and body weight at first estrus, Figure 17C ($Kiss1^{GRKO}$: 16.09 +/- 0.31 grams vs. $GR^{flox/flox}$: 15.93 +/- 0.29 grams, p: 0.69).

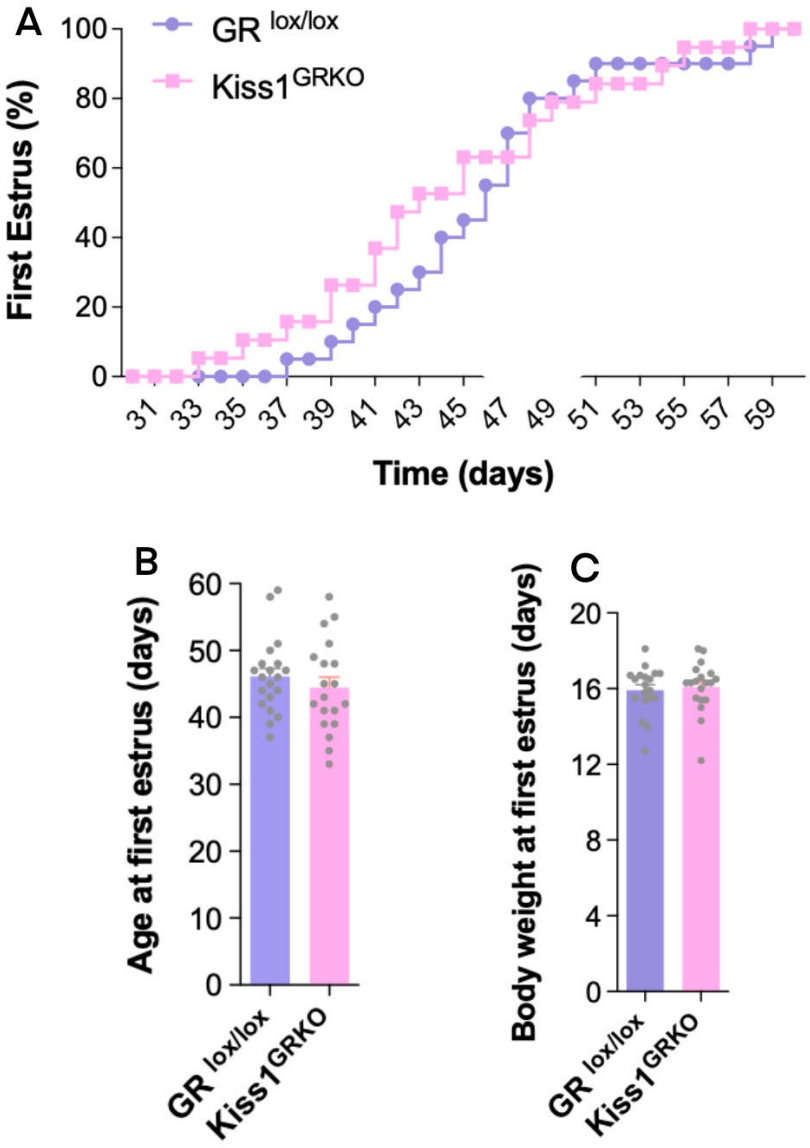

Figure 17. First estrus of Kiss1^{GRKO} mice compared to GR$^{flox/flox}$ control mice. (A) Percentage of females at PND age of first estrus from PND-31 to PND-59. (B) PND age when first estrus occurred, n= 19-20. (C) Body weight when first estrus occurred, n= 19-20. Data represent mean +/- SEM by unpaired t test.

