Identification of hypothalamic sites that control puberty onset and sexual maturation

by

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Basic research is what I'm doing when I don't know what I'm doing.

- Wernher von Braun

Dedication

Dedicated to my amazing husband, James.

I am eternally grateful for your never-ending love, understanding and encouragement.

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Table of Contents

List of Figures

Chapter 1

Chapter 2

Chapter 3

Chapter 4

Chapter 1 - Introduction

Puberty is the period of time during which a child transitions into a sexually mature adult capable of reproduction. From a biological perspective, it is the activation of the hypothalamic-pituitary-gonadal (HPG) axis, culminating in the maturation of the brain, pituitary and gonads. Throughout puberty, there is a complex interplay between environmental and endogenous factors that regulate the HPG axis. Despite decades of research, there are still large gaps in our understanding of puberty.

How is the timing of puberty onset regulated?

By which hormones?

What are the underlying neural circuits important for puberty onset?

Where do these hormones act in the brain to modulate the timing of puberty onset?

This literature review summarizes existing studies of how two important hormones- estradiol and leptin- produced in the periphery by the ovaries and white adipose tissue respectively, act in the brain to affect the timing of puberty onset and subsequent sexual maturation.

Puberty onset and sexual maturation

Primates

Although functional by the end of gestation, the ovaries and testes become relatively inactive for a brief period around the time of birth and stop secreting gonadal steroid hormones. Within a few days, however, they become active again and remain active until several months after birth when they become quiescent again and stop secreting significant amounts of hormones. This suppression of activity is referred to as the *juvenile pause* and is thought to be the result of inhibitory mechanisms in the brain and to a lesser extent at the level of the pituitary gland, a small endocrine organ located below the base of the brain. The regulation of gonadal activity throughout childhood is ultimately controlled by the brain, and more specifically by an area of the brain called the hypothalamus, which functions to link the neuroendocrine system to peripheral organ systems via the pituitary gland. Feedback loops exist between the hypothalamus, pituitary gland and the gonads, which together are called the HPG axis (**Figure 1**). During the juvenile pause, neurons in the brain that produce gonadotropin-releasing hormone (GnRH) are relatively inactive and produce very little GnRH. Since pulsatile GnRH secretion is required for the production and release of specific hormones from the pituitary, low levels of the pituitary-derived gonadotropins luteinizing hormone (LH) and follicle-stimulating hormone (FSH) are characteristic of the period of time prior to puberty onset [1,2]. Before the discorvery of FSH, GnRH was often termed luteinizing hormone releasing hormone (LHRH).

As an organism matures and approaches puberty, GnRH neurons become more active, resulting in increased secretion of GnRH and causing the gonadotropes in the anterior pituitary to also become more active. While young prepubertal boys and girls do not exhibit pulsatile LH release, nocturnal LH pulses are readily detected in older prepubertal children; the increased nocturnal LH levels are characteristic of human puberty onset [1,2]. During puberty, both LH and FSH increase in overall levels, pulse frequency and amplitude [1–5].

The increased pulsatile release of the gonadotropins from the anterior pituitary triggers effects in the gonads, including the production of gonadal hormones, termed androgens or estrogens. In females, LH acts on the theca cells of the ovaries, resulting in the production of androgenic precursors of estradiol, a potent estrogen. In males, LH acts on the Leydig cells of the testes, leading to production of testosterone, a potent androgen. FSH acts on granulosa cells in the ovaries and Sertoli cells in the testes to stimulate gametogenesis and gonadal growth. Additionally, in females FSH acts within the granulosa cells to promote the aromatization of thecal androgens to estradiol. With continued stimulation by the gonadotropins LH and FSH, the peripubertal gonads grow and secrete sex hormones at steadily increasing rates.

During puberty, the effects of the rising hormone levels can be seen in a variety of tissues in a sex-specific manner. In females, estrogens are responsible for growth of the breasts and maturation of the female genitalia, while circulating androgens control the growth of pubic and axillary hair. In males, androgens control not only the development of genitalia and growth of

body hair, but also deepen the voice by enlarging the larynx and laryngeal muscles. The development of these secondary sexual characteristics does not occur at precisely the same chronological age in all individuals, but the *sequence* of changes is characteristic for each sex and generally the same across individuals.

Two mechanisms for the control of puberty onset

It has been hypothesized that the suppression of GnRH release prior to puberty onset is the result of two different mechanisms (which may not be equally important or prominent across species). The first mechanism relies on the fact that prior to puberty, the hypothalamus and the pituitary gland are extraordinarily sensitive to the negative feedback effect of gonadal steroid hormones, which inhibit GnRH and LH release and keep the HPG axis quiescent. If this system becomes less sensitive to gonadal hormone feedback, gonadotropin secretion can escape from negative feedback, allowing gonadal hormone levels to rise as puberty progresses. This mechanism is often referred to as the 'gonadostat hypothesis,' and has been attributed to the endocrinologist Melvin Grumbach, although his initial description of the phenomenon was "gonadal steroid-dependent LHRH increase" [6]. The second mechanism is independent of gonadal hormone feedback and instead postulates that there are neural mechanisms that restrain the activity of GnRH neurons prior to puberty, resulting in low gonadotropin and gonadal hormone levels in prepubertal organisms. This is referred to as the "central drive" hypothesis. It's possible that

in addition to active restraint of the GnRH neuron there may also be a lack of stimulatory inputs to GnRH neurons during this time. Under this hypothesis, puberty begins when the inputs to GnRH neurons change and allow more GnRH to be secreted.

The primary mechanism of puberty initiation seems to vary between species and sometimes even between sexes of the same species. In most species, however, a combination of both mechanisms is likely responsible for the initiation of puberty. It has been proposed that the hormone independent mechanism may provide coarse control over pubertal timing, whereas the hormone dependent mechanism is responsible for fine control. In addition to the two systems described above, there are also numerous other signals that must be integrated to permit the pubertal increase in GnRH secretion. Leptin, a fat-derived hormone and an indicator of the amount of energy stored in the periphery, is one such permissive signal and will be described later in this review.

In humans, the suppression of GnRH secretion appears to be mostly independent of gonadal hormone feedback. Even though agonadal infants have elevated levels of LH and FSH during the first few years of life, they still exhibit the same qualitative pattern of quiescence that is observed in gonad-intact children in the years preceding puberty onset; levels of gonadotropins decrease during the juvenile pause compared to levels at birth [7,8]. Since these individuals lack gonads, gonadal steroid hormone feedback can not be responsible for this juvenile decrease.

Around the age at which puberty would normally occur, gonadotropin

levels rise in these individuals, despite the fact that they lack gonads and thus gonadal hormones. This suggests a significant contribution of the gonadal hormone independent mechanism in the restraint and subsequent activation of the GnRH pulse generator in humans.

Rodents

Researchers are limited in the manipulations that can be done in humans to study the regulation of puberty onset and sexual maturation. Although they have more freedom in non-human primates, the size, cost and relatively long pre-pubertal juvenile period of non-human primates have led to the development and use of other animal models in which to study puberty and reproductive maturation. Mice, rats, and sheep have all been popular models for researchers studying the HPG axis.

Rats are an attractive animal model for studying puberty and reproduction due to their rapid growth and sexual maturation as well as easily detectable external signs of sexual maturity. During the first half of the 20th century, studies in rats provided insight into the neuroendocrinology of puberty, including the identification of 'substances' released from the ovaries and the pituitary that could accelerate the maturation of juvenile female rats or their gonads (reviewed elsewhere [9]). In female rodents, the most common measures of sexual maturation are age at vaginal opening and then the presence of cornified epithelial cells in a vaginal smear. Vaginal opening is an estrogen-dependent phenomenon and precedes the first appearance of cornified epithelial cells in a

vaginal smear, which indicates that circulating estradiol was recently at levels capable of causing ovulation. In male rodents, the separation of the foreskin of the penis from the glans, called balanopreputial separation, is generally used as a measure of puberty onset [10].

Although rats have proved useful in the field of reproductive biology over the last century, the advent of modern molecular biology techniques has led to an increase in the use of laboratory mice. The laboratory mouse is not without limitations as a model of HPG axis regulation, but advances in mouse genetics have allowed researchers to investigate reproductive biology in ways that are impossible in other organisms. Before the 1990s, the 'knockout' mice available to researchers were spontaneous knockouts; that is, mice with naturally occurring alterations in specific genes that render the resulting gene product non-functional. With the introduction of gene-targeting technology and transgenic techniques, researchers can now manipulate genes for which spontaneous mutations had never been discovered. Additionally, in mice, the conditional deletion of receptors or proteins from specific cell types or specific areas of the body (termed conditional knockouts) has even further revolutionized the field of reproductive biology.

Estrogen and its receptors

Investigations into the gonadal hormone dependent mechanism of puberty onset regulation have focused mainly on estrogen action in females. Two nuclear estrogen receptors exist, encoded by two separate genes. In mice, the

genes *Esr1* and *Esr2* encode estrogen receptor α (ERα) and estrogen receptor β (ERβ) respectively; both receptors are class I members of the superfamily of nuclear hormone receptors and they share significant sequence homology. Each receptor is composed of 6 functional domains, including a DNA-binding domain and a domain capable of both ligand binding and activation of gene transcription. The mechanism of action is the same for both receptors: when the receptor binds to its ligand, it forms a dimer and migrates from the cell's cytosol into the nucleus where it affects transcription of target genes. Although their sequence is similar, for the most part the two estrogen receptors are expressed in different areas of the body, or in different cell types within the same tissue. Detailed descriptions of the distribution of the two receptors have been previously reported [11,12].

After the *Esr1* and *Esr2* genes were cloned (*Esr1* in 1986 and *Esr2* in 1996), researchers disrupted the normal gene sequence by inserting a copy of the neomycin-resistance gene (*neo*) into one exon of each gene [13–15]. The insertion of *neo* prevents normal expression of the functional receptor throughout the entire body, creating global knockout mice. Since the initial reports of the αERKO (lacking ERα globally) and βERKO (lacking ERβ globally) mice, numerous studies have investigated the effects of loss of estrogen action on specific reproductive tissues, as well as on overall reproductive maturation and fertility. It is important to note that more than one knockout mouse line exists for each receptor, and slight differences in the resulting phenotypes have been reported.

While the loss of either receptor is not lethal, both αERKO and βERKO

mice have reproductive impairments [13–16]. Female αERKO mice have normal ovaries during the neonatal period and prior to puberty, and mature oocytes can be generated in young animals with a pharmacologic hormonal manipulation, but the success of this manipulation is dependent on which αERKO mouse line is used [14,16–18]. As adults, αERKO females have enlarged cystic ovaries that lack corpora lutea, the structure that develops from a recently ruptured ovarian follicle [14–16,19]. The presence of corpora lutea in the ovaries indicates that ovulation has recently occurred and that the mouse is fertile. In αERKO mice, folliculogenesis proceeds through the early stages as primary and secondary follicles are visible, but they never fully mature and rupture [14–16,19]. Circulating levels of both LH and estradiol are profoundly increased in αERKO females [11,19–21]. The elevated levels of LH may be the direct cause of some aspects of the αERKO phenotype as mice that have targeted transgenic overexpression of LH are also anovulatory and share the ovarian phenotype of αERKO females [22,23]. The uterus of αERKO females is significantly smaller than a wild-type uterus and is unresponsive to the elevated circulating estrogen levels characteristic of these mice [14–16]; in a wild-type female mouse, elevated estrogen levels cause uterine enlargement and fluid accumulation. The mammary glands of αERKO mice are rudimentary and are also unresponsive to estrogenic compounds [15,24].

Female βERKO mice also have impaired overall fertility, but the effect of the mutation on the female reproductive axis is quite different from that observed in αERKO females [13]. The uterus and the vagina of βERKO females are normal and undergo the expected cyclic changes associated with estrogen exposure [13].

At a gross level, the ovaries of βERKO females are not different from those of wild-type females. Histological analysis reveals an increased number of early atretic (degenerating) follicles and very few corpora lutea, indicating subfertility and incomplete folliculogenesis [13]. Not surprisingly, given the reduced number of corpora lutea, βERKO females produce fewer litters and less pups per litter than wild-type females [13]. In contrast with the reported phenotype of the αERKO females, estradiol levels are unaffected, LH levels are only slightly elevated, and mammary glands are normal in females lacking ERβ globally [13,20,21]. Elevated estradiol levels in αERKO females but unaffected levels in βERKO females indicate a requirement of ERα, but not ERβ, in estrogen negative feedback.

While much information has been gleaned from these whole-animal knockouts of ERα and ERβ, the development of new genetic technologies has allowed for more site-specific investigation into the differential roles of these two receptors. As noted previously, ERα and ERβ are expressed in many different tissues of the body. For example, ERα mRNA can be found in the pituitary, ovary, uterus, oviduct and mammary gland, as well as in multiple locations within the brain [25]. The receptors have distinct physiological roles in different tissues and the following section of this review will focus exclusively on the central role of ERα in estrogen feedback and the use of site-specific ERα deletions to better elucidate its function in the brain.

Estrogen feedback control of LH and FSH

As described earlier, gonad-derived steroid hormones play an important

role in the regulation of GnRH and gonadotropin secretion. These hormones have a predominantly inhibitory role (termed negative feedback), but in post-pubertal females estrogen also has a stimulatory function (positive feedback), but only during a specific time of the rodent estrous cycle (similar to the human menstrual cycle). The feedback regulation translates into differential LH secretion patterns: tonic release (in males and females) and a surge-like secretion (only in females prior to ovulation). Estrogens have an essential role in these feedback loops, but the exact site of estrogen action and the neurons responsible for the feedback regulation have remained elusive.

Since a preovulatory LH surge can be generated in βERKO but not αERKO females and estrogen negative feedback is lost in αERKO but not βERKO females [19,20,26], research has focused on locating and characterizing the ERα-expressing neurons responsible for estrogen feedback. To do this, researchers have looked for ways to selectively ablate ERα from increasingly more specific sets of cells. With specific deletion studies, one can determine the physiological function and necessity of ERα in circumscribed subsets of cells, either in the central nervous system (CNS) or in the periphery.

This type of research has been made possible through the use of cre recombinase, an enzyme that *in vivo* can be used to catalyze site-specific DNA recombination between DNA sequence repeats called loxP sites. For instance, when the expression of cre recombinase is driven by the endogenous *CamKIIα* promoter (which is highly expressed in forebrain neurons), the recombinase will excise any DNA between loxP sites in forebrain neurons. This mouse line

(*CamKIIα*-cre mice) was bred to a second mouse line that has two loxP sites flanking the third exon of *Esr1*, the murine gene that encodes ERα [26]. This breeding scheme results in the excision of the coding sequence for the DNA binding domain of ERα from forebrain neurons and leads to production of nonfunctional ERα only in those cells in the conditional knockout mice.

Ablation of functional ERα from all *CamKIIα*-expressing neurons results in infertility [26]; knockout female mice exhibited abnormalities in their reproductive organs, including grossly enlarged and fluid-filled uteri, and their ovaries lacked corpora lutea, suggesting abnormally high estrogen levels and a failure to ovulate. Persistently high estradiol levels are indicative of impaired estrogen negative feedback. Additionally, an LH surge could not be generated in the knockout animals using a well-established surge-induction paradigm. At the time of the LH surge in a wild-type female mouse, the product of the immediate early gene c-Fos can be readily detected in GnRH neurons and also in neurons located in the anteroventral periventricular nucleus (AVPV; an area of the brain thought to play a significant role in the generation of the LH surge), indicating recent activation of these neurons. Although there were no changes in the number or distribution of GnRH-positive neurons in the knockouts, there was a lack of c-Fos immunoreactivity in GnRH neurons after the surge induction treatment [26]. C-Fos immunoreactivity was also absent from the AVPV. Together, this suggests that estrogen feedback (both positive and negative) relies on estrogen action via ERα-expressing forebrain neurons. Since GnRH neurons don't seem to produce functional ERα [27], there must be neurons located

upstream of them that do express ERα and are capable of transmitting information about changing estrogen levels to GnRH neurons. Recent research indicates that those neurons may be hypothalamic kisspeptin neurons.

Kisspeptin

Evidence from numerous studies points to a connection between the neuropeptide kisspeptin (product of the gene *Kiss1*) and reproductive function. In 2003, two independent studies illustrated this point, describing absent puberty, infertility and hypogonadotropic hypogonadism in humans with loss-of-function mutations in the kisspeptin receptor GPR54, product of the gene *Kiss1r* [28,29]. Subsequent reports of loss of functional kisspeptin itself have found a similar phenotype [30]. The reproductive phenotype described in human patients has been reproduced in mice lacking functional GPR54 or kisspeptin, indicating a common function of kisspeptin across species [31]. In contrast, activating *Kiss1* and *Kiss1r* mutations in humans lead to the opposite reproductive phenotype- precocious puberty [32,33]. In addition to its role in regulating puberty onset, kisspeptin/GPR54 signaling is also essential for the preovulatory LH surge and blockade of GPR54 signaling with a kisspeptin antagonist suppresses LH pulses in sheep [34–37].

Within the hypothalamus, there are two distinct populations of kisspeptin-synthesizing neurons. The larger population of kisspeptin neurons, located in the arcuate nucleus (ARC), expresses ERα and two additional neuropeptides, neurokinin B (product of the gene *Tac2*) and dynorphin A (product of the gene

Pdyn). These neurons have been termed KNDy neurons, reflecting the expression of these three peptides (kisspeptin, neurokinin B and dynorphin A) in this population of kisspeptin neurons in both rodents and sheep [38–40]. The kisspeptin neurons in the second hypothalamic population do not express *Tac2* or *Pdyn*, and are found predominantly in the anteroventral periventricular nucleus (AVPV) but extend caudally along the third ventricle into the periventricular nucleus (PeN). The majority of AVPV/PeN kisspeptin neurons also express ERα, and are activated around the time of the preovulatory LH surge [41].

There is evidence that both populations of kisspeptin neurons synapse on GnRH neurons, but the ARC kisspeptin neurons also communicate extensively with each other within the ARC [40,42]. What further distinguishes these two populations and points to their potential role in estrogen feedback is the effect of changing estrogen levels on *Kiss1* expression in the two populations. In a gonad-intact adult female, *Kiss1* mRNA levels are quite low in the ARC while levels are much more robust in the AVPV/PeN [43]. If the ovaries are removed, thus significantly lowering circulating estrogen levels, *Kiss1* mRNA levels rise in the ARC [43], indicating an inhibition of *Kiss1* by estrogen in intact females. If the ovariectomized female undergoes estrogen replacement, *Kiss1* mRNA levels drop in the ARC [43,44]. The opposite effect is observed in the AVPV/PeN: *Kiss1* mRNA levels drop significantly after ovariectomy but return to normal levels if the ovariectomized female is treated with estradiol [43,44].

This differential gene expression response of ARC and AVPV/PeN kisspeptin neurons to gonadectomy is also evident in male mice [45]. These

effects of changing estrogen levels on *Kiss1* mRNA are absent in female αERKO mice, suggesting a critical role for ERα in this phenomenon [43]. Interestingly, the overall effect is still present in male mice lacking ERα, perhaps suggesting that in males the androgen receptor (AR) is capable of effecting the same changes in *Kiss1* expression as ERα is able to in females.

Based on their response to changing estradiol levels and the fact that the AVPV/PeN kisspeptin neurons are active around the time of the LH surge, many researchers have hypothesized that estrogen action on kisspeptin neurons is required for estrogen feedback and more specifically that the AVPV/PeN kisspeptin neurons are responsible for estrogen positive feedback and the ARC KNDy neurons are responsible for estrogen negative feedback.

To test whether estrogen action via ERα in kisspeptin neurons is truly necessary for estrogen feedback, Mayer and colleagues generated a *Kiss1* cell-specific ERα knockout mouse (termed KERKO mice) [46]. Without ERα in kisspeptin-producing cells, puberty onset is dramatically advanced, indicating that estrogen signaling in kisspeptin neurons acts as a "pubertal brake." Since female KERKO mice don't ovulate or achieve estrous cyclicity, the researchers concluded that ERα in kisspeptin neurons is required for complete sexual maturation. Although LH levels in the knockouts are significantly higher than wild-type controls at early ages, this difference dissipates as KERKO females enter adulthood. The number of kisspeptin-immunoreactive cell bodies is diminished in the AVPV/PeN, but *Kiss1* mRNA levels in the ARC are significantly increased, providing further support for the idea that estrogen action through ERα

stimulates AVPV/PeN *Kiss1* expression while inhibiting it in the ARC. A second group of researchers performed a similar deletion study and found very comparable results [44]. Furthermore, they also describe elevated estradiol levels in the knockout females and the expected increase in uterine weight resulting from high estrogen levels.

While these two studies point to a critical role for estrogen action via ERα in kisspeptin neurons in the regulation of the timing of puberty onset and subsequent sexual maturation, they do little to clarify the differential role of the two kisspeptin populations. Taking a different approach, Rance and colleagues capitalized on the fact that ARC KNDy neurons (but not AVPV/PeN kisspeptin neurons) express *Tacr3*, the gene that encodes the neurokinin B receptor; they ablated all neurons that express NK3r and observed reduced negative feedback in their female mice [47]. The ablation did not, however, result in a complete loss of negative feedback, likely due to other hypothalamic circuits or unaffected negative feedback at the level of the pituitary. While this is the best evidence to date that ARC KNDy neurons are required for estrogen negative feedback, it doesn't definitively implicate estrogen action via ERα as necessary in these neurons.

In order to test the hypothesis that estrogen action via ERα in ARC KNDy neurons is absolutely required for estrogen negative feedback, a method for eliminating ERα from these neurons (while leaving AVPV/PeN kisspeptin neurons unaltered) must be developed. Capitalizing on the fact that *Tac2* is expressed in ARC KNDy neurons but not in AVPV/PeN kisspeptin neurons, I

investigated the necessity of estrogen action via ERα in ARC KNDy neurons by performing a genetic ablation of ERα from all *Tac2*-expressing cells and comparing the resulting phenotype to that of female mice lacking ERα in all *Kiss1* neurons (discussed in detail in **Chapter 3**).

The reproductive phenotype observed in male αERKO mice (reduced testes weight, reduced sperm count, reduced fertility) suggests a role for estrogen action via ERα in the control of the male reproductive axis as well [14]. Similar to females, male mice have ERα-expressing KNDy neurons in the ARC and *Kiss1* expression within these cells is negatively regulated by estradiol treatment [48]. The post-gonadectomy rise in LH can be attenuated by estrogen treatment, indicating a role for estrogen in negative feedback in males.

The requirement of estrogen action via ERα in kisspeptin neurons in male has been investigated previously [46]. Although the reported assessment of the male phenotype is extraordinarily brief, they found no effect on basal LH levels and no changes in testes weight in male KERKO mice. We sought to more completely characterize male mice lacking ERα in all kisspeptin neurons as well as compare their phenotype to that of males lacking ERα only in the ARC kisspeptin (KNDy) neurons. I employed the same genetic deletion strategy described above to investigate the effect of deletion of ERα from *Kiss1*- or *Tac2*-expressing neurons in the male mouse (discussed in detail in **Chapter 4**).

While gonad-derived hormones clearly play an important role in the

regulation of the HPG axis, they are not the only hormones that regulate reproduction and puberty onset; other circulating hormones also function to modulate the HPG axis. Although estrogen is a well-known stimulatory signal for puberty onset in females, other signals are considered to be permissive and function to fine-tune the timing of puberty onset and sexual maturation. These signals do not dramatically advance puberty onset like estrogen does, but they are still necessary regulatory components of the reproductive system. Identification of these permissive signals and their neural substrates will be necessary if researchers are to fully understand the regulation of mammalian puberty onset and sexual maturation.

The link between energy balance and reproduction

During times of undernutrition or food deprivation, all organisms must halt energy-intensive but non-critical activities and direct all their energy toward survival until food is readily available again. These energy-intensive but non-critical physiological activities include growth, immune function and reproduction. The regulatory mechanisms underlying these activities must be able to respond to changing levels of metabolic cues. One such metabolic cue is leptin, a hormone secreted by white adipose tissue in proportion to the amount of fat stored in the body. Thus, circulating leptin levels generally reflect the amount of energy that an organism has stored in the form of fat. Leptin acts in the brain to mediate not only energy balance and food consumption, but also a variety of neuroendocrine processes, including reproduction. Without leptin or its receptor,

energy balance is severely disrupted, resulting in profound obesity and diabetes. Additionally, immune function, growth and fertility are negatively impacted. The reported reproductive impairment of humans and mice that have mutations in the gene encoding either leptin or its receptor has led to the hypothesis that the hormone leptin and its receptor are critical for normal reproductive function.

Mice

A naturally-occurring single gene mutation causing profound obesity and hyperphagia in mice was first described in the literature in 1950 by researchers at what would eventually become the Jackson Laboratory [49]. The gene product and the site of its synthesis would remain unknown until the mid-1990s. The mutated gene causing the obesity, *Lep*, was eventually mapped and cloned, and its gene product was named leptin [50,51]. Mice lacking either leptin (called *ob/ob* mice) or its receptor (*db/db* mice) are profoundly obese, hyperphagic, hyperglycemic, cold-intolerant and have impaired immune function. Treating *ob/ob* mice with leptin rapidly and significantly reduces their food intake and body weight, confirming that the hyperphagia and obesity are a direct result of the lack of circulating leptin [51,52].

In addition to the impairments described above, homozygous *ob/ob* mice are also infertile, although heterozygote animals have unaffected fertility [49]. The reproductive failure in male mice lacking leptin has been attributed to insufficient production of LH and testosterone, resulting in increased FSH levels, and reduced testes weight [53,54]. The sterility in male *ob/ob* mice can be reversed with

leptin treatment or food restriction- thwarting the *ob/ob*'s natural predilection for over-eating [52,55,56]. While the degree of infertility of the *ob/ob* mouse is somewhat strain-dependent, the fact that it can be reversed with leptin treatment indicates a critical role for leptin in the regulation of male reproductive function.

Similar to their male counterparts, the sterility of female *ob/ob* mice can be corrected with chronic leptin treatment, but can not, however, be reversed with food restriction [55,57,58]. Uterine weight, ovarian weight, total follicle number and serum LH are all significantly increased in *ob/ob* adult females after chronic leptin treatment [55]. The reproductive axis of *ob/ob* females responds positively to a crude GnRH extract made from pituitary stalk/median eminence, evidence that the pituitary gland is functional but lacks appropriate stimulation by secretions from the hypothalamus [59]. Ovulation is only possible in *ob/ob* females after gonadotropin treatment, providing further evidence that the reproductive impairment in *ob/ob* females is due to a central deficit in leptin action and not an impairment in either the pituitary or gonads [54,60,61]. Together, these data reveal an important role for leptin in the central regulation of the female reproductive axis in mice.

As illustrated above, adult mice with congenital deficits in leptin signaling have severe reproductive impairments. Additionally, leptin has an effect on pre-pubertal animals; leptin treatment slightly accelerates puberty onset in wild-type female mice [62,63]. Similar to leptin treatment, transgenic leptin overexpression also leads to slightly advanced puberty onset in females [64]. While increasing circulating levels of leptin in prepubertal animals – either genetically or

pharmacologically – does accelerate puberty onset, the advancement is very small and may suggest that in mice, sufficient leptin levels are one of the final permissive signals needed for puberty onset. The magnitude of advancement of puberty in these two cases is incomparable to the advancement observed when, for example, estrogen feedback to the hypothalamus is disrupted [44,46]. Hence, leptin is an important permissive signal for puberty onset, but should not be classified as a stimulatory signal.

Humans

Additional evidence of leptin's regulation of the reproductive axis has been gleaned from rare occurrences of *LEP* or *LEPR* (the human genes for leptin and its receptor) mutations in the human population. Although initial mutational screenings of large groups of obese individuals failed to identify any individuals with mutations in the genes for leptin or the leptin receptor [65,66], there are a scant number of documented occurrences of patients with mutations in these genes. The first report described two young cousins from a consanguineous family who were homozygous for a single nucleotide deletion in *LEP*, resulting in severe obesity and hyperphagia [67]. In these young patients, chronic leptin treatment reduced energy intake during a test meal (indicating reduced hyperphagia) and produced a remarkable reduction in fat mass [68,69]. The young age of these affected individuals initially prevented researchers from drawing any conclusions as to leptin's role in human puberty or fertility, but continued observation and treatment did eventually inform researchers of leptin's role in human reproductive

axis function.

To determine the effect of leptin on the onset of puberty, three pre- or peripubertal individuals underwent chronic leptin replacement [69]. Chronic treatment of the two prepubertal children previously described did not lead to elevation of basal LH and FSH levels and sex steroid levels above the prepubertal range. In contrast, chronic leptin treatment of the third peripubertal individual resulted in a gradual increase in gonadotropin and estradiol levels, and eventually she developed secondary sexual characteristics and began menstruating regularly. In an additional peripubertal individual with a leptin mutation, leptin treatment beginning at age 14 years and 9 months led to a rapid rise in basal and stimulated LH and FSH from prepubertal to pubertal levels [70]. After chronic leptin treatment, her uterine volume and estradiol levels increased substantially. Taken together, data from these four leptin-deficient patients (two prepubertal and two peripubertal) reveal that in humans, leptin treatment does not stimulate precocious puberty in prepubertal individuals but instead acts in a permissive manner; leptin allows puberty to commence at an appropriate time and only when all other signals that affect pubertal timing are present.

Consistent with the notion that leptin is a permissive but necessary signal that allows puberty to commence, adults with either leptin or leptin receptor mutations not only exhibit profound obesity but also hypogonadotropic hypogonadism with severely delayed or absent puberty [71,72]. In one case, three sisters in a consanguineous Algerian family all possessed the same single base substitution in exon 16 of *LEPR*, the leptin receptor gene [71]. None of the affected

sisters had entered puberty despite the fact that their ages were between 13 and 19 years. They had no mammary gland development, sparse pubic hair and no axillary hair- all secondary sex characteristics that develop during puberty- and they were amenorrhoeic. Along with their low estradiol and LH levels, all three fit the criteria for central hypogonadism. This absence of sexual maturation due to leptin deficiency provides further evidence of the necessity of leptin action for normal reproductive function in human females.

Leptin signaling is also necessary for human male puberty commencement and sexual maturation. The report of an adult male (age 22) with a *LEP* mutation describes hypogonadism in addition to obesity and hyperinsulinemia; he never entered into puberty and lacked normal secondary sexual characteristics such as pubic and axillary hair [72]. Treatment of this affected individual with either human chorionic gonadotropin or GnRH significantly increased circulating levels of testosterone and FSH and LH, respectively, leading to a correction of his central hypogonadism.

Additional studies have described other affected adults with hypogonadism and delayed/absent puberty who lack secondary sexual characteristics as a result of leptin receptor mutations, although due to the nature of the mutation (affecting the receptor instead of leptin production), leptin administration would be ineffective as a treatment for the hypogonadism [69,71,73]. Due to their increased fat mass, these individuals already have elevated leptin levels, but without a functional leptin receptor, the circulating leptin is ineffective at regulating either energy balance or reproduction.

In agreement with findings from animal models of impaired leptin action, most evidence indicates that in both male and female humans, leptin is necessary for puberty commencement and full sexual maturation. However, a report of pregnancy in a woman with a known LEPR mutation has recently called into question the necessity of leptin signaling for human fertility [74].

In both mice and humans, the threshold for the amount of leptin needed for normal reproductive capacity and puberty onset seems relatively low compared to other physiological functions that require leptin signaling. Despite higher fat mass and decreased circulating leptin, mice that are heterozygous for the *ob* mutation (*ob*/+ mice) are fertile [75]. This suggests that while one wild-type copy of the gene encoding leptin is not sufficient to properly regulate energy balance, it is able to support normal function of the reproductive axis. An additional model of decreased leptin action lends support to the previous report of spared fertility in mice with low leptin levels; when *ob/ob* mice were bred to a mouse strain that expresses a weak human leptin transgene, the offspring were fertile despite the fact that their leptin levels were approximately half that of wild-type mice [76]. Some but not all other neuroendocrine effects were also corrected in the transgenic *ob/ob* animals, supporting the hypothesis that not all physiologic functions that require leptin signaling require the same degree of normal leptin action. In humans, an individual with a heterozygous *LEP* or *LEPR* mutation is clearly fertile since the homozygous affected individuals described above have parents who are unaffected heterozygous carriers of the mutation.

Insights into leptin's role in the regulation of the reproductive axis also

come from patients with congenital generalized lipodystrophy, a condition characterized by a lack of adipose tissue and thus extremely low leptin levels. Women with congenital generalized lipodystrophy are infertile; and suffer from reproductive symptoms that include hyperandrogenism, oligomenorrhea or amenorrhea, and polycystic ovaries [77,78]. Severe insulin resistance is also a hallmark of congenital generalized lipodystrophy and is likely to be the main cause of the excessive ovarian androgen production, whereas the lack of leptin may be responsible for the reduced pulsatile gonadotropin release. Leptin administration is a common treatment for affected individuals and corrects not only the severe insulin resistance and resulting hyperandrogenism, but also improves menstrual cyclicity [77,78]. Chronic leptin treatment has been shown to be sufficient to restore fertility and support pregnancy in a lipodystrophic female patient [79].

An additional opportunity to study leptin's effect on fertility comes from situations where leptin levels fall acutely. A laboratory-controlled starvation experiment in adult males illustrated the ability of leptin replacement to strongly regulate the HPG axis; leptin treatment was able to fully restore LH pulsatility and testosterone levels, despite a 72-hour fast that resulted in a dramatic decrease in circulating leptin [80]. In females, amenorrhea due to negative energy balance can also be corrected with leptin treatment, as can the reduced LH levels, ovarian volume and estradiol levels associated with the reduced leptin levels resulting from negative energy flux [81].