DISCUSSION

Using a mouse model with the specific deletion of GR from Kiss1 neurons, we sought out to determine if GRs on Kiss1 neurons directly mediate HPA axis regulation of reproduction depression of the HPG axis. To determine if the removal of GR from Kiss1 neurons affected pubertal development, we first completed the phenotypic characterization of $Kiss1^{GRKO}$ mice and their littermate controls**,** $GR^{flox/flox}$. In this study we found the deletion of GR from Kiss1 neurons did not significantly alter male and female body weight, or onset of preputial separation, vaginal opening, and first estrus. Peripuberty and pubertal development are critical not only for the development of the HPG axis (Meek et al 1997) and HPA axis (Romero et al, 2005 and Romero et al, 2006) but also for the establishment of sexual behavior and reproductive processes in adulthood (Romero et al, 2002a, Romero et al. 2002b, Schulz et al 2004). Therefore, it is imperative to become more informed about what influences affect pubertal development and the means by which they do so. Laroche et al, 2009 found that exposure to stressors including caloric restriction, restraint, light, and *Escherichia coli* physiologic injections, during development lead to short-term and long-term physiological and behavioral alterations. Peripubertal stress has been significantly linked to body weight changes as well as changes in estradiol and progesterone tissue responses mediated by increased corticosterone levels. Both body weight and exposure to estradiol and progesterone directly affect pubertal onset and therefore it should be elucidated the mechanism of action stress (HPA axis) has on pubertal development (HPG axis).

Upon analysis, it was determined that there was no significant difference in male mice body weight during week three to week fourteen of life when comparing the two groups, $GR^{flox/flox}$ and $Kiss1^{GRKO}$. In addition, it was also determined that there was no significant difference in female mice body weight during week three to week fourteen of life also when comparing the two groups, $Kiss1^{GRKO}$ and $GR^{flox/flox}$. Body weight is correlated to pubertal onset, as body weight rises pubertal development occurs (Laroach et al, 2009). Nieves et al 2019 found that animals exposed to caloric, physical restraint, and LPS stress early in development weighed significantly less. We would have expected $Kiss1^{GRKO}$ mice to be larger in size, and weigh more, due to their lack of ability to experience stress. Our revised hypothesis suggests that GCs signaling through GR in Kiss1 neurons is not required for body weight changes in mice. These findings suggest a role of GCs acting on GR above or upstream of Kiss1 neurons to affects body weight. Targets upstream of these neurons must be established and studies executed to elucidate this mechanism.

To further evaluate if GR deletion in Kiss1 neurons affects pubertal development the weight, age, and percentage of animals that experienced preputial separation were recorded. Preputial separation is a time-tested means of determining the onset of puberty in male mice (Korenbrot et al, 1977). The separation of prepuce from the glans penis is completely dependent on the presence of androgens. The presence of androgens and consequently motile sperm production has been established and therefore is a marker of phenotypic pubertal onset (Korenbrot et al, 1977). Between the $Kiss1^{GRKO}$ and $GR^{flox/flox}$ groups, we found there was no significant difference in the percentage of mice from each

group that experienced preputial separation. There was also no significant difference in the age or weight at which preputial separation occurred between both groups. Due to androgens' ability to blunt stress-like behaviors in males (Wright et al, 2020), we would have expected male Kiss1^{GRKO} mice to develop preputial separation slightly earlier than their control littermates, GR$^{flox/flox}$. Our study did not show any male precocious or delayed phenotypic differences when GR was removed from Kiss1 neurons.

In females, we recorded the day of vaginal opening for both groups, Kiss1^{GRKO} and GR$^{flox/flox}$ mice, and their corresponding weight and age. There was no significant difference in the percentage of mice from each group that experienced vaginal opening as well as no difference in the age or weight of the animals at the vaginal opening. The vaginal opening is apoptosis mediated (Rodriguez et al, 1997) and occurs due to a rise in circulating estradiol (Caligioni, 2009). It is therefore an external sign of puberty onset in female mice. It was found in Nieves et al 2019, that onset of vaginal opening was significantly delayed when animals were exposed to stress via elevated plus mazes at PND – 28, 35, 50, and 75-110 as well as peripubertal stress associated with limited bedding between ages PND-4 to PND-11. Both of these types of stresses during different times in development induced lower than normal levels of progesterone and estradiol in these mice (Cora et al 2015). From these two studies, we initially hypothesized Kiss1^{GRKO} mice will experience an early vaginal opening when compared to their control littermates, GR$^{flox/flox}$. Our study however did not yield these results and our hypothesis was not supported. These results from male and female Kiss1^{GRKO} mice lead us to surmise that GC signaling through GR in Kiss1 neurons is not an inhibitor of preputial

separation or vaginal opening during pubertal development in mice in the absence of stress-induced GC release. These findings suggest that there could be a role of GCs and their GRs upstream to the Kiss1 neurons that affects preputial separation and vaginal opening.

To further determine if the female phenotypic characterization was altered by the deletion of GR in the Kiss1 neurons, vaginal smears were taken each day after vaginal opening occurred to determine the timing of the animal's first estrus. Documenting the ovulatory stages via microscopic evaluation of the types of cells present in these mice is a time-honored means of assessing the functional status of the HPG axis (Cora et al, 2015). According to Goldman, Murr, and Cooper 2007, estrus cycling is the principal measure to determine reproduction and ovulation. The levels of progesterone and estradiol change every four to five days in rats and mice which influences the proportions of each cell type present in the vaginal canal (Cora et al 2015). The cells that are differentiated between to determine a first estrus were pro-estrus, estrus, metestrus, and diestrus cells. By determining the majority of which type of cell is present, the timing of the secretion of an ovarian follicle can be evaluated. Between the two groups, $Kiss1^{GRKO}$ and $GR^{flox/flox}$, there was no significant difference in the percentage of animals in each group that experienced first estrus as well as the age or weight at which first estrus was recorded. Determining when first estrus occurs in these peripubertal mice is an important and unequivocal marker of pubertal onset and first ovulation or first estrus (Gaytan et al, 2017). There are many factors that can affect the vaginal opening and the first estrus in mice including age, temperature, nutrition, and stress (Li and Davis, 2007; Nelson et al,

1981; Campbell, Ryan, and Schwartz, 1976; Campbell and Schwartz, 1980; Nelson et al, 1982; Whitten, 1956; Goldman, Murr, and Cooper, 2007). We would expect Kiss1^{GRKO} mice to develop pubertal onset earlier than controls, GR$^{flox/flox}$, due to their "inability" to experience GCs signaling from GR in Kiss1 neurons. According to our hypothesis, removing GR from Kiss1 neurons would not allow GC signaling from these neurons to suppress the HPG axis initiation. Since this phenotypic pubertal characteristic was not altered in the Kiss1^{GRKO} mice, we now hypothesize the HPA axis may influence puberty onset upstream of Kiss1 neurons and their GRs.

Overall, these pubertal phenotypic studies did not yield any significant differences in puberty onset between Kiss1^{GRKO} and GR$^{flox/flox}$ mice. In relation to pubertal development, the removal of GR from Kiss1 neurons had no effect. Further research is needed to clearly define the mechanism of stress on the HPG axis as well as the effects of a deletion of GR from Kiss1 neurons. Research is also required to determine the effects of stress during pubertal development in animals with the GR deletion from Kiss1 neurons.

CONCLUSION

This project is currently in the process of starting stimulation trials in male and female Kiss1^{GRKO} and GR$^{flox/flox}$ mice with and without restraint stress. These additional experiments will further determine if the HPA axis is able to depress the HPG axis post-puberty in both female and male mice. We expect Kiss1^{GRKO} mice to be more resistant to stress due to their lack of an ability to experience stress through GRs. Both trials will involve restrained and unrestrained reproductive and physiological stress. Female mice of

both groups, Kiss1^{GRKO} and GR$^{flox/flox}$, cycles will be followed with and without caloric and restraint stress.

REFERENCES

Amoss, M. S., Burgus, R., Blackwell, R. J., Vale, W., Fellows, R. J., & Guillemin, R. (1971). Purification, amino acid composition and N-terminus of the hypothalamic luteinizing hormone releasing factor (LRF) of ovine origin. *Biochemical and Biophysical Research Communications*, *44*(1), 205–210. https://doi.org/10.1016/s0006-291x(71)80179-1

Caligioni, C. S. (2009). Assessing Reproductive Status/Stages in Mice. *Current Protocols in Neuroscience*, *48*(1). https://doi.org/10.1002/0471142301.nsa04is48

Campbell C. S., Ryan K. D., Schwartz N. B. (1976). Estrus cycle in the mouse: Relative influence of continuous light and the presence of a male. *Biological Reproduction* 14, 292–99.