Leptin receptor signaling

The gene encoding the leptin receptor was cloned and identified shortly after leptin, its only known ligand, was cloned and identified [82]. Several leptin receptor isoforms exist, all derived from alternative splicing of the *Lepr* mRNA. Five isoforms have been identified in the mouse, differing at the C terminus but identical in their ligand-binding and membrane-spanning domains. Only the isoform called Ob-rb or LepRb has a significant intracellular domain (approximately 300 amino acids); this intracellular domain contains all the protein motifs capable of activating the Jak-STAT signal transduction pathway. Transgenic expression of LepRb in the brains of mice lacking all five LepR isoforms corrects most of the *db/db* phenotype [83], confirming the importance of this isoform and its central action. Leptin binding to its receptor activates the LepRb-associated Jak2 tyrosine kinase and promotes its autophosphorylation. This in turn phosphorylates three intracellular LepRb tyrosine residues: Tyr_{985}, Tyr_{1077} and Tyr_{1138}. Although the Jak2 tyrosine kinase activity is necessary for LepRb signaling, it is not sufficient to mediate most leptin action as LepRb mutant mice that can bind and activate Jak2 but lack all three tyrosine residues and other intracellular LepRb motifs (termed $LepRb^{\Delta 65}$ mice) are virtually indistinguishable from *db/db* mice [84]. Each tyrosine phosphorylation site recruits specific SH2 domain-containing effector proteins.

Phosphorylation of Tyr_{985} leads to recruitment of SHP2 and SOCS3. During leptin signaling in cultured cells, SHP2 participates in ERK activation [85,86]. *In vitro*, SOCS3 functions to attenuate LepRb signaling [85–87]. Phosphorylation of

Tyr_{985} does not appear to play a significant role in LepRb's control of metabolism and neuroendocrine physiology as mice homozygous for a Tyr→Leu mutation at 985 (named *l/l* mice) are lean, fertile and are more sensitive to leptin than controls [88]. They show no neuroendocrine impairment and actually exhibit protection from high-fat diet-induced obesity. Both male and female *l/l* mice are fertile. Thus LepRb signaling via phosphorylation of Tyr_{985} is not necessary for normal fertility.

Phosphorylation of Tyr_{1077} leads to the activation of the latent transcription factor signal transducer and activator of transcription-5 (STAT5). Mice homozygous for a Tyr→ Phe mutation at Tyr_{1077} (named *f/f* mice), exhibit only a very mild metabolic phenotype consisting mainly of increased fat mass and an increase in food consumption when maintained on a high-fat diet [89], suggesting that phosphorylation Tyr_{1077} plays only a minor role in leptin's control of metabolism. *f/f* mice do, however, have slightly impaired reproductive function, with females exhibiting long intervals between estrus cycles.

Phosphorylation of Tyr_{1138} leads to the activation of the latent transcription factor STAT3. Mice homozygous for a Tyr→ Ser mutation at Tyr1138 (named *s/s* mice) exhibit hyperphagia and obesity similar to that of *db/db* animals, but unlike *db/db* mice, they are fertile, have increased body length compared to controls and have only slightly elevated glucose levels [90]. In addition to the hyperphagia and obesity, *s/s* mice share other aspects of the *db/db* phenotype: they have difficulty maintaining body temperature in response to acute cold exposure (although not to the same degree as *db/db* animals), equivalently decreased

expression of uncoupling protein-1 (UCP1) in brown adipose tissue (BAT), and a similar repression of thyroid axis function [91]. Thus, leptin-induced phosphorylation of Tyr_{1138} is indispensable for many physiological functions, but not overall fertility.

In summary, it appears that the three identified tyrosine residues that are found on the intracellular domain of the leptin receptor are each responsible for different aspects of the physiology of leptin action. Mice completely devoid of leptin signaling are obese, hyperphagic, infertile, and exhibit a wide array of neuroendocrine deficits. Based on the phenotype of the *l/l* mice, it appears that signaling via Tyr_{985} does not play a large role in either metabolic or neuroendocrine control by leptin. Based on the phenotype of the *f/f* mice, signaling via Tyr_{1077} plays a role in leptin's control of the reproductive axis, but only a very minor role in leptin's control of metabolism. And finally, based on the observed phenotype of the *s/s* mice, signaling via Tyr_{1138} seems to be integral to the obesity and hyperphagia phenotype of *db/db* mice, as well as many of the their neuroendocrine impairments such as decreased thyroid axis function, but not their infertility.

Central leptin action

The major effects of leptin action on metabolism and reproduction appear to be mediated by the central nervous system [92–97]. Neurons expressing the long form of the leptin receptor, LepRb, are found throughout the brain [98–101], within discrete hypothalamic nuclei and also in circumscribed areas of the midbrain and

brainstem. In the hypothalamus, there are a large number of LepRb-expressing neurons in the arcuate nucleus (ARC), dorsomedial hypothalamus (DMH), lateral hypothalamus (LHA), ventromedial hypothalamus (VMH), posterior hypothalamus (PH) and the ventral premammillary nucleus (PMv).

Although some brain areas that contain LepRb-expressing neurons are known to be involved in regulation of the reproductive axis, GnRH neurons do not express LepRb [102–104]. Therefore, there must be LepRb-expressing neurons located upstream of GnRH neurons, the final output to the neuroendocrine reproductive axis. Since ARC *Kiss1* expression is reduced in *ob/ob* mice and leptin treatment ameliorates this deficit, some have postulated that direct leptin action on ARC kisspeptin neurons may be the mechanism by which leptin regulates the HPG axis [105]. There is some debate over the extent to which kisspeptin neurons express LepRb [103,105], but regardless, a genetic deletion of LepRb from all *Kiss1*-expressing neurons did not alter puberty onset or fertility [106]. This indicates that direct leptin action through LepRb on kisspeptin neurons is not the main mechanism for leptin's effect on puberty regulation or adult fertility.

Using a mouse model that allows us to visualize neurons that are in synaptic contact with GnRH neurons, we determined that LepRb-expressing neurons in the PMv and the striohypothalamic nucleus (StHy) contact GnRH neurons [103]. Although the LepRb-expressing neurons in the StHY have not been previously reported to be involved in the control of reproduction, the PMv is known to play an important role in the control of the reproductive axis. Neurons

in the PMv project to both GnRH and kisspeptin neurons and lesions to the PMv disrupt estrous cyclicity [106–110]. Lesions to the PMv also alter the changes in *Kiss1* and *Gnrh1* expression that are normally part of the proestrus → estrus transition in female rodents [111].

Although these studies point to an important role for the PMv in modulating the reproductive axis, they don't necessarily implicate leptin action in the PMv as a required component of this regulation. As many of the LepRb-expressing neurons in the PMv also express neuronal nitric oxide synthase (nNOS, product of the *Nos1* gene), we performed a *Nos1*-specific ablation of LepRb [112]. Excision of *Lepr* exon 17 by cre recombinase causes a frameshift in the last exon producing a premature stop codon and a null leptin receptor only in cells that express the gene *Nos1*. We investigated the metabolic and neuroendocrine effects of this *Nos1* cell-specific ablation of LepRb (discussed in detail in **Chapter 2**). Our results suggest that while leptin action in *Nos1*-expressing neurons is not necessary for overall female fertility, it does play an important role in energy balance and pubertal maturation. Future studies will be needed to determine whether there is a population of LepRb-expressing neurons that is absolutely crucial for sexual maturation and if it does exist, where in the brain those neurons reside. It is possible, however, that no such circumscribed population exists and that instead, leptin action in a distributed network of neurons is responsible for the metabolic control of puberty onset and fertility in mice.

Puberty and sexual maturation are highly regulated and incredibly important aspects of mammalian physiology. It is imperative that an organism not begin to reproduce until all levels of the HPG axis (the hypothalamus, the pituitary and the gonads) are fully mature and the organism has sufficient energy stores to support gametogenesis and sexual reproduction. Estrogen and leptin are two crucial hormonal signals that function within the hypothalamus to regulate the timing of puberty onset and also the completion of puberty. A better understanding of where these signals act and what exactly their function is within specific subsets of hypothalamic neurons is necessary if we are to implement better treatments for disorders such as precocious or delayed puberty, or other diseases with symptoms that include impaired pubertal onset or maturation.

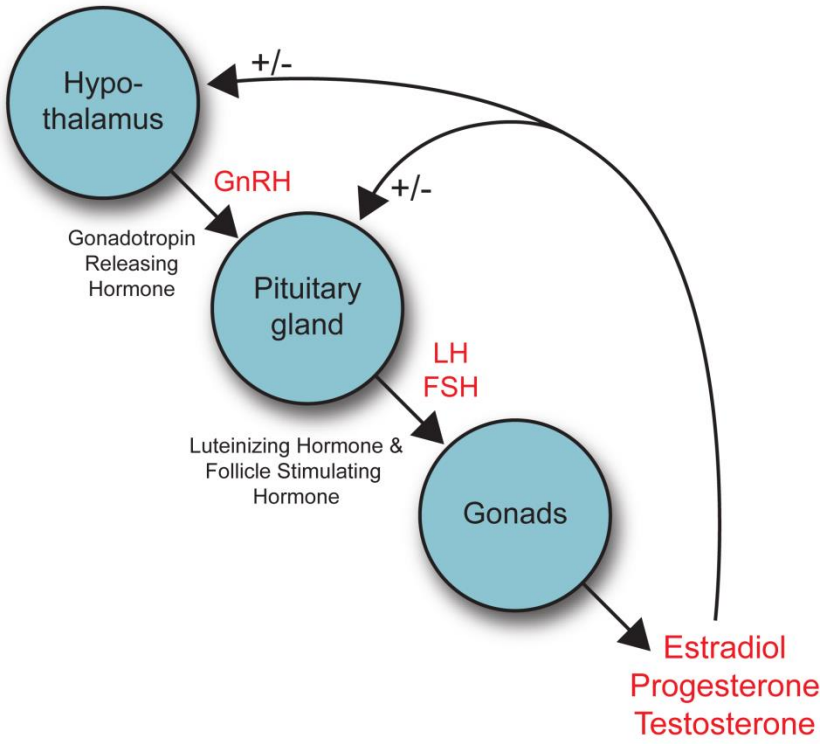

Figure 1- The hypothalamic-pituitary-gonadal (HPG) axis

Gonadotropin-releasing hormone (GnRH) is released in a pulsatile manner from GnRH neurons residing in the hypothalamus into the hypophyseal portal system where it can reach the gonadotropes in the anterior pituitary. The pituitary gonadotropes produce the gonadotropins follicle-stimulating hormone (FSH) and luteinizing hormone (LH). The effects of the gonadotropins on the gonads depend on the organism's sex and the pattern of release of the gonadotropins from the pituitary. Gonadal hormones (as well as many other signals) feed back to the hypothalamus and the pituitary gland to modulate the axis.

Chapter 2 - Genetic ablation of LepRb from *Nos1*-expressing neurons [*]

The fat-derived hormone leptin controls a diverse array of physiological processes. Loss-of-function mutations in the gene for either leptin or its receptor (product of the gene *Lepr*) reveal that leptin action is necessary for proper regulation of feeding, energy balance and a variety of other neuroendocrine functions [113–115]. Conditional knockout mice have allowed researchers to begin to determine the importance and necessity of individual subsets of *Lepr*-expressing neurons. Genetic ablation of *Lepr* from all hypothalamic neurons, for example, leads to increased body mass, adiposity and food intake, confirming the importance of hypothalamic leptin action in the regulation of energy balance [116]. More restricted deletions have uncovered subsets of *Lepr*-expressing neurons that are responsible for satiety [117,118] and *Lepr*-expressing neurons that play a role in dopamine-mediated behaviors [119–122]. While the neurons responsible for some aspects of leptin action have been identified, the neuronal

[*] The work described here has been previously published [112]. The targeting construct used to generate the $Nos1^{IRES\text{-}cre}$ mice was the work of Yusong Gong. $Lepr^{Nos1}$KO mice were first generated and studied by Dr. Rebecca Leshan. Dr. Leshan also performed the validation of the $Nos1^{IRES\text{-}cre}$ mouse line.

populations that control some well-established functions of leptin action remain elusive.

Global *Lepr* mutants (*db/db* mice) or *Lep* mutants (*ob/ob* mice) of both sexes have severe reproductive impairments. The obesity that results from the loss of leptin action is not the cause of the infertility as reducing obesity with food restriction does not reverse infertility in *ob/ob* female mice [57]. In *db/db* mice, infertility can be completely rescued with transgenic expression of the long isoform of the leptin receptor (LepRb) exclusively in neurons [97]. While it's clear that the reproductive problems in *ob/ob* and *db/db* mice are due to a central deficit in leptin action, the location of the LepRb-expressing neurons that modulate the reproductive axis has not yet been determined.

LepRb-expressing neurons are found in many hypothalamic nuclei that have a known role in regulating the HPG axis. While much attention is paid to LepRb neurons in the arcuate nucleus (ARC), selective expression of LepRb in the ARC of leptin receptor null mice does not rescue their reproductive function [123], indicating that other brain areas mediate the effects of leptin action on reproduction. Re-expression of LepRb in the ventral premammillary nucleus (PMv), however, improves several aspects of reproductive function [106]. There is a large population of LepRb-expressing neurons in the PMv, a hypothalamic nucleus reciprocally connected with brain regions that are central to reproduction including the preoptic nucleus (POA) and the anteroventral periventricular nucleus (AVPV) [107,108,124–126]. Lesions to the PMv disrupt the estrous cycle and prevent the activation of the HPG axis that occurs at the time of the preovulatory

LH surge [109]. Additionally, neurotoxic lesions to the PMv of female *ob/ob* mice prevented leptin from stimulating LH secretion, indicating that direct leptin action in the PMv is an important modulator of the reproductive axis [106].

Based on these data implicating PMv LepRb neurons as an integral component in leptin's regulation of the reproductive axis, we have developed a genetic approach to test the necessity of direct leptin action in the PMv. Using a unique genetic marker of PMv LepRb neurons (including a small number of LepRb neurons elsewhere in the hypothalamus), we have ablated LepRb from a subset of hypothalamic neurons and investigated the effects of the deletion on reproduction, energy balance and neuroendocrine function. Our results suggest that direct leptin action on *Nos1*-expressing neurons is necessary for normal pubertal maturation and energy balance, but plays only a minor role in neuroendocrine function.

Methods

Animals

All animals were bred in our colony in the Unit for Laboratory Animal Medicine at the University of Michigan. All animals and procedures used were in accordance with the guidelines and approval of the University Committee on the Care and Use of Animals.

The generation of the $Nos1^{IRES\text{-}cre}$ mice has been previously reported [112]. Briefly, an IRES-cre cassette was inserted downstream of the STOP codon of the *Nos1* gene. $Lepr^{flox/flox}$ mice [127], which were initially provided by Dr. Streamson Chua and subsequently propagated in our colony, were crossed with $Nos1^{IRES\text{-}cre}$ mice to produce *Nos1*-specific *Lepr* knockout animals [112]. Cre expression during early development in some animals led to recombination globally and resulted in a Δ (or null) *Lepr* allele. Cre-mediated excision of exon 17 causes a frameshift in subsequent exons, resulting in obesity that is indistinguishable from the phenotype of *db/db* mice. $Nos1^{IRES\text{-}cre/+}$;$Lepr^{\Delta/+}$ or $Nos1^{IRES\text{-}cre/+}$;$Lepr^{flox/+}$ mice were bred to $Lepr^{flox/flox}$ mice in order to produce experimental ($Nos1^{IRES\text{-}cre/+}$;$Lepr^{\Delta/flox}$ "$Lepr^{Nos1}$KO") and control ($Lepr^{\Delta/flox}$ and $Nos1^{IRES\text{-}cre/+}$;$Lepr^{\Delta/+}$ "Control", $Nos1^{IRES\text{-}cre/+}$;$Lepr^{\Delta/\Delta}$ "LeprKO") animals. Genotyping was performed as described previously [112].

Phenotypic studies

Beginning at weaning (PND 21), mice were housed individually. Female mice were monitored daily for vaginal opening and then vaginal cytology was

assessed daily to determine estrous cyclicity. Body mass was measured and recorded prior to dissection when mice were 7-8 weeks old. After rapid decapitation without anesthesia, blood was collected and allowed to clot at room temperature for 30 to 90 minutes. Serum was isolated by centrifugation at 2,000xg for 15 minutes and stored at -20° C.

An additional cohort of female mice was generated to assess overall fertility. 7-8 week old females were housed with naïve C57BL/6J males purchased from Jackson Laboratory. For each female mouse, latency to produce pups was recorded; females were deemed infertile if no pups were born within 90 days of mating.

Brain microdissection and analysis by RT-qPCR

During the week preceding dissection, mice were briefly handled each day. All dissections took place between 13:00 and 16:00. At the time of dissection, mice were quickly decapitated and the brain was removed from the skull. The brain was placed in a rodent coronal brain matrix (1 mm divisions). The ARC and an area of the rostral hypothalamus that includes the AVPV/PeN were dissected and immediately frozen in separate tubes on dry ice.