Campbell, C., & Schwartz, N. B. (1980). The Impact of Constant Light on the Estrus Cycle of the Rat. *Endocrinology*, *106*(4), 1230–1238. https://doi.org/10.1210/endo-106-4-1230

Chandran, U. R., Attardi, B., Friedman, R. H., Dong, K., Roberts, J. M., & DeFranco, D. B. (1994). Glucocorticoid receptor-mediated repression of gonadotropin-releasing hormone promoter activity in GT1 hypothalamic cell lines. *Endocrinology*, *134*(3), 1467–1474. https://doi.org/10.1210/endo.134.3.8119188

Cheng, G., Coolen, L. M., Padmanabhan, V., Goodman, R. L., & Lehman, M. N. (2010). The kisspeptin/neurokinin B/dynorphin (KNDy) cell population of the arcuate nucleus: sex differences and effects of prenatal testosterone in sheep. *Endocrinology*, 151(1), 301–311. doi:10.1210/en.2009-0541

Chung, D. D., Pinson, M. R., Bhenderu, L. S., Lai, M. S., Patel, R. A., & Miranda, R. C. (2021). Toxic and teratogenic effects of prenatal alcohol exposure on fetal development, adolescence, and adulthood. *International Journal of Molecular Sciences*, 22(16), 8785. doi:10.3390/ijms22168785

Clarkson, J., Han, S. Y., Piet, R., McLennan, T., Kane, G. M., Ng, J., … Herbison, A. E. (2017). Definition of the hypothalamic GnRH pulse generator in mice. *Proceedings of the National Academy of Sciences of the United States of America*, 114(47), E10216–E10223. doi:10.1073/pnas.1713897114

Colledge, W. H. (2004). GPR54 and puberty. *Trends in Endocrinology and Metabolism*, *15*(9), 448–453. https://doi.org/10.1016/j.tem.2004.09.008

Cora, M. C., Kooistra, L., & Travlos, G. (2015). Vaginal cytology of the laboratory rat and mouse: Review and criteria for the staging of the estrus cycle using stained vaginal smears. *Toxicologic Pathology*, 43(6), 776–793. doi:10.1177/0192623315570339

Dubey, A. K., & Plant, T. M. (1985). A Suppression of Gonadotropin Secretion by Cortisol in Castrated Male Rhesus Monkeys (Macaca Mulatta) Mediated by the Interruption of Hypothalamic Gonadotropin-Releasing Hormone Release. *Biology of Reproduction*, *33*(2), 423–431. https://doi.org/10.1095/biolreprod33.2.423

Gaytan, F., Morales, C. P., Leon, S., Heras, V., Barroso, A., Avendaño, M. S., Vázquez, M. J., Castellano, J. M. P., Roa, J. C., & Tena-Sempere, M. (2017). Development and validation of a method for precise dating of female puberty in laboratory rodents: The puberty ovarian maturation score (Pub-Score). *Scientific Reports*, *7*(1). https://doi.org/10.1038/srep46381

Goldman, J. M., Murr, A. S., & Cooper, R. L. (2007). The rodent estrus cycle: characterization of vaginal cytology and its utility in toxicological studies. Birth Defects Research. Part B, *Developmental and Reproductive Toxicology*, 80(2), 84–97. doi:10.1002/bdrb.20106

Goodman, R., & Inskeep, E. K. (2015). Control of the Ovarian Cycle of the Sheep. *Elsevier EBooks*, 1259–1305. https://doi.org/10.1016/b978-0-12-397175-3.00027-2

Gottsch, M. L., Cunningham, M. E., Smith, J. C., Popa, S. M., Acohido, B. V., Crowley, W. F., Seminara, S. B., Clifton, D. K., & Steiner, R. D. (2004). A Role for Kisspeptins in the Regulation of Gonadotropin Secretion in the Mouse. *Endocrinology*, *145*(9), 4073–4077. https://doi.org/10.1210/en.2004-0431

Grachev, P., Li, X., & O'Byrne, K. T. (2013). Stress Regulation of Kisspeptin in the Modulation of Reproductive Function. *Advances in Experimental Medicine and Biology*, 431–454. https://doi.org/10.1007/978-1-4614-6199-9_20

Herbison, A. E. (2015). Physiology of the Adult Gonadotropin-Releasing Hormone Neuronal Network. *Elsevier EBooks*, 399–467. https://doi.org/10.1016/b978-0-12-397175-3.00011-9

Herbison, A. E. (2016). Control of puberty onset and fertility by gonadotropin-releasing hormone neurons. *Nature Reviews. Endocrinology*, *12*(8), 452-466. https://doi.org/10.1038/nrendo.2016.70

Kegami, K., Watanabe, Y., Nakamura, S., Goto, T., Inoue, N., Uenoyama, Y., & Tsukamura, H. (2022). Cellular and molecular mechanisms regulating the KNDy neuronal activities to generate and modulate GnRH pulse in mammals. *Frontiers in Neuroendocrinology*, 64(100968), 100968. doi:10.1016/j.yfrne.2021.100968

Korenbrot, C. C., Huhtaniemi, I. T., & Weiner, R. I. (1977). Preputial separation as an external sign of pubertal development in the male Rat1. *Biology of Reproduction*, 17(2), 298–303. doi:10.1095/biolreprod17.2.298

Kim, H., Kim, M., Im, S.-K., & Fang, S. (2018). Mouse Cre-LoxP system: general principles to determine tissue-specific roles of target genes. *Laboratory Animal Research*, 34(4), 147–159. doi:10.5625/lar.2018.34.4.147

Kinsey-Jones, J. S., Li, X., Knox, A. M. I., Wilkinson, E. S., Zhu, X., Chaudhary, A. A., Milligan, S., Lightman, S. L., & O'Byrne, K. T. (2009). Down-Regulation of Hypothalamic *Kisspeptin* and its Receptor, *Kiss1r*, mRNA Expression is Associated with Stress-Induced Suppression of Luteinizing Hormone Secretion in the Female Rat. *Journal of Neuroendocrinology*, *21*(1), 20–29. https://doi.org/10.1111/j.1365-2826.2008.01807.x

Laroche, J., Gasbarro, L., Herman, J. P., & Blaustein, J. D. (2009). Enduring influences of peripubertal/adolescent stressors on behavioral response to estradiol and progesterone in adult female mice. *Endocrinology*, 150(8), 3717–3725. doi:10.1210/en.2009-0099

Lee, J. M., Miele, M. E., Hicks, D. J., Phillips, K. S., Trent, J., Weissman, B. E., & Welch, D. R. (1996). KiSS-1, a Novel Human Malignant Melanoma Metastasis-Suppressor Gene. *Journal of the National Cancer Institute*, *88*(23), 1731–1737. https://doi.org/10.1093/jnci/88.23.1731

Lehman, M. N., Merkley, C. M., Coolen, L. M., & Goodman, R. L. (2010). Anatomy of the kisspeptin neural network in mammals. *Brain Research*, 1364, 90–102. doi:10.1016/j.brainres.2010.09.020

Li, S., & Davis, B. (2007). Evaluating rodent vaginal and uterine histology in toxicity studies. *Teratology*, *80*(3), 246–252. https://doi.org/10.1002/bdrb.20120

Luo, E., Stephens, S. W., Chaing, S., Munaganuru, N., Kauffman, A. S., & Breen, K. M. (2016). Corticosterone Blocks Ovarian Cyclicity and the LH Surge via Decreased Kisspeptin Neuron Activation in Female Mice. *Endocrinology*, *157*(3), 1187–1199. https://doi.org/10.1210/en.2015-1711

Magiakou, M. A., Mastorakos, G., Webster, E., & Chrousos, G. P. (1997). The Hypothalamic-Pituitary-Adrenal Axis and the Female Reproductive

System. *Annals of the New York Academy of Sciences*, *816*(1 Adolescent Gy), 42–56. https://doi.org/10.1111/j.1749-6632.1997.tb52128.x

Matsuo, H., Baba, Y., Nair, R., Arimura, A., & Schally, A. V. (1971). Structure of the porcine LH- and FSH-releasing hormone. I. The proposed amino acid sequence. *Biochemical and Biophysical Research Communications*, *43*(6), 1334–1339. https://doi.org/10.1016/s0006-291x(71)80019-0