RNA was extracted from microdissected tissue using Trizol (Invitrogen) and then converted to cDNA using SuperScript First-Strand Synthesis system for reverse transcriptase PCR. cDNA was analyzed in triplicate by quantitative realtime-PCR on an Applied Biosystems StepOnePlus Real-Time PCR System for *Gapdh* (endogenous control) and each of the following: *Pomc, Agrp, Npy,*

Socs3 and *Kiss1.* All Taqman assays were acquired from Applied Biosystems (Foster City, CA).

Hormone analysis

Serum thyroxine levels were analyzed in duplicate by the Michigan Diabetes Research and Training Center (MDRTC) Chemistry Laboratory using radioimmunoassay (TKT41, Siemens). Serum corticosterone levels were analyzed in duplicate using an enzyme immunoassay kit (K014-H1, Arbor Assays). Absorbance was measured using a Tecan Infinite F200 plate reader.

Image collection, data analysis and statistics

Gene expression levels were calculated using the $2^{-\Delta\Delta CT}$ method, normalized to expression in control animals. Corticosterone levels were determined by carrying out four parameter logistic curve (4PLC) fitting using Magellan Data Analysis Software (Tecan). One-way ANOVA with Bonferroni post-hoc analysis was used to test for significant differences between genotypes. Vaginal opening, first estrus and parturition datasets were analyzed using a Mantel-Cox (logrank) test. Differences were deemed significant if $p<.05$. Data are presented as mean±SEM.

Results

Generation of mice lacking LepRb in all Nos1-expressing neurons

In order to determine the physiological role of PMv LepRb-expressing neurons, we generated mice in which cre recombinase was inserted into the *Nos1* locus by homologous recombination (**Figure 2 a**). The restriction of cre expression to *Nos1*-expressing neurons has been previously confirmed [112]. The co-expression of cre (*Nos1)* and LepRb was determined and revealed that nearly all PMv LepRb-expressing neurons are *Nos1*-positive. *Nos1*/LepRb neurons are also found in the ARC and in the dorsomedial nucleus of the hypothalamus (DMH) [112].

$Nos1^{IRES\text{-}cre}$ mice were bred to Lepr-flox mice to eventually generate *Nos1* cell-specific LepRb knockout animals (**Figure 2 b**). Intermittent expression of cre during early development occasionally led to early recombination (producing what we refer to as a "Δ" *Lepr* allele) around the LoxP sites in the modified *Lepr* allele, precluding the use of $Nos1^{IRES\text{-}cre/+}$;$Lepr^{flox/flox}$ mice in the following studies. Instead, we compared experimental ($Nos1^{IRES\text{-}Cre/+}$;$Lepr^{\Delta/flox}$ "$Lepr^{Nos1}$KO") and control ($Lepr^{\Delta/flox}$ and $Nos1^{IRES\text{-}Cre/+}$;$Lepr^{\Delta/+}$ "Control") animals as well as global *Lepr* knockouts ($Nos1^{Cre}$ $Lepr^{\Delta/\Delta}$ "LeprKO") in some analyses.

Ablation of LepRb from Nos1 neurons results in a reproductive phenotype in female mice

Since the PMv has previously been implicated in the regulation of the

reproductive axis [106,107,109–111], we investigated several reproductive parameters in our female mice (**Figure 3**); age at vaginal opening, age at first vaginal estrus, and the latency to produce pups were all determined. While we did not observe any differences in the age at vaginal opening when comparing Lepr^{Nos1}KO females to their littermate controls (**Figure 3 a**), there was a significant effect of the deletion on the age at which females had their first estrus (**Figure 3 b**). Despite the observed delay in reaching reproductive maturity, overall fertility was not impaired and Lepr^{Nos1}KO females were capable of producing pups (**Figure 3 c**).

Reports suggest that the neuropeptide kisspeptin may be integral in relaying energy balance information to the HPG axis in a variety of species [103,128–130]. Since *Kiss1* expression is regulated by both acute and chronic alterations in leptin levels [129,130], we investigated whether *Kiss1* levels were altered in our Lepr^{Nos1}KO mice. In the ARC, *Kiss1* levels were completely unchanged by ablation of LepRb from *Nos1*-expressing neurons (**Figure 3 d**). We found that AVPV/PeN *Kiss1* levels were significantly reduced in the global LeprKOs compared to controls, but there was no significant alteration in Lepr^{Nos1}KOs (**Figure 3 e**). While the delay in first estrus suggests a slight deficit in pubertal maturation, the spared overall fertility indicates that either leptin action in *Nos1*-expressing neurons is not necessary for fertility or that other neural circuits are able to compensate for the loss of direct leptin action on *Nos1* neurons.

Deletion of LepRb from Nos1 neurons results in impaired energy balance in both male and female mice

Lepr-expressing neurons in many different areas of the brain play a role in the regulation of energy balance. Although there are no prior studies implicating the PMv in energy balance regulation, *Nos1*/LepRb neurons are found in more areas than just the PMv, leading us to investigate other physiological parameters in the Lepr^{Nos1}KO animals. We found that female Lepr^{Nos1}KO mice are heavier than their littermate controls, but not as heavy as the global LeprKOs (**Figure 4 a**). Compared to their littermate controls, the male Lepr^{Nos1}KOs are also obese, but more closely resemble global LeprKOs (although they are still significantly lighter) (**Figure 4 b**).

To determine whether the classical regulatory neuropeptides produced in the ARC were affected by the deletion of LepRb from *Nos1* neurons, we next examined mRNA levels of *Npy*, *Agrp*, *Pomc* and *Socs3* in the ARC of control, Lepr^{Nos1}KO and LeprKO mice (**Figure 5**). In agreement with previous reports [90,97,131], global loss of leptin signaling resulted in increased expression of *Npy* and *Agrp*, but reduced expression of *Pomc* in the ARC (**Figure 5 a-c**). Compared to controls, Lepr^{NOS1}KOs had unchanged *Npy* and *Agrp* expression, but significantly reduced *Pomc*, such that they were not different compared to LeprKOs. Expression of *Socs3*, a negative regulator of LepRb signaling is significantly decreased in global LeprKOs, but not significantly altered by ablation of LepRb from *Nos1*-expressing neurons (**Figure 5 d**).

The obesity (and its severity) observed in Lepr^{Nos1}KO mice was surprising

since the majority of *Nos1*/LepRb neurons are found in the PMv, an area without a known role in energy balance. Deletion of LepRb from GABAergic neurons causes profound obesity, but deletion from glutamatergic neurons has only a very minor effect on body mass [132]; since the PMv is a mostly glutamatergic nucleus, it is likely that extra-PMv GABAergic *Nos1*/LepRb neurons are responsible for the obesity observed in our Lepr^{Nos1}KO mice.

The neuroendocrine axis is affected in male but not female Nos1-specific Lepr knockouts

Hypothalamic-pituitary-adrenal (HPA) axis over-activation and elevated serum corticosterone levels are known effects of either leptin or leptin receptor deficiency [133–136]. The *Lepr*-expressing neurons responsible for this effect have not previously been reported, so we assessed HPA axis activity in our mice lacking LepRb in *Nos1*-expressing neurons. We found that in females, corticosterone levels were not significantly altered in either Lepr^{Nos1}KO or global LeprKOs compared to controls (**Figure 6 a**). This is in contrast with previous work that has found elevated corticosterone levels in female *db/db* mice [133]. We did, however, find corticosterone levels to be significantly elevated in male global LeprKOs, but not Lepr^{Nos1}KOs (**Figure 6 b**). Thus, direct leptin action on *Nos1* neurons is not required for proper regulation of the HPA axis in either females or males.

The hypothalamic-pituitary-thyroid (HPT) axis is also affected by deficient leptin signaling; decreased thyroxine (T4) levels have been reported previously in

mice lacking either leptin or its receptor [91,137]. Since the neuronal population responsible for this effect has not yet been determined, we investigated whether serum T4 levels were significantly altered as a result of ablation of LepRb from *Nos1*-expressing neurons. As expected, we observed significantly lower serum T4 levels in LeprKO mice of both sexes compared to littermate controls (**Figure 6 c, d**). T4 levels in female $Lepr^{Nos1}$KO mice were not, however, significantly changed compared to their littermate controls (**Figure 6 c**). In male $Lepr^{Nos1}$KOs, T4 levels were significantly lower than those of controls, but not as low as what we observed in the global LeprKOs (**Figure 6 d**). This sexual dimorphism in thyroxine deficiency suggests either a sex difference in the neural regulation of thyroid function, or that the greater obesity of male $Lepr^{Nos1}$KOs has a secondary effect on the thyroid gland.

Conclusions & Discussion

When energy stores are limited, organisms must prioritize physiological functions. Immune function, growth and reproduction are often suspended while all energy is shifted to food acquisition and consumption. The suspension of reproductive axis function has been described and studied for many decades. The hypothesis that a certain body mass is necessary for normal functioning of the reproductive axis has largely been supported by research in both humans and rodents since its introduction in the 1970s [138]. For example, despite their substantial energy stores, *db/db* mice lack the necessary receptor isoform (LepRb) to inform the central nervous system of the amount of energy stored in the periphery. As a result, they exhibit deficits in many of the systems described above- immune function, growth and reproduction. We set out to identify the neuronal population which functions to transmit information about energy stores to the hypothalamic output neurons of the reproductive axis- the GnRH neurons.

The leptin receptor isoform LepRb is found throughout the hypothalamus, in several nuclei that are known to regulate the reproductive axis. We chose to investigate the necessity of direct leptin action on ventral premammillary nucleus (PMv) neurons since numerous studies have identified the PMv as an important component of the neural circuitry connecting energy balance to reproduction [106,109–111]. Because *Nos1* is expressed in most PMv LepRb neurons, we developed and utilized a $Nos1^{IRES\text{-}cre}$ mouse to selectively ablate LepRb from all *Nos1*-expressing neurons.

Female mice lacking LepRb in all *Nos1* neurons (Lepr^{Nos1}KO mice) exhibited a mild impairment in reproductive maturation; although age at vaginal opening (puberty onset) wasn't affected by our genetic manipulation, Lepr^{Nos1}KO females were older than their littermate controls when they first went into estrus (completion of puberty). While this indicates a delay in reaching reproductive maturity, adult knockouts are fertile as evidenced by their ability to successfully produce live litters. Thus, direct leptin action in *Nos1* neurons appears to be unnecessary for fertility, but does modulate sexual maturation of female mice. Given the importance of reproduction to all organisms, it is not surprising that there are redundant neuronal pathways or perhaps even pathways that are plastic enough to compensate for this loss of direct leptin action on *Nos1*-expressing neurons.

The mechanism by which *Nos1*/LepRb neurons modulate the reproductive axis does not appear to involve changes in expression of the gene that encodes the neuropeptide kisspeptin; although decreased *Kiss1* expression has been postulated to contribute to the reproductive impairments associated with leptin-deficient states [105], we found ARC *Kiss1* levels to be normal in both Lepr^{Nos1}KO females and mice lacking LepRb globally (LeprKO mice). Kisspeptin neurons are also found in the AVPV/PeN, but again, we found unchanged *Kiss1* levels in that area of Lepr^{Nos1}KO females. Thus, it appears that *Nos1*/LepRb neurons may modulate the GnRH neuron directly and don't rely on kisspeptin neurons as an interneuron or that the mechanism doesn't involve changes in *Kiss1* gene expression.

While the reproductive phenotype in mice lacking LepRb in *Nos1*-expressing neurons was not as striking as we had expected, these mice did exhibit a dramatic metabolic phenotype. Both male and female Lepr^{Nos1}KO mice are profoundly obese, although not quite as obese as mice lacking LepRb globally. As the PMv has no known role in energy balance regulation (despite the number of LepRb neurons found there), we believe that non-PMv *Nos1*/LepRb neurons are responsible for this metabolic phenotype. Additionally, the loss of LepRb from glutamatergic neurons does not significantly alter energy balance and the PMv is a mostly glutamatergic nucleus [132]. As such, GABAergic *Nos1*/LepRb neurons are likely critical regulators of energy balance and should be the focus of future investigations into hypothalamic leptin action.

Leptin deficiency causes significant and well-characterized gene expression changes in the ARC. We assessed what effect, if any, deletion of LepRb from *Nos1*-expressing neurons had on the expression of ARC *Agrp*, *Pomc*, *Npy* and *Socs3*. While *Agrp*, *Npy* and *Socs3* expression was unchanged in Lepr^{Nos1}KO mice compared to littermate controls, we found a significant decrease in *Pomc* expression in Lepr^{Nos1}KO mice. *Pomc*/LepRb neurons do not express *Nos1*, so LepRb has not been genetically ablated from them; the change in *Pomc* expression is not an effect of the loss of direct leptin action on ARC Pomc neurons. The change in expression, then, is due to either the effect of high circulating leptin in these animals [112], or is an effect of altered neuronal inputs to the Pomc neuron as a result of the deletion.

Other hypothalamic-pituitary axes in addition to the HPG axis are affected

by leptin deficiency, so we assessed whether our deletion had any significant effects on them as well. We found no effect of our deletion on circulating corticosterone levels, indicating a normally functioning hypothalamic-pituitary-adrenal (HPA) axis in both sexes. While global LeprKO males exhibited the expected increase in corticosterone as a result of deficient leptin action [133–136,139], we did not see the same effect in females, indicating a possible sexual dimorphism in the effect of leptin deficiency on the HPA axis. Activity of the hypothalamic-pituitary-thyroid (HPT) axis is suppressed in states of leptin deficiency [134,139], so we measured thyroxine levels in Lepr^{Nos1}KO mice of both sexes. LeprKO male and female mice have diminished thyroxine levels, but we saw an effect of our conditional deletion only in males. This likely reflects a sexual dimorphism in leptin's regulation of the HPT axis, or is an effect of the greater obesity in male Lepr^{Nos1}KO mice.

Using a conditional deletion of LepRb from all *Nos1*-expressing neurons, we have uncovered a requirement of leptin action on *Nos1* neurons for proper energy balance regulation but not for fertility. Although in males they contribute modestly to neuroendocrine function, their main function appears to be control of energy balance. As the distribution of *Nos1*/LepRb neurons expands into multiple hypothalamic nuclei, future studies should focus on further dissection of this neuronal population and characterization of individual subpopulations of *Nos1*/LepRb neurons within the hypothalamus.

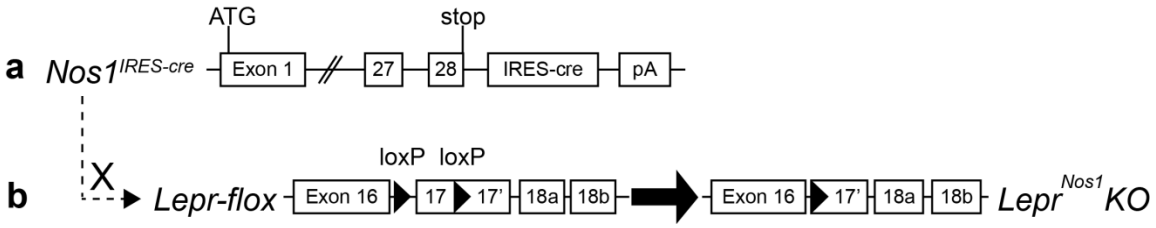

Figure 2 - Generation of Lepr^{Nos1}KO mice

(**a**) An IRES-driven cre coding sequence was inserted in the final *Nos1* exon between the stop codon and the pA site. A neomycin cassette has previously been removed from the Nos1$^{IRES\text{-}cre}$ mice. (**b**) Nos1$^{IRES\text{-}cre}$ mice were mated with Lepr$^{flox/flox}$ mice[127] to eventually generate Lepr^{Nos1}KO animals and littermate controls. ATG, start codon; IRES, internal ribosome entry site; pA, polyadenylation.