Meek, L. R., Romeo, R. D., Novak, C. M., & Sisk, C. L. (1997). Actions of Testosterone in Prepubertal and Postpubertal Male Hamsters: Dissociation of Effects on Reproductive Behavior and Brain Androgen Receptor Immunoreactivity. *Hormones and Behavior*, *31*(1), 75–88. https://doi.org/10.1006/hbeh.1997.1371

Navarro, Víctor M. (2020). Metabolic regulation of kisspeptin - the link between energy balance and reproduction. *Nature Reviews. Endocrinology*, 16(8), 407–420. doi:10.1038/s41574-020-0363-7

Navarro, Victor M., Gottsch, M. L., Chavkin, C., Okamura, H., Clifton, D. K., & Steiner, R. A. (2009). Regulation of gonadotropin-releasing hormone secretion by kisspeptin/dynorphin/neurokinin B neurons in the arcuate nucleus of the mouse. *The Journal of Neuroscience*, 29(38), 11859–11866. doi:10.1523/JNEUROSCI.1569-09.2009

Navarro, Víctor M., & Kaiser, U. B. (2013). Metabolic influences on neuroendocrine regulation of reproduction. *Current Opinion in Endocrinology, Diabetes, and Obesity*, 20(4), 335–341. doi:10.1097/MED.0b013e32836318ce

Nelson, J. L., Felicio, L. S., Osterburg, H. H., & Finch, C. E. (1981). Altered Profiles of Estradiol and Progesterone Associated with Prolonged Estrus Cycles and Persistent Vaginal Cornification in Aging C578L/6J Mice1. *Biology of Reproduction*, *24*(4), 784–794. https://doi.org/10.1095/biolreprod24.4.784

Nelson, J. L., Felicio, L. S., Randall, P. K., Sims, C., & Finch, C. E. (1982). A Longitudinal Study of Estrus Cyclicity in Aging C57BL/6J Mice: I. Cycle Frequency, Length and Vaginal Cytology1. *Biology of Reproduction*, *27*(2), 327–339. https://doi.org/10.1095/biolreprod27.2.327

Nieves G., Schilit Nitenson, A., Lee, H.-I., Gallo, M., Aguilar, Z., Johnsen, A., … Bath, K. G. (2019). Early life stress delays sexual maturation in female mice. *Frontiers in Molecular Neuroscience*, 12, 27. doi:10.3389/fnmol.2019.00027

Oakley, A. E., Clifton, D. K., & Steiner, R. D. (2009). Kisspeptin Signaling in the Brain. *Endocrine Reviews*, *30*(6), 713–743. https://doi.org/10.1210/er.2009-0005

Outsourced PCR genotyping services. (n.d.). Retrieved March 5, 2023, from TransnetYX website: https://www.transnetyx.com/

Pinilla, L., Aguilar, E., Dieguez, C., Millar, R. P., & Tena-Sempere, M. (2012). Kisspeptins and Reproduction: Physiological Roles and Regulatory Mechanisms. *Physiological Reviews*, *92*(3), 1235–1316. https://doi.org/10.1152/physrev.00037.2010

Pintér, O., Beda, Z., Csaba, Z., & Gerendai, I. (2007). Differences in the onset of puberty in selected inbred mouse strains. *9th European Congress of Endocrinology*, *14*. https://www.endocrine-abstracts.org/ea/0014/ea0014p617

Xie, Q., Kang, Y., Zhang, C., Xie, Y., Wang, C., Liu, J., Yu, C., Zhao, H., & Huang, D. (2022). The Role of Kisspeptin in the Control of the Hypothalamic-Pituitary-Gonadal Axis and Reproduction. *Frontiers in Endocrinology*, *13*. https://doi.org/10.3389/fendo.2022.925206

Ralph, C. R., Lehman, M. N., Goodman, R., & Tilbrook, A. J. (2016). Impact of psychosocial stress on gonadotrophins and sexual behaviour in females: role for cortisol? *Reproduction*, *152*(1), R1–R14. https://doi.org/10.1530/rep-15-0604