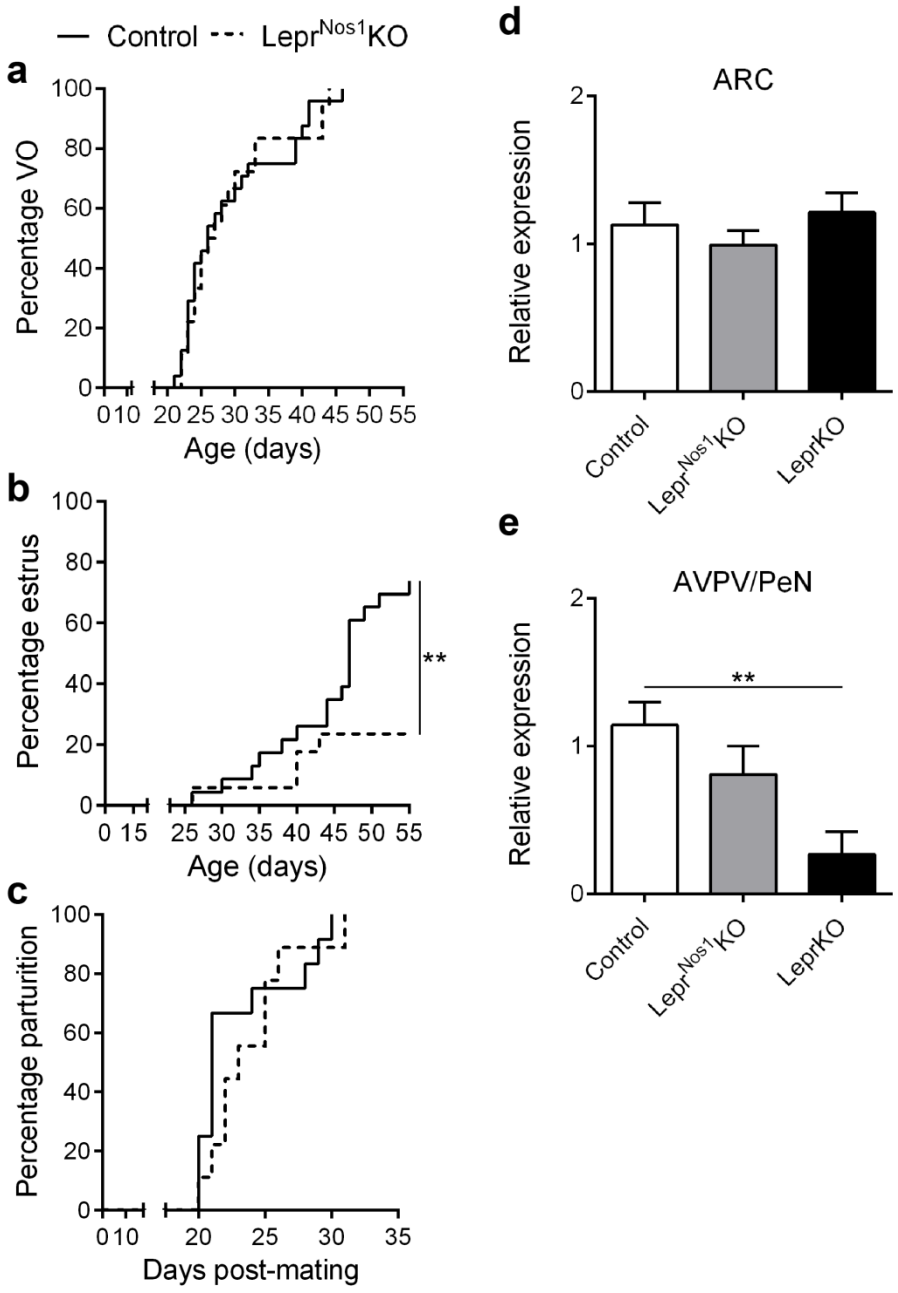

Figure 3 - Reproductive effects of ablation of LepRb from *Nos1*-expressing neurons

Percentage of female Lepr^{Nos1}KO and littermate control mice that have undergone (**a**) vaginal opening (n = 18-24) and (**b**) first vaginal estrus (n = 17-23). Mantel-Cox (logrank) test: **, p<.01. (**c**) Percentage of adult female Lepr^{Nos1}KO and littermate control mice that have produced a live litter of pups (n = 9-12). Relative *Kiss1* expression in the (**d**) ARC (n = 9-13) and (**e**) AVPV/PeN (n = 10-15) of adult female controls, Lepr^{Nos1}KOs and global LeprKOs. One-way ANOVA, Bonferroni post-hoc: **, p<.01. VO, vaginal opening.

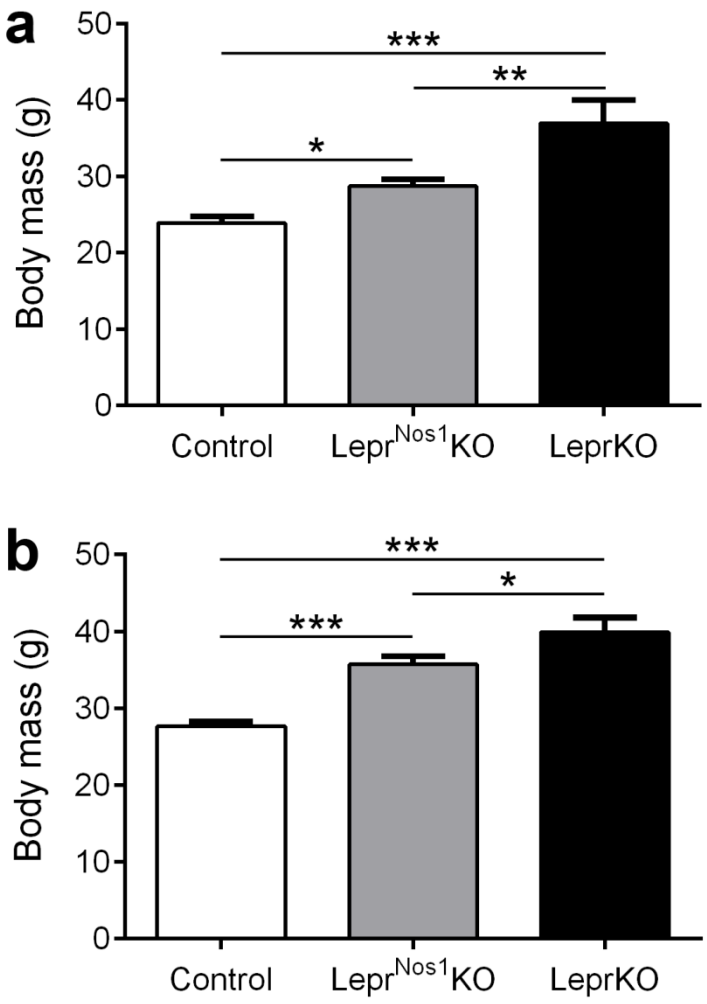

Figure 4 - Body mass is increased in both female and male Lepr^{Nos1}KO mice

Body mass of (**a**) female (n = 9-18) and (**b**) male (n = 12-25) adult littermate control, Lepr^{Nos1}KO and global LeprKO mice. One-way ANOVA, Bonferroni post-hoc: *, p<.05; **, p<.01; ***, p<.001.

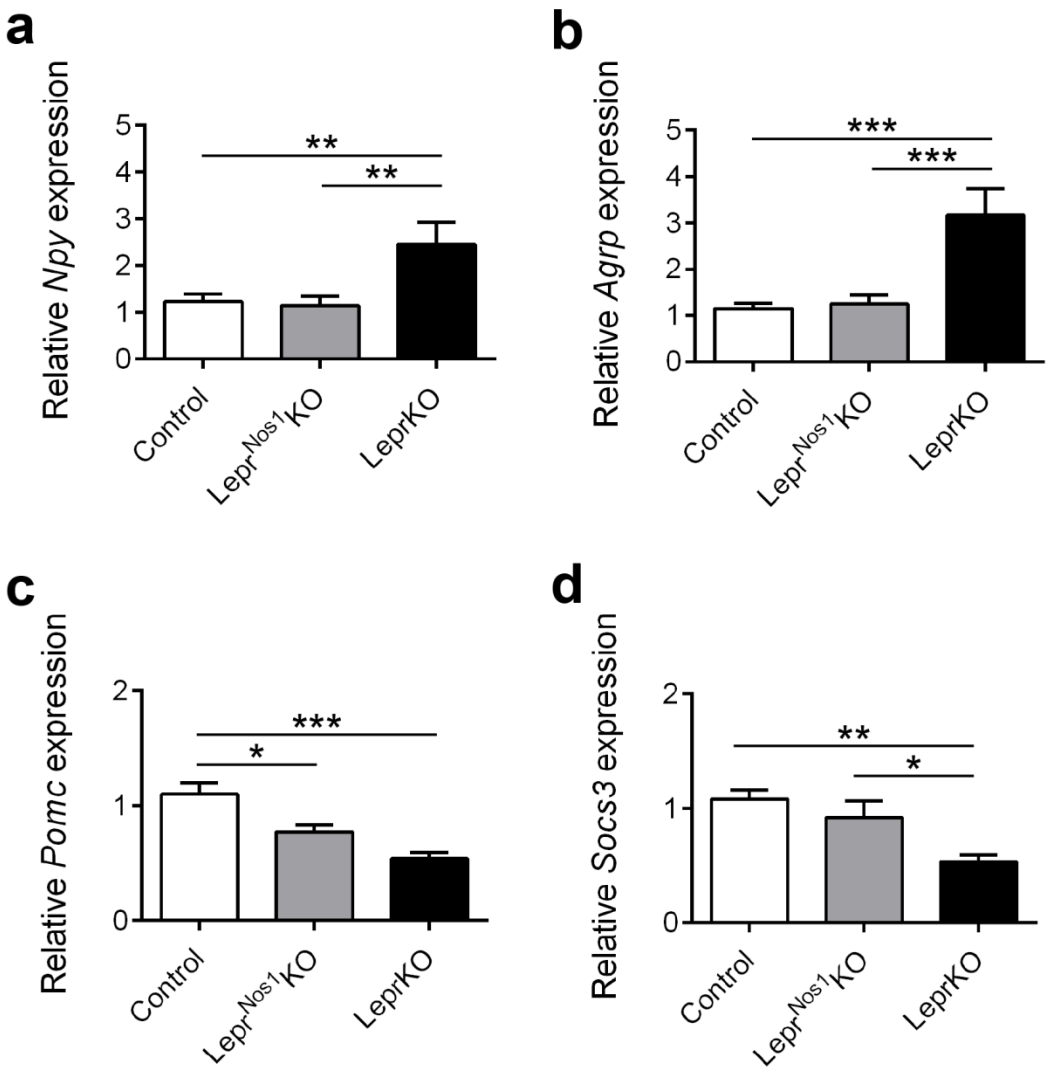

Figure 5 - ARC *Pomc* gene expression is altered in Lepr^{Nos1}KO mice

Relative expression levels of (**a**) *Npy* (n = 14-25), (**b**) *Agrp* (n = 14-25), (**c**) *Pomc* (n = 15-25) and (**d**) *Socs3* (n = 15-24) in the ARC of littermate control, Lepr^{Nos1}KO and global LeprKO mice. One-way ANOVA, Bonferroni post-hoc: *, p<.05; **, p<.01; ***, p<.001.

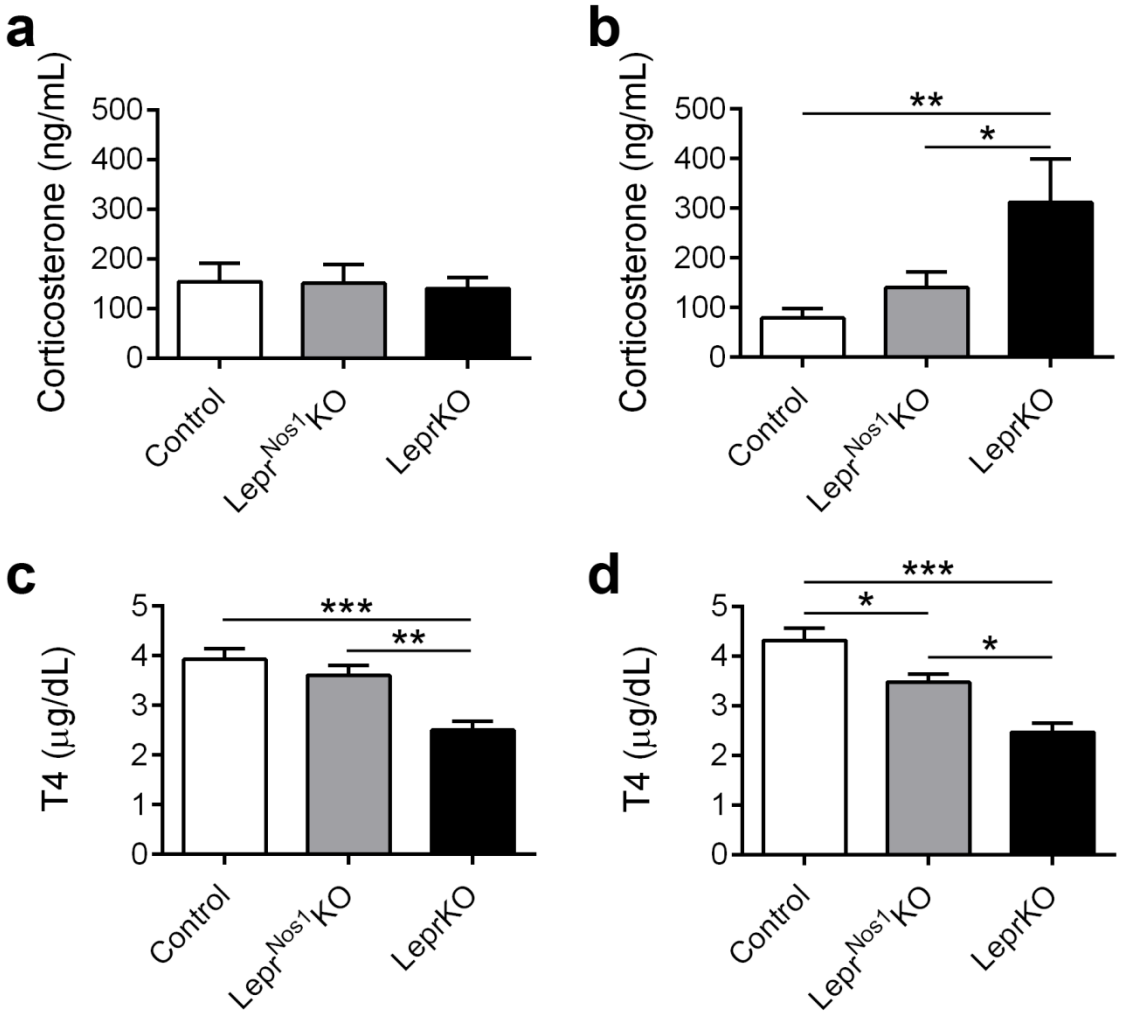

Figure 6 - Sexually dimorphic alteration in T4 levels in Lepr^{Nos1}KO mice

Serum corticosterone levels in (**a**) female (n = 8-14) and (**b**) male (n = 6-12) littermate control, Lepr^{Nos1}KO and global LeprKO mice. Serum T4 levels in adult (**c**) females (n = 8-16) and (**d**) males (n = 6-12). One-way ANOVA, Bonferroni post-hoc: *, p<.05; **, p<.01; ***, p<.001. T4, thyroxine.

Chapter 3 - Estrogen acts via ERα in kisspeptin/neurokinin B neurons to mediate feedback on the reproductive axis and control the onset of puberty in female mice[†]

Neurons producing the neuropeptide kisspeptin mediate important aspects of the neural control of the reproductive axis. Humans with *Kiss1* or *Kiss1r* (the kisspeptin receptor) mutations present with hypothalamic infertility [28,29], prompting a multitude of experiments in rodents as well as other mammals investigating the precise physiological role of kisspeptin neurons. Experiments in rodents illustrate a critical role for kisspeptin and its receptor in puberty regulation and fertility; the importance of kisspeptin neurons in the anteroventral periventricular nucleus/periventricular nucleus (AVPV/PeN) in estrogen positive feedback and generation of the pre-ovulatory luteinizing hormone (LH) surge has been previously reported [41,140]. Kisspeptin-producing neurons are also found in the hypothalamic arcuate nucleus (ARC) but the function of these kisspeptin neurons is less clear. Although some have concluded from mRNA experiments that ARC kisspeptin neurons control estrogen negative feedback [43],

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[†] In these studies, Dr. Courtney Marsh assisted with collecting the data presented in Figure 10-Figure 12 and performed some of the quantification of data presented in Figure 9. Drs. Carol Elias and Roberta Cravo generated the data presented in Figure 8.

electrophysiological assessments of this population find no evidence of their role in negative feedback [141]. Ablation of ARC kisspeptin neurons does, however, result in altered feedback on the reproductive axis, providing some evidence that they may play a role in estrogen negative feedback [47].

Both populations of kisspeptin neurons express estrogen receptor alpha (ERα), the receptor necessary for both positive and negative estrogen feedback [40,43]. Manipulation of the kisspeptin neuron's response (or ability to respond) to circulating estrogen will reveal their role in estrogen feedback. Not surprisingly, genetic ablation of ERα from all *Kiss1*-expressing neurons in the mouse results in a dramatic reproductive phenotype; *Kiss1* cell-specific ERα knockout females have precocious vaginal opening but never reach reproductive maturity as they don't have estrous cycles and are anovulatory [46]. One limitation of this model, however, is that ERα was ablated from both populations of kisspeptin neurons and doesn't allow for specific delineation of the function of each population individually.

Kiss1 gene expression is regulated by estrogen differently in the two neuronal populations; AVPV/PeN *Kiss1* expression is positively regulated by estrogen while ARC *Kiss1* expression is negatively regulated by estrogen treatment [43]. The populations differ not only in their response to circulating estrogen and their location in the brain, but also in the other neuropeptides that they produce. ARC kisspeptin neurons also produce neurokinin B (NKB), a product of the gene *Tac2*, and dynorphin A, an endogenous opioid peptide produced by the gene *Pdyn*. These neurons have been nicknamed KNDy

neurons to reflect the coexpression of these three peptides and distinguish them from the kisspeptin neurons residing in the AVPV/PeN which do not produce NKB or dynorphin A.

In the present study, we capitalized on the fact that ARC kisspeptin neurons express the gene *Tac2* and AVPV/PeN kisspeptin neurons do not. We generated a mouse that expresses cre recombinase under the control of the endogenous *Tac2* promoter and used this mouse line to ablate ERα from the ARC KNDy neurons without disturbing estrogen action in the AVPV/PeN kisspeptin neurons. We hypothesized that ERα in ARC KNDy neurons is required for restraint of the reproductive axis prior to puberty onset and also for normal estrogen negative feedback in adult female mice. Indeed, our results suggest that direct estrogen action on ARC KNDy neurons via ERα is required for 1) restraint of the reproductive axis prior to puberty and 2) for proper estrogen negative feedback in adult female mice.

Methods

Animals

All animals were bred in our colony in the Unit for Laboratory Animal Medicine at the University of Michigan in accordance with the guidelines and approval of the University Committee on the Care and Use of Animals. The *Esr1*-flox mouse line has been previously described [142]. Genotyping primers for this mouse line are as follows: For, 5'- aaggctgcaaggctttcttt – 3'; Rev, 5' – gaaattcttagccacagcttc – 3'. Early recombination around the loxP sites of this allele did occasionally occur and could be detected in a tail biopsy using a different forward primer: 5' – gaatccaccagctgctgtag – 3' and the reverse primer described above.

Generation of Kiss1$^{IRES\text{-}cre}$ and Tac2$^{IRES\text{-}cre}$ mice

5.6 kb of genomic DNA containing portions of exon 2 and the 3' UTR of the murine *Kiss1* gene was amplified by PCR from R1 ES cells and cloned into a plasmid containing a FRT-NEO-FRT-IRES-CRE cassette such that the cassette deleted a small sequence located 3' to the *Kiss1* STOP codon but 5' to the polyadenylation site. The targeting construct was linearized using the NotI restriction enzyme and electroporated into R1 mouse embryonic stem cells at the University of Michigan Transgenic Animal Model Core. Neomycin-resistant clones were analyzed by quantitative real-time PCR [143] and further confirmed by Southern blotting using the restriction enzyme HindIII and an external 580 bp

probe. Correctly-targeted ES cells were injected into C57Bl/6J blastocysts to generate chimeras. Male chimeras were then bred to C57Bl/6J females and pups were genotyped to confirm insertion of IRES-cre into the *Kiss1* locus. Primers used for genotyping are as follows: WT for, 5' – ccaaggcagggagcttcta – 3'; common rev, 5' – gagggtgtgggcatatgagt – 3'; Cre for, 5' – acctggcctggtctggacac - 3'. To remove the FRT-flanked NEO cassette, mice were bred to a Flp recombinase deleter mouse line (Jax 012930) and excision was confirmed by PCR.

5.2 kb of genomic DNA containing portions of exon 6 and the 3' UTR of the *Tac2* gene was amplified by PCR from R1 ES cells and cloned into the same FRT-NEO-FRT-IRES-CRE containing plasmid as described above. Electroporation occurred as described above and resistant clones were analyzed by quantitative real-time PCR and then Southern blotting using the restriction enzyme EcoRV and an external 560 bp probe. The targeting construct was linearized with NotI. Chimeras were generated and bred as described above, and pups were genotyped for insertion of IRES-cre into the *Tac2* locus. Primers used for genotyping: WT for, 5' – cccccagctttggcatcctc – 3'; common rev, 5' – gcgaatgacagaccatctctcc – 3'; Cre for, 5' - acctggcctggtctggacac – 3' (same as what is used for $Kiss1^{IRES\text{-}cre}$ genotyping). Removal of the FRT-flanked NEO cassette was the same as described above.

Mouse model validation

$Kiss1^{IRES\text{-}cre/+}$ and $Tac2^{IRES\text{-}cre/+}$ male mice were bred to homozygous cre-

inducible eYFP reporter females (Jax 006148) to generate Kiss1^{eYFP} and Tac2^{eYFP} animals respectively, allowing visualization and quantification of cre-expressing cells as eYFP expression is restricted to cells that express cre recombinase.

Dual-label immunohistochemistry/in situ hybridization

Dual-label immunohistochemistry/*in situ* hybridization (IHC/ISH) was performed as previously described [144] on brains from adult female Kiss1^{eYFP} and Tac2^{eYFP} mice. eYFP was detected using a chicken anti-GFP primary antibody (Abcam, 1:5000) and riboprobes were generated using the following primers: *Kiss1* F: 5'- AATTAACCCTCACTAAAGGGAGACTGTGTCGCCACCTATGG - 3'; *Kiss1* R: 5'- TAATACGACTCACTATAGGGAGATCTAGAAGCTCCCTGCCTTG - 3'; *Tac2* F: 5'- AATTAACCCTCACTAAAGGGAGAGCTGTCCTCGCCCTCAGCT TGGCTTGGA - 3'; *Tac2* R: 5'- TAATACGACTCACTATAGGGAGATTTGAGGA TGCCAAAGCTGGGGGTG - 3'

Perfusion and immunohistochemistry

Blood was drawn from the heart of female mice (age 21 days or 9-10 weeks) after receiving an overdose of sodium pentobarbital. They were then perfused transcardially with phosphate-buffered saline (PBS, pH 7.4) followed by 10% formalin. Blood was allowed to clot for 30 minutes at room temperature and then centrifuged for 15 minutes at 2,000 x g and the serum was removed and stored at -20° C. The brain was removed and postfixed in 10% formalin for 2-4

hours and then dehydrated in 30% sucrose in PBS until the time of sectioning. Brains were cut in 30 µm coronal sections on a sliding microtome, collected in four representative series, and stored at -20° C in cryoprotectant.

For kisspeptin immunostaining, free-floating brain sections were pretreated in 1% H_2O_2 in PBS, then blocked in 3% normal donkey serum (NDS)/3% Triton X-100 in PBS and then incubated with primary antibody (rabbit anti-kisspeptin, Millipore, 1:2000) overnight. Sections were thoroughly washed with PBS to remove the cryoprotectant, incubated in biotinylated donkey-anti-rabbit (Jackson ImmunoResearch, 1:200) for 2 hours, followed by avidin-biotin-complex labeling (Vectastain Elite kit, Vector Laboratories). Signals were developed with diaminobenzidine resulting in a brown precipitate.

For eYFP and ERα immunostaining, sections were blocked as described above and then incubated in primary antibodies (chicken anti-GFP, Abcam, 1:1000; rabbit anti-ERα, Santa Cruz, 1:1000) overnight. Brain sections were washed and then incubated with Alexa Fluor-conjugated secondary antibodies (goat anti-chicken 488 and donkey anti-rabbit 568, Invitrogen, 1:250). Sections were mounted onto gelatin-coated slides and coverslipped with ProLong Antifade mounting medium (Invitrogen).

Longitudinal study

Cre-positive males (either $Kiss1^{IRES\text{-}cre/+}$ or $Tac2^{IRES\text{-}cre/+}$) were bred to $ERα^{flox/flox}$ females. The resulting $Kiss1^{IRES\text{-}cre/+}/ERα^{flox/+}$ or $Tac2^{IRES\text{-}cre/+}/ERα^{flox/+}$ males and $ERα^{flox/+}$ females were bred to each other to generate *Kiss1*- or *Tac2*-

specific ERα knockouts (Kiss1$^{IRES\text{-}cre/+}$/ERα$^{flox/flox}$, "ERα^{Kiss1}KO" or Tac2$^{IRES\text{-}cre/+}$/ERα$^{flox/flox}$, "ERα^{Tac2}KO") and their littermate controls ERα$^{flox/flox}$ and Kiss1$^{IRES\text{-}cre/+}$ or Tac2$^{IRES\text{-}cre/+}$.

Females were identified using toe-clipping and checked daily for vaginal opening beginning at PND10. Mice were individually housed beginning at PND21 and body weight was recorded weekly. After vaginal opening, estrous cycle phase was determined daily based on vaginal cytology [145]. At 8 weeks of age, body composition was determined by a NMR-based Minispec LF90II (Bruker Optics) analyzer. 9-week-old mice were dissected (some controls in diestrus and some in estrus, based on vaginal cytology). Blood was collected after an overdose of sodium pentobarbital was given and allowed to clot for 30 minutes at room temperature. It was then centrifuged for 15 minutes at 2,000 x g and the serum was collected and stored at -20° C. Using a mouse brain matrix, the ARC and a triangle of tissue containing the AVPV/PeN as well as the preoptic area were dissected and individually snap-frozen on dry ice. Brain tissue was stored at -80° for later RNA extraction. Ovaries and uterus were removed, weighed, and fixed overnight in Z-fix (Anatech) and then transferred to 70% ethanol for storage. Ovaries were sectioned and stained with hematoxylin and eosin stain (H&E) by the University of Michigan Comprehensive Cancer Center Tissue Core. The total number of follicles or corpora lutea (both ovaries, one representative section per ovary) were counted by an individual blind to the genotype.

RNA was extracted from microdissected brain tissue using TRIzol

(Invitrogen) and then converted to cDNA using the iScript cDNA Synthesis Kit (Bio-Rad). Gene expression was analyzed in triplicate by quantitative RT-PCR using *Gapdh* (endogenous control)*, Kiss1, Pdyn, Tac2* and *Gnrh1* Taqman assays (Invitrogen). Relative mRNA expression values were calculated by the $2^{-\Delta\Delta Ct}$ method, with normalization of each sample's ΔCt value to the average ΔCt of the intact control samples.

Ovariectomy and estradiol treatment

Nine week old female mice ($ER\alpha^{Kiss1}KO$, $ER\alpha^{Tac2}KO$ and their littermate controls) were ovariectomized ("OVX") under isofluorane anesthesia. Blood was collected in a heparinized capillary tube from the tail vein prior to ovariectomy. Estradiol treatment was based on a previously established negative feedback induction paradigm [146]; after a 12-day recovery from OVX, blood was collected again from the tail vein and mice received a sub-cutaneous injection of β-Estradiol 3-benzoate (1µg/20g body mass, Sigma Aldrich) in sesame oil. 3 hours later, blood was collected again. All blood samples were centrifuged and plasma was frozen at -20° C for later analysis. Seven days after estradiol treatment, all mice were dissected and the brains were microdissected as previously described.

Microscopy and image analysis

Microscopic images were obtained using an Olympus BX-51 microscope with a DP30BW camera (Olympus) or a Nikon Eclipse 90i microscope with a DS-

Fi1 color camera (Nikon) (for color images [ovaries histology, dual IHC/ISH]). Images from fluorescent labeling experiments were pseudo-colored and merged using Adobe Photoshop.

Hormone analysis

Serum or plasma was analyzed by the University of Virginia Center for Research in Reproduction Ligand Assay and Analysis Core or the University of Michigan Chemistry Core. Estradiol levels were determined by ELISA (Calbiotech) while LH and FSH levels were assessed using the Millipore MILLIPLEX MAP rat pituitary panel.

Statistics

Student's *t*-test was used when only two groups were compared. One-way ANOVA with Bonferroni post-hoc analysis was used when comparing three or more groups. Repeated measures two-way ANOVA was used to analyze body weight and serum gonadotropin levels in the ovariectomy experiment. Data were analyzed and graphs were generated using either OriginPro 8 or GraphPad Prism software. Differences were deemed significant if $p < .05$. Data are presented as mean±SEM.

Results

Generation and validation of Tac2 and Kiss1 cell-specific mouse lines

To study the effect of the loss of ERα from ARC KNDy neurons, we first used gene targeting in embryonic stem cells to develop a knock-in mouse line that expresses cre recombinase under the control of the endogenous murine *Tac2* promoter (**Figure 7 a**). An internal ribosome entry site (IRES) followed by cre recombinase cDNA was inserted downstream of the *Tac2* STOP codon. In $Tac2^{IRES\text{-}cre}$ mice, transcription of the modified *Tac2* allele yields a bicistronic mRNA from which neurokinin B and cre recombinase are independently translated. $Tac2^{IRES\text{-}cre}$ mice were first crossed to a cre-activated fluorescent reporter mouse line (ROSA26-EYFP mice), generating $Tac2^{eYFP}$ mice and allowing us to determine sites of cre expression (**Figure 7 b**). To specifically ablate ERα from *Tac2*-expressing neurons, we used a mouse strain in which exon 3 of the *Esr1* gene, which encodes the ERα binding domain, is flanked by loxP sites (*Esr1*-flox mice [142], **Figure 7 c**). Breeding this mouse line to the $Tac2^{IRES\text{-}cre}$ line described above generated $ERα^{Tac2}KO$ animals.

We also developed a *Kiss1* knock-in IRES-cre line (**Figure 7 d**) in order to compare the effect of ERα ablation from just a subset of kisspeptin neurons (using the $Tac2^{IRES\text{-}cre}$ mice) to the effect of loss of ERα from all kisspeptin neurons (using the $Kiss1^{IRES\text{-}cre}$ mice). $Kiss1^{IRES\text{-}cre}$ mice were also crossed to the ROSA26-EYFP line, generating $Kiss1^{eYFP}$ mice, and to the *Esr1*-flox line, generating $ERα^{Kiss1}KO$ animals.

To verify that cre expression was restricted to *Kiss1*- and *Tac2*-expressing cells in the two novel mouse lines, we performed dual-label *in situ* hybridization/immunohistochemistry on hypothalamic sections from Kiss1^{eYFP} and Tac2^{eYFP} female mice. In the ARC of a Tac2^{eYFP} female mouse, most eYFP cells express *Tac2* mRNA (**Figure 8 a**) and also *Kiss1* mRNA (**Figure 8 b**). Although we observed strong *Kiss1* hybridization lining the third ventricle in the AVPV of a female Tac2^{eYFP} female, we found no eYFP cells in that area (**Figure 8 c**), confirming that AVPV/PeN kisspeptin neurons do not express *Tac2*. There was a high degree of co-localization between hybridized *Kiss1* probe and eYFP immunoreactivity in the ARC of a Kiss1^{eYFP} mouse (**Figure 8 d**) confirming that cre expression was limited to kisspeptin neurons.

Using Kiss1^{eYFP} female mice, we determined that in the ARC, nearly all eYFP cells express ERα (93±0.8%; *n*=3) and in the AVPV/PeN, ~65% (66.2±7.2%; *n*=3) of eYFP cells express ERα (**Figure 9 a-b**). In Tac2^{eYFP} female mice, nearly all ARC eYFP cells express ERα (96.6±0.3%; *n*=3) but we detected no eYFP/ERα cells in the AVPV/PeN (**Figure 9 c-d**). While we found scattered eYFP/ERα cells elsewhere in the brain of Tac2^{eYFP} mice, they were few in number and the ARC eYFP/ERα cells represent 88% of all eYFP/ERα cells.

Generation and initial characterization of ERα^{Kiss1}KO and ERα^{Tac2}KO females

To generate *Kiss1*-specific ERα knockout mice, we first crossed Kiss1$^{IRES\text{-}cre}$ heterozygotes to Esr1-flox homozygotes (ERα$^{flox/flox}$) and then crossed the resultant Kiss1$^{IRES\text{-}cre/+}$/ERα$^{flox/+}$ males to ERα$^{flox/+}$ females. The F2 progeny

consisted of Kiss1$^{IRES\text{-}Cre/+}$/ERα$^{flox/flox}$ ("ERα^{Kiss1}KO") mice, ERα$^{flox/flox}$ mice (without the modified *Kiss1* allele) and Kiss1$^{IRES\text{-}Cre/+}$ mice (without a modified *Esr1* allele). The latter two genotypes were used as littermate controls to the ERα^{Kiss1}KO animals in all studies. When appropriate, Kiss1$^{IRES\text{-}cre/+}$ and ERα$^{flox/flox}$ female mice were combined into one larger control group. The same strategy was used to generate *Tac2*-specific ERα knockout mice, eventually generating littermate Tac2$^{IRES\text{-}Cre/+}$/ERα$^{flox/flox}$ ("ERα^{Tac2}KO") mice, ERα$^{flox/flox}$ mice (without the modified *Tac2* allele) and Tac2$^{IRES\text{-}Cre/+}$ mice (without a modified *Esr1* allele).

The body mass of ERα^{Kiss1}KO females was not significantly altered compared to littermate controls at early ages, but at 8 weeks of age ERα^{Kiss1}KO females were very slightly but significantly heavier than control littermates (**Figure 10 a**). A similar effect was observed in ERα^{Tac2}KO females; they were slightly but significantly heavier than their littermate controls at 5, 6, 7, and 8 weeks of age (**Figure 10 c**).

Body composition analysis of 8-week-old females revealed that both ERα^{Kiss1}KO and ERα$^{flox/flox}$ mice both had lower body fat percentage than Kiss1$^{IRES\text{-}cre/+}$ mice and also that ERα^{Kiss1}KO females have increased fluid percentage compared to both littermate control genotypes (**Figure 10 b**). The same effect was observed in ERα^{Tac2}KO mice and their littermate controls; Tac2$^{IRES\text{-}cre/+}$ mice have significantly higher fat percentage compared to both ERα$^{flox/flox}$ and ERα^{Tac2}KO animals and we found increased fluid percentage in ERα^{Tac2}KO females compared to both littermate control genotypes (**Figure 10 d**).