Rance, N. E., Krajewski, S. J., Smith, M. D., Cholanian, M., & Dacks, P. A. (2010). Neurokinin B and the hypothalamic regulation of reproduction. *Brain Research*, *1364*, 116–128. https://doi.org/10.1016/j.brainres.2010.08.059

Rivier, C., & Rivest, S. (1991). Effect of Stress on the Activity of the Hypothalamic-Pituitary-Gonadal Axis: Peripheral and Central Mechanisms1. *Biology of Reproduction*, *45*(4), 523–532. https://doi.org/10.1095/biolreprod45.4.523

Rivier, C., Rivier, J., & Vale, W. (1986). Stress-Induced Inhibition of Reproductive Functions: Role of Endogenous Corticotropin-Releasing Factor. *Science*, *231*(4738), 607–609. https://doi.org/10.1126/science.3003907

Rodriguez, I., Ody, C., Araki, K., Garcia, I. T. S., & Vassalli, P. (1997). An early and massive wave of germinal cell apoptosis is required for the development of functional spermatogenesis. *The EMBO Journal*, *16*(9), 2262–2270. https://doi.org/10.1093/emboj/16.9.2262

Romeo, R. D., Bellani, R., & McEwen, B. S. (2005). Stress-induced progesterone secretion and progesterone receptor immunoreactivity in the paraventricular nucleus are modulated by pubertal development in male rats. *Stress*, *8*(4), 265–271. https://doi.org/10.1080/10253890500489320

Romeo, R. D., Karatsoreos, I. N., & McEwen, B. S. (2006). Pubertal maturation and time of day differentially affect behavioral and neuroendocrine responses following an acute stressor. *Hormones and Behavior*, *50*(3), 463–468. https://doi.org/10.1016/j.yhbeh.2006.06.002

Romeo, R. D., Richardson, H. N., & Sisk, C. L. (2002). Puberty and the maturation of the male brain and sexual behavior: recasting a behavioral potential. *Neuroscience & Biobehavioral Reviews*, *26*(3), 381–391. https://doi.org/10.1016/s0149-7634(02)00009-x

Romeo, R. D., Wagner, C. K., Jansen, H. T., Diedrich, S. L., & Sisk, C. L. (2002). Estradiol induces hypothalamic progesterone receptors but does not activate mating behavior in male hamsters (Mesocricetus auratus) before puberty. *Behavioral Neuroscience*. https://doi.org/10.1037/0735-7044.116.2.198

Saketos, M., Sharma, N., & Santoro, N. (1993). Suppression of the Hypothalamic-Pituitary-Ovarian Axis in Normal Women by Glucocorticoids1. *Biology of Reproduction*, *49*(6), 1270–1276. https://doi.org/10.1095/biolreprod49.6.1270

Schulz, K. M., Richardson, H. N., Zehr, J. L., Osetek, A. J., Menard, T. A., & Sisk, C. L. (2004). Gonadal hormones masculinize and defeminize reproductive behaviors during puberty in the male Syrian hamster. *Hormones and Behavior*, *45*(4), 242–249. https://doi.org/10.1016/j.yhbeh.2003.12.007

Smith, S. M., & Vale, W. W. (2006). The role of the hypothalamic-pituitary-adrenal axis in neuroendocrine responses to stress. *Dialogues in Clinical Neuroscience*, 8(4), 383–395. doi:10.31887/dcns.2006.8.4/ssmith

Seminara, S. B., Messager, S., Chatzidaki, E. E., Thresher, R. R., Acierno, J. S., Shagoury, J. K., Bo-Abbas, Y., Kuohung, W., Schwinof, K. M., Hendrick, A. G., Zahn, D., Dixon, J., Kaiser, U. B., Slaugenhaupt, S. A., Gusella, J. F., O'Rahilly, S., Carlton, M. B. L., Crowley, W. F., Aparicio, S., & Colledge, W. H. (2003). The*GPR54* Gene as a Regulator of Puberty. *The New England Journal of Medicine*, *349*(17), 1614–1627. https://doi.org/10.1056/nejmoa035322