Puberty onset and reproductive phenotype of ERα^{Kiss1}KO and ERα^{Tac2}KO females

Vaginal opening is an external signal of puberty onset in rodents and can be significantly accelerated by a variety of different manipulations, including treatment with exogenous kisspeptin or estrogenic compounds [147–149]. We found vaginal opening to be significantly and profoundly advanced to a similar extent in mice lacking ERα in either *Kiss1* (**Figure 11 a**) or *Tac2* (**Figure 11 f**) neurons compared with their littermate controls. Vaginal cytology revealed a lack of estrous cyclicity in ERα^{Kiss1}KOs; they spent significantly less time in diestrus or metestrus and significantly more time in estrus than their littermate controls (**Figure 11 b & e**). While Kiss1$^{IRES\text{-}cre/+}$ (**Figure 11 c**) and ERα$^{flox/flox}$ (**Figure 11 d**) showed all stages of estrous cyclicity, ERα^{Kiss1}KO females exhibited constant vaginal cornification for the entire extent of the study (**Figure 11 e**). A similar lack of estrous cyclicity was also observed in ERα^{Tac2}KO females (**Figure 11 g & j**); they exhibited persistent vaginal cornification while Tac2$^{IRES\text{-}cre/+}$ (**Figure 11 h**) and ERα$^{flox/flox}$ (**Figure 11 i**) mice showed all stages of estrous cyclicity.

In addition to their impaired estrous cyclicity, both ERα^{Kiss1}KO and ERα^{Tac2}KO females exhibit the same striking abnormalities in their reproductive organs (**Figure 12**). The uterus of ERα^{Kiss1}KO animals was significantly enlarged and filled with fluid (**Figure 12 a**). Additionally, in these animals we observed an increase in the total number of follicles within the ovary but a stark reduction in the number of corpora lutea (**Figure 12 b**). Histological analysis of the ovary from a representative control adult female (**Figure 12 c**) revealed several corpora

lutea while the representative ovary from an age-matched ERα^{Kiss1}KO female (**Figure 12 d**) contained a hemorrhagic cyst and no corpora lutea.

The same effect was observed in the ERα^{Tac2}KO females. We found enlarged, fluid-filled uteri in the knockouts (**Figure 12 e**), an increase in the total number of follicles and a nearly complete lack of corpora lutea (**Figure 12 f**). Histological analysis of representative ovary sections from a control adult female (**Figure 12 g**) revealed a typical-looking ovary with several corpora lutea while a section from an ERα^{Tac2}KO animal (**Figure 12 h**) contained an increased number of follicles, several hemorrhagic cysts, and no corpora lutea.

Ablation of ERα from either *Kiss1*- or *Tac2*-expressing neurons results in accelerated puberty onset but incomplete sexual maturation. Their ovarian and uterine histology suggest a hyperstimulation of the HPG axis, possibly due to reduced negative feedback on gonadotropin secretion.

Gonadotropins and gonadal hormones

To assess whether deletion of ERα from either *Kiss1* or *Tac2* neurons altered feedback on gonadotropin and gonadal hormone levels, we analyzed serum from adult (9-week-old) (**Figure 13**) female knockouts and their littermate controls. Due to the fact that both ERα^{Kiss1}KO and ERα^{Tac2}KO adult females showed persistent vaginal cornification, a group of littermate controls were dissected in estrus and analyzed alongside the knockouts and the controls dissected in diestrus. We found LH levels to be slightly higher in ERα^{Kiss1}KO adult females only when compared to estrus controls but not when compared to

diestrus controls (**Figure 13 a**). FSH levels were completely unchanged (**Figure 13 b**). Estradiol levels in ERα^{Kiss1}KO adult females were approximately three times higher than levels in either control group (**Figure 13 c**).

A similar but slightly more profound effect was observed in ERα^{Tac2}KO females. When gonadotropin levels were assessed in adult females, we found a significant elevation in serum LH in the ERα^{Tac2}KOs compared to both control groups (**Figure 13 d**). As we saw in the ERα^{Kiss1}KOs, FSH levels were not significantly changed between ERα^{Tac2}KOs and the two control groups (**Figure 13 e**). The estradiol levels of the ERα^{Tac2}KO females were, however, significantly elevated (**Figure 13 f**), and to the same extent as what we observed in the ERα^{Kiss1}KO females.

While the increase in LH levels in the ERα^{Tac2}KO adults was significant, the small magnitude of the difference led us to investigate the hormonal milieu in animals around the time of puberty; it is possible that developmental compensation may explain the relatively small elevation in LH levels in our adult ERα^{Tac2}KOs and the surprisingly normal levels in ERα^{Kiss1}KOs.

When gonadotropin levels were assessed in juvenile animals, we saw a profound elevation in the ERα^{Kiss1}KO females' serum LH concentration compared to littermate controls (**Figure 14 a**), but no effect on FSH (**Figure 14 b**). Not surprisingly, given their precocious vaginal opening, juvenile ERα^{Kiss1}KO females also had significantly higher estradiol levels than their littermate controls (**Figure 14 c**). We observed the same effect on serum LH (**Figure 14 d**) and estradiol (**Figure 14 f**) in the ERα^{Tac2}KO females, but unlike ERα^{Kiss1}KO juveniles, they

also had significantly elevated FSH levels (**Figure 14 e**).

While these data suggest reduced negative feedback on the reproductive neuroendocrine system, we also wanted to assess the effect of ovariectomy and acute estradiol treatment in the knockout females (**Figure 15**). Since ovariectomy is a complete removal of estrogen feedback, the extent of the disrupted negative feedback in our knockout females could be determined by comparing their gonadotropin levels to those of ovariectomized females. As a result of ovariectomy, both LH (**Figure 15 a**) and FSH (**Figure 15 b**) levels rose in both ERα^{Kiss1}KOs and their littermate controls, although the elevation of FSH was significantly blunted in the conditional knockouts. Acute estradiol treatment was effective in reducing LH levels equally well in both ERα^{Kiss1}KOs and their littermate controls, but FSH levels remained lower than those of controls.

The same effect was observed in ERα^{Tac2}KOs; LH (**Figure 15 c**) and FSH (**Figure 15 d**) rose in both the knockouts and controls after ovariectomy, but the effect on FSH was blunted in ERα^{Tac2}KOs compared to controls. Acute estradiol treatment reduced LH levels similarly in both ERα^{Tac2}KOs and controls, although FSH levels remained lower than littermate controls. Thus, despite our observations of reduced estrogen negative feedback in both ERα^{Kiss1}KO and ERα^{Tac2}KO intact females, ovariectomy revealed that neither knockout had completely lost negative feedback on gonadotropin secretion. Both knockout mouse lines were also still sensitive to the negative feedback effects of acute estradiol treatment.

Hypothalamic gene expression

ERα belongs to a class of receptors that when bound to their ligands act as transcription factors, translocating to the nucleus and regulating gene expression. In both the ARC and the AVPV/PeN, expression of *Kiss1* is affected by fluctuating estrogen levels [43,44]. To determine how gene expression was affected by ablation of ERα from either *Kiss1*- or *Tac2*-expressing neurons, we extracted RNA from microdissected tissue (ARC and rostral hypothalamus), converted it to cDNA and then determined relative gene expression levels. We found that ablation of ERα from kisspeptin neurons significantly altered the expression of the genes encoding the three neuropeptides found in ARC KNDy neurons, all of which are known to be estrogen-responsive (**Figure 16**). Relative expression of *Kiss1* (**Figure 16 a**), *Tac2* (**Figure 16 b**) and *Pdyn* (**Figure 16 c**) were all significantly higher in the ARC of ERα^{Kiss1}KO adult females compared to littermate controls dissected in either diestrus or estrus.

The same effect was observed when ERα was ablated from all *Tac2*-expressing neurons; relative expression of *Kiss1* (**Figure 16 d**), *Tac2* (**Figure 16 e**) and *Pdyn* (**Figure 16 f**) in the ARC were all also significantly elevated in ERα^{Tac2}KO adult females compared to controls. The relatively small magnitude of the difference in *Tac2* levels between ERα^{Tac2}KOs and controls appears to be due to an effect of the insertion of the IRES-cre sequence into the *Tac2* gene as expression was diminished in Tac2$^{IRES-cre/+}$ controls relative to ERα$^{flox/flox}$ controls (data not shown). Since the changes in ARC gene expression are the same between ERα^{Kiss1}KOs and ERα^{Tac2}KOs, we believe this effect to be due to loss of

ERα from ARC KNDy neurons since this is common to both knockout lines studied here.

Gene expression in the rostral hypothalamus, where the AVPV/PeN kisspeptin neurons and GnRH neurons are found, was altered by ablation of ERα from all *Kiss1*-expressing neurons but not by ablation of ERα from *Tac2*-expressing neurons (**Figure 17**). In stark contrast to what we observed in the ARC, relative expression of AVPV/PeN *Kiss1* in ERα^{Kiss1}KO females was substantially diminished compared to controls (**Figure 17 a**). Despite all indications that the reproductive axis in these mice is overactive, we found no changes in *Gnrh1* gene expression (**Figure 17 b**).

Unlike what we observed in ERα^{Kiss1}KO mice, ERα^{Tac2}KO females exhibited no changes in *Kiss1* expression in the AVPV/PeN (**Figure 17 c**). Again we saw no effect of ablation of ERα from *Tac2* neurons on *Gnrh1* expression (**Figure 17 d**). Since we saw a change in AVPV/PeN *Kiss1* expression in the ERα^{Kiss1}KOs but not in the ERα^{Tac2}KOs, we believe this effect to be due to the ablation of ERα from AVPV/PeN *Kiss1*-expressing neurons.

While the ablation of ERα from either *Kiss1*- or *Tac2*-expressing neurons prevents direct estrogen action, we wanted to assess whether the gene expression changes we observed in intact ERα^{Kiss1}KO and ERα^{Tac2}KO were identical to the changes that would occur as a result of ovariectomy, a complete loss of estrogen negative feedback. To do this, we compared the expression of hypothalamic *Kiss1*, *Tac2*, *Pdyn* and *Gnrh1* in intact controls to levels in ovariectomized controls, intact knockouts and ovariectomized knockouts.

We found that in the ARC, *Kiss1* expression rose dramatically as a result of ovariectomy, to levels that were indistinguishable from those of intact and ovariectomized ERα^{Kiss1}KO females (**Figure 18 a**). Although we previously were able to detect a significant increase in ARC *Tac2* in the ERα^{Kiss1}KO mice (**Figure 16 b**), we found no significant changes in *Tac2* when comparing intact controls to ovariectomized controls, intact knockouts or ovariectomized knockouts due to problems specifically with the *Tac2* assay (**Figure 18 b**). ARC *Pdyn* expression was elevated in ovariectomized control animals to the same extent as intact ERα^{Kiss1}KOs (**Figure 18 c**). Of the genes that we analyzed, none were significantly different between ovariectomized controls and intact ERα^{Kiss1}KOs, suggesting that the alterations in ARC gene expression resulting from ovariectomy are due to loss of direct estrogen action on *Kiss1*-expressing neurons.

ARC *Kiss1* was elevated in ovariectomized controls to the same extent as what we observed in both intact and ovariectomized ERα^{Tac2}KO females (**Figure 18 d**). Again, even though we had previously detected a significant increase in ARC *Tac2* expression in ERα^{Tac2}KO females compared to littermate controls (**Figure 16 e**), a problem with the assay prevented us from finding any possible differences when comparing intact controls to ovariectomized controls, intact knockouts or ovariectomized knockouts (**Figure 18 e**). There was a significant increase in *Pdyn* expression as a result of ovariectomy, but no difference between ovariectomized controls and intact ERα^{Tac2}KOs and no additive effect of ovariectomy on *Pdyn* levels in the knockouts (**Figure 18 f**). Furthermore, the fact

that expression of all three of these genes are altered similarly between ovariectomized controls and intact ERα^{Tac2}KOs suggests that the increases in ARC gene expression resulting from ovariectomy are due to loss of direct estrogen action from not just *Kiss1*-expressing neurons but more specifically from the loss of ERα from ARC KNDy neurons specifically.

We next wanted to determine the effect of ovariectomy on gene expression in the rostral hypothalamus of control and *Kiss1*- or *Tac2*-specific ERα knockouts (**Figure 19**). As we found previously, AVPV/PeN *Kiss1* expression was severely diminished in ERα^{Kiss1}KO females, but we did not observe a significant reduction in controls as a result of ovariectomy as has been previously reported [43] (**Figure 19 a**). Ovariectomy did, however, reduce AVPV/PeN *Kiss1* levels in ERα^{Kiss1}KO females even further to undetectable levels. *Gnrh1* expression was not affected by ovariectomy or by ablation of ERα from all *Kiss1* neurons (**Figure 19 b**). The substantial reduction in AVPV/PeN *Kiss1* as a result of either ovariectomy or ERα deletion confirms the loss of positive regulation by direct estrogen action on AVPV/PeN kisspeptin neurons.

We detected a profound and significant decrease in *Kiss1* expression as a result of ovariectomy when we analyzed the littermate controls of the ERα^{Tac2}KOs, but as we found before, ablation of ERα from *Tac2* neurons does not significantly affect AVPV/PeN *Kiss1* expression (**Figure 19 c**). Our analysis revealed that *Gnrh1* expression was unaffected by ovariectomy or by the ablation of ERα from *Tac2* neurons (**Figure 19 d**). The reduction in AVPV/PeN *Kiss1* levels in ERα^{Kiss1}KO but not ERα^{Tac2}KO mice provides even more evidence that

1) our deletion is specific for ARC kisspeptin neurons and doesn't directly affect AVPV/PeN kisspeptin neurons, and 2) the severe reproductive phenotype of ERα^{Tac2}KO females is a result of affected ARC kisspeptin neuron function.

Analysis of kisspeptin peptide levels in the ARC and AVPV/PeN

The dramatic changes in hypothalamic *Kiss1* gene expression that we discovered in the conditional ERα knockouts prompted us to investigate whether these changes led to significant alterations in kisspeptin peptide as well. In young (21-day-old) animals, we found that ablation of ERα from all kisspeptin neurons had no effect on ARC kisspeptin peptide immunoreactivity (**Figure 20 a & b**). Despite the fact that *Kiss1* mRNA levels are dramatically increased in ERα^{Kiss1}KO adult females, we found no changes in ARC kisspeptin peptide staining in adults (~ 9 weeks old) (**Figure 20 e & f**). Similarly, in both young and adult ERα^{Tac2}KOs, we observed no changes in ARC kisspeptin staining (**Figure 20 c & d, g & h**).

In the AVPV/PeN, we saw the expected changes in kisspeptin peptide staining based on previously published data and our own gene expression findings [46] (**Figure 21**). In young ERα^{Kiss1}KOs and their littermate controls, we saw very little kisspeptin immunoreactivity (**Figure 21 a & b**), but kisspeptin staining was significantly diminished in adult ERα^{Kiss1}KO females compared to littermate controls, in agreement with our gene expression data (**Figure 21 e & f**). In young ERα^{Tac2}KO females, however, we saw a slight increase in AVPV/PeN kisspeptin immunoreactivity compared to their littermate controls

(**Figure 21 c & d**). In contrast with the diminished amount of kisspeptin in the AVPV/PeN of ERα^{Kiss1}KO adult females, there was no effect of ERα ablation from *Tac2* neurons on adult AVPV/PeN kisspeptin staining (**Figure 21 g & h**).

In summary, while we saw no effect of either deletion on the amount of ARC kisspeptin peptide immunoreactivity, AVPV/PeN kisspeptin immunoreactivity was diminished in ERα^{Kiss1}KO adults and increased in young ERα^{Tac2}KOs.

Conclusions & Discussion

While substantial progress has been made in understanding how the female HPG axis functions, there are still many unanswered questions. Central estrogen action via ERα is required for normal reproductive function in female rodents; without it, female mice exhibit reproductive organ anomalies and impaired estrogen feedback, resulting in anovulation and infertility [26]. Although the GnRH neurons are the final output of the hypothalamus, regulating the reproductive axis, they don't express detectable levels of ERα. Thus, there must be ERα-expressing neurons that reside upstream of the GnRH neurons that are critical for normal regulation of the female reproductive axis. The location and identity of those neurons are one of those remaining unanswered questions in the field of reproductive biology.

We used a genetic approach to test the necessity of direct estrogen action via ERα in either all *Kiss1*-expressing neurons or within a subset of kisspeptin neurons (the ARC KNDy neurons). Ablation of ERα from all *Kiss1* neurons or from only the ARC KNDy neurons resulted in the same dramatic acceleration of puberty onset in female mice, in agreement with previous reports that ablated ERα from all kisspeptin neurons [44,46]. As adults, both conditional knockout genotypes exhibited a complete lack of estrous cyclicity and reduced ovarian function; we found few corpora lutea and an increased total number of follicles in addition to a number of hemorrhagic cysts. Although the $ERα^{Kiss1}KO$ and $ERα^{Tac2}KO$ females entered puberty earlier than littermate controls, their lack of

cyclicity or ovulation suggests a failure to reach sexual maturity. The nearly identical phenotype suggests that the defect in sexual maturation is due to the loss of ERα from the ARC KNDy neurons since this is common to both knockout lines.