Spiga, S., Talani, G., Mulas, G., Licheri, V., Fois, G. R., Muggironi, G., Masala, N., Cannizzaro, C., Biggio, G., Sanna, E., & Diana, M. (2014). Hampered long-term depression and thin spine loss in the nucleus accumbens of ethanol-dependent rats. *Proceedings of the National Academy of Sciences of the United States of America*, *111*(35). https://doi.org/10.1073/pnas.1406768111

Takumi, K., Iijima, N., Higo, S., & Ozawa, H. (2012). Immunohistochemical analysis of the colocalization of corticotropin-releasing hormone receptor and glucocorticoid

receptor in kisspeptin neurons in the hypothalamus of female rats. *Neuroscience Letters*, *531*(1), 40–45. https://doi.org/10.1016/j.neulet.2012.10.010

Tena-Sempere, M & Roa, J. (2010). Energy balance and puberty onset: emerging role of central mTOR signaling. *Trends in Endocrinology and Metabolism: TEM*, 21(9), 519–528. doi:10.1016/j.tem.2010.05.003

Terasawa, E., Guerriero, K. A., & Plant, T. M. (2013). Kisspeptin and Puberty in Mammals. *Springer New York EBooks*, 253–273. https://doi.org/10.1007/978-1-4614-6199-9_12

Tilbrook, A. J., Turner, A. M., & Clarke, I. J. (2002). Stress and Reproduction: Central Mechanisms and Sex Differences in Non-rodent Species. *Stress*, *5*(2), 83–100. https://doi.org/10.1080/10253890290027912

Us, A. (2021, September 22). Introducción al eje hipotalámico-pituitario-gonadal. Retrieved March 5, 2023, from Advanced Orthomolecular Research website: https://aor.us/introduccion-al-eje-hipotalamico-pituitario-gonadal/?lang=es

Wagenmaker, E. R., Breen, K. M., Oakley, A. E., Tilbrook, A. J., & Karsch, F. J. (2009). Psychosocial stress inhibits amplitude of gonadotropin-releasing hormone pulses independent of cortisol action on the type II glucocorticoid receptor. *Endocrinology*, 150(2), 762–769. doi:10.1210/en.2008-0757

Wakabayashi, Y., Nakada, T., Murata, K., Ohkura, S., Mogi, K., Navarro, V. M., … Okamura, H. (2010). Neurokinin B and dynorphin A in kisspeptin neurons of the arcuate nucleus participate in generation of periodic oscillation of neural activity driving pulsatile gonadotropin-releasing hormone secretion in the goat. *The Journal of Neuroscience: The Official Journal of the Society for Neuroscience*, 30(8), 3124–3132. doi:10.1523/JNEUROSCI.5848-09.2010

Whirledge, S., & Cidlowski, J. A. (2010). Glucocorticoids, stress, and fertility. *Minerva Endocrinologica*.

Whitten, W. K. (1956). MODIFICATION OF THE OESTRUS CYCLE OF THE MOUSE BY EXTERNAL STIMULI ASSOCIATED WITH THE MALE. *Journal of Endocrinology*, *13*(4), 399-404. https://doi.org/10.1677/joe.0.0130399

Williams, N. I., Berga, S. L., & Cameron, J. L. (2007). Synergism between psychosocial and metabolic stressors: impact on reproductive function in cynomolgus monkeys. *American Journal of Physiology-Endocrinology and Metabolism*, *293*(1), E270–E276. https://doi.org/10.1152/ajpendo.00108.2007

Wright, E. C., Culkin, H. I., Sekar, S., Kapoor, A., Corbett, C., & Trainor, B. C. (2020). Pubertal androgens reduce the effects of social stress on anxiety-related behaviors in California mice. *Developmental Biology*, doi:10.1101/2020.12.02.408526

Yang, J. H., Song, C. I., Hughes, J., Kreisman, M. J., Parra, R. A., Haisenleder, D. J., Kauffman, A. S., & Breen, K. M. (2017). Acute Psychosocial Stress Inhibits LH Pulsatility and Kiss1 Neuronal Activation in Female Mice. *Endocrinology*, *158*(11), 3716–3723. https://doi.org/10.1210/en.2017-00301

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