When we investigated the hormonal milieu in these mice around the time of puberty onset, we found that both ERα^{Kiss1}KO and ERα^{Tac2}KO juvenile females had elevated LH and estradiol levels. While the increased estradiol explains the early vaginal opening, elevated LH levels in the face of elevated circulating estradiol indicates impaired estrogen negative feedback in both knockout mouse lines. Serum analysis was performed in adult females as well; again, we found elevated LH levels in ERα^{Tac2}KOs, but the LH levels in adult ERα^{Kiss1}KOs were now unchanged compare to littermate controls. Since estradiol levels were profoundly elevated in both adult ERα^{Kiss1}KO and adult ERα^{Tac2}KO female mice, the unchanged and elevated LH levels, respectively, suggest a reduction in estrogen negative feedback.

Ovariectomy results in a complete loss of estrogen negative feedback and gonadotropin levels rise quickly and dramatically. Even though LH levels were significantly elevated in ERα^{Tac2}KO adult females, they were not as high as what we observed in ovariectomized females and in fact LH levels in these animals rose even further upon ovariectomy. After ovariectomy, acute administration of exogenous estradiol suppressed gonadotropin levels in both conditional knockouts. This implies additional redundant or compensatory negative feedback systems which can partially restrain LH levels in an intact animal and

upon acute estradiol treatment after ovariectomy, but are not able to properly restrain puberty onset or allow normal estrous cyclicity. Thus, while estrogen action via ERα in ARC KNDy neurons may not be the only estrogen negative feedback system in the mouse, its loss can not be completely compensated for by other systems under normal physiological conditions.

mRNA levels of several genes within the hypothalamus of the mouse are sensitive to estrogen feedback. Ablation of ERα from either all kisspeptin neurons or only ARC kisspeptin neurons resulted in a complete loss of negative feedback on ARC *Kiss1* and *Pdyn* gene expression, mimicking the levels found in ovariectomized animals. While we could detect a significant increase in ARC *Tac2* expression in both ERα^{Kiss1}KO and ERα^{Tac2}KO mice compared to littermate controls, technical difficulties with the gene expression assay prevented us from determining whether those levels were comparable to what is observed in an ovariectomized mouse. These findings indicate that while additional negative feedback systems may exist for the restraint of LH levels, loss of ERα from ARC KNDy neurons results in a complete loss of regulation of *Kiss1* and *Pdyn* and possibly *Tac2* as well.

In the rostral hypothalamus, AVPV/PeN kisspeptin neurons reside in close proximity to GnRH neurons. From the same microdissected tissue sample, we were able to assess expression levels of both *Kiss1* and *Gnrh1*. While *Kiss1* expression in the AVPV/PeN was not affected by loss of ERα from ARC KNDy neurons, it was significantly decreased as a result of ablation of ERα from all kisspeptin neurons. This reduction mimics what we observed in ovariectomized

mice, indicating the necessity of direct estrogen action via ERα on AVPV/PeN kisspeptin neurons for proper maintenance of *Kiss1* expression in those neurons.

While we saw dramatic increases in adult ARC *Kiss1* expression in both conditional knockout mouse lines, this effect was not apparent at the peptide level when we performed immunohistochemistry. This may be due to limitations in the ability to quantify IHC staining, so future studies should address whether ARC kisspeptin neurons produce and release more kisspeptin as a result of ERα ablation. In the AVPV/PeN we observed the expected decrease in kisspeptin in ERα^{Kiss1}KO but not ERα^{Tac2}KO females. While there appeared to be a slight increase in kisspeptin immunoreactivity in the AVPV/PeN of juvenile ERα^{Tac2}KOs, IHC is likely not the best way to quantify an increase in peptide levels and future studies are needed to confirm this finding. Regardless, this potential increase in kisspeptin peptide is not likely to be the cause of the precocious vaginal opening or elevated LH and estradiol in juvenile animals since these effects were common to both knockout lines and we didn't see the same peptide change in juvenile ERα^{Kiss1}KO females.

Interestingly, the phenotype that we have described here in the ERα^{Kiss1}KO and ERα^{Tac2}KO female mice is quite similar to that described by Singh and colleagues when they performed a selective deletion of ERα from the pituitary [150]. They found fewer corpora lutea and more atretic follicles and cysts in the ovary, as well as elevated LH and estradiol levels. They similarly found no change in *Gnrh1* expression despite an obvious reduction in estrogen negative feedback. Interestingly, while this selective deletion of ERα from the pituitary

resulted in decreased estrogen negative feedback, a different group performing a similar genetic deletion found no evidence of impaired estrogen feedback in their mice [151].

While we are the first to perform a genetic ablation of ERα selectively from ARC KNDy neurons, others have investigated the overall necessity of those neurons [47]. Surprisingly, mice lacking ARC KNDy neurons don't exhibit elevated basal LH levels, implying intact estrogen negative feedback. Taking into account our data which illustrates a reduction in negative feedback when ARC KNDy neurons lose ERα, this implies that the increase in LH as a result of decreased estrogen negative feedback requires ARC KNDy neurons. The precise manner by which the unrestrained KNDy neurons affect GnRH output to increase LH levels was not investigated here but should be the focus of future work.

Here, we show for the first time a clear reduction in estrogen negative feedback after loss of direct estrogen action via ERα in ARC KNDy neurons. This is in contrast with only a slight reduction in negative feedback on gonadotropin secretion in mice lacking ERα in <u>all</u> kisspeptin neurons. Despite the differences in the magnitude of feedback reduction between the two conditional knockout mouse lines, the overall reproductive phenotype is nearly identical. The regulation of puberty onset and sexual maturation is extremely sensitive to alterations in gonadal steroid feedback, and ERα in ARC KNDy neurons is a critical aspect of this system.

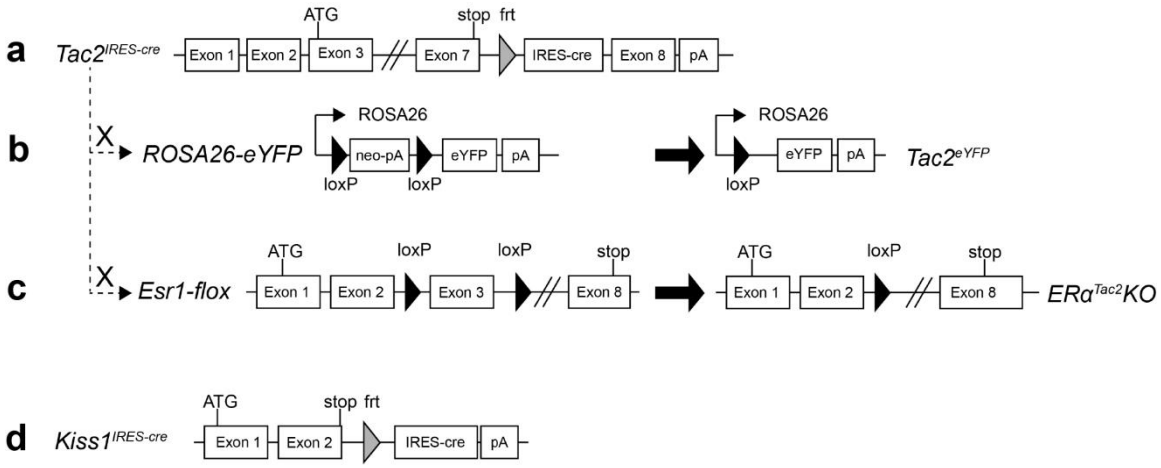


Figure 7- Generation of mice to study *Kiss1*- and *Tac2*-expressing neurons

(**a**) Tac2$^{IRES\text{-}cre}$ mice carry an IRES-cre cassette in the 3' UTR of the endogenous murine *Tac2* gene. (**b**) Tac2^{eYFP} mice were generated by crossing the Tac2$^{IRES\text{-}cre}$ mouse to a commercially available ROSA26 fluorescent reporter strain (Jax 006148), which after recombination around the loxP sites results in eYFP production in all cre-expressing cells and thus fluorescent labeling of *Tac2*-expressing cells. (**c**) *Tac2*-specific ERα knockout animals ("ERα^{Tac2}KO") were generated by crossing the Tac2$^{IRES\text{-}cre}$ mice to *Esr1*-flox mice which have loxP sites flanking exon 3 which contains the sequence for the DNA binding domain of the receptor [142]. Cre-mediated recombination around the loxP sites results in a *Tac2* cell-specific ERα null mouse. (**d**) Kiss1$^{IRES\text{-}cre}$ mice carry the same IRES-cre cassette in the 3' UTR of the murine *Kiss1* gene. They were used to generate Kiss1^{eYFP} and ERα^{Kiss1}KO mice. IRES, internal ribosome entry site; pA, polyadenylation site; frt, flippase recognition target.

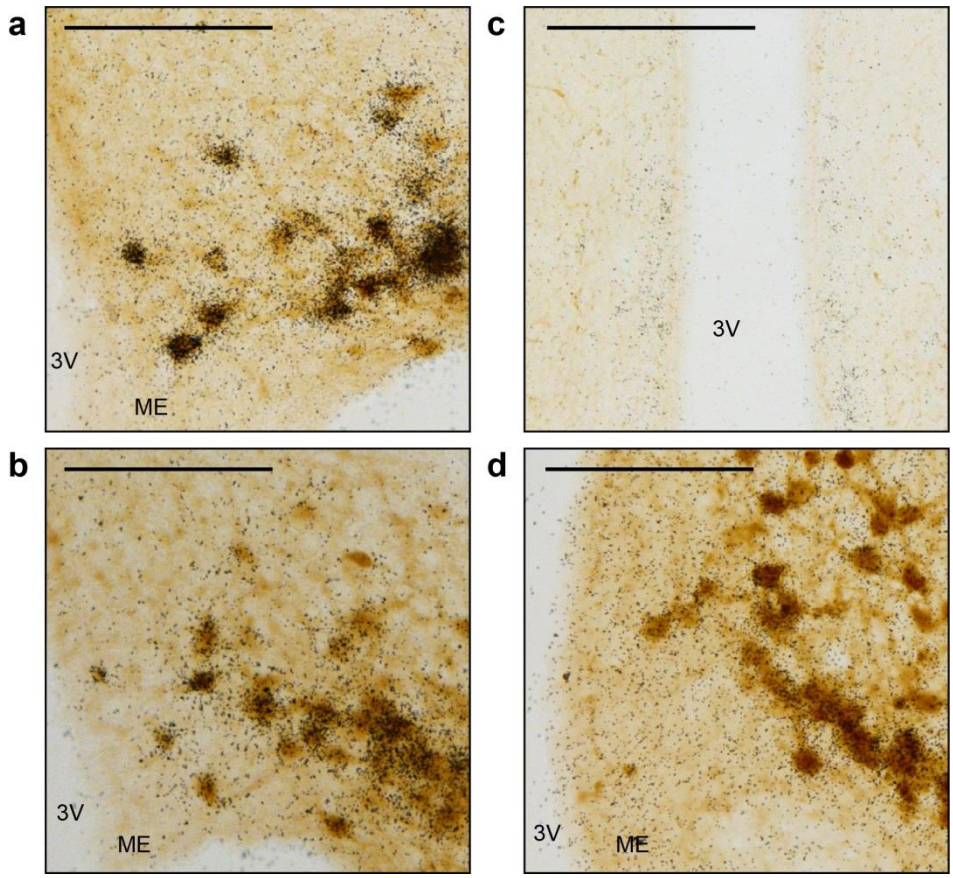


Figure 8 – Dual-label *in situ* hybridization/immunohistochemistry in Tac2^{eYFP} and Kiss1^{eYFP} female mice

(**a**) Most eYFP-ir (brown) neurons in the ARC of a female Tac2^{eYFP} mouse express *Tac2* mRNA (black dots) and (**b**) *Kiss1* mRNA (black dots). (**c**) In the AVPV/PeN of a female Tac2^{eYFP} mouse, there are no eYFP-ir neurons (brown) co-localizing with the *Kiss1* mRNA (black dots) along the third ventricle. (**d**) In the ARC of a Kiss1^{eYFP} mouse, most eYFP-ir (brown) neurons express *Kiss1* mRNA. Scale bars in (**a,b,d**) are 100 µm. Scale bar in (**c**) is 200 µm. 3V, third ventricle; ME, median eminence.

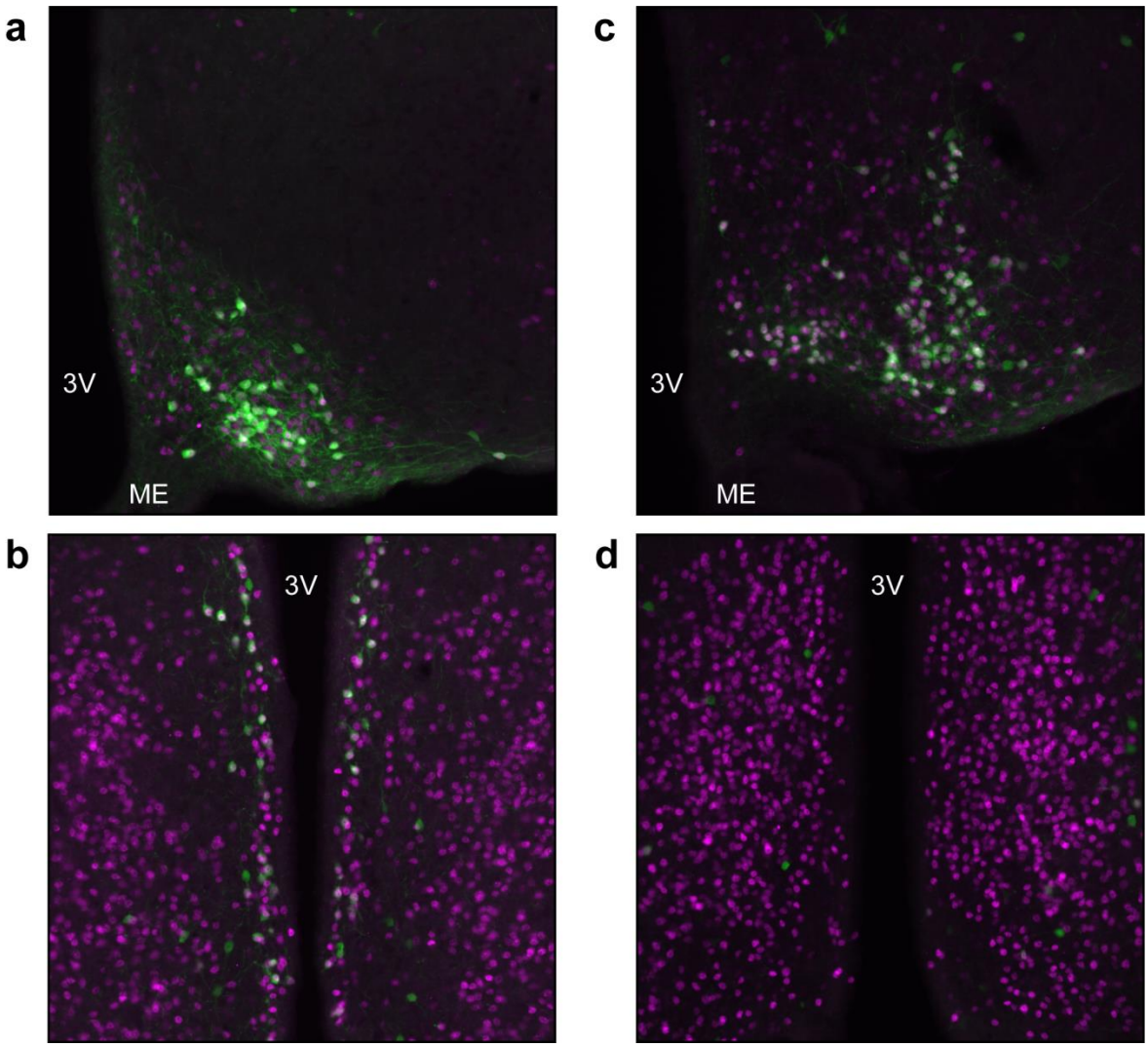


Figure 9 – Co-expression of ERα and eYFP in Tac2^{eYFP} and Kiss1^{eYFP} female mice

eYFP and ERα immunoreactivity (pseudocolored green and purple respectively) in the (**a**) ARC and the (**b**) PeN of a Kiss1^{eYFP} mouse. eYFP and ERα immunoreactivity in the (**c**) ARC and (**d**) PeN of a Tac2^{eYFP} mouse. Neurons expressing both eYFP and ERα appear white. ARC, Arcuate nucleus; PeN, periventricular nucleus; 3V, third ventricle; ME, median eminence.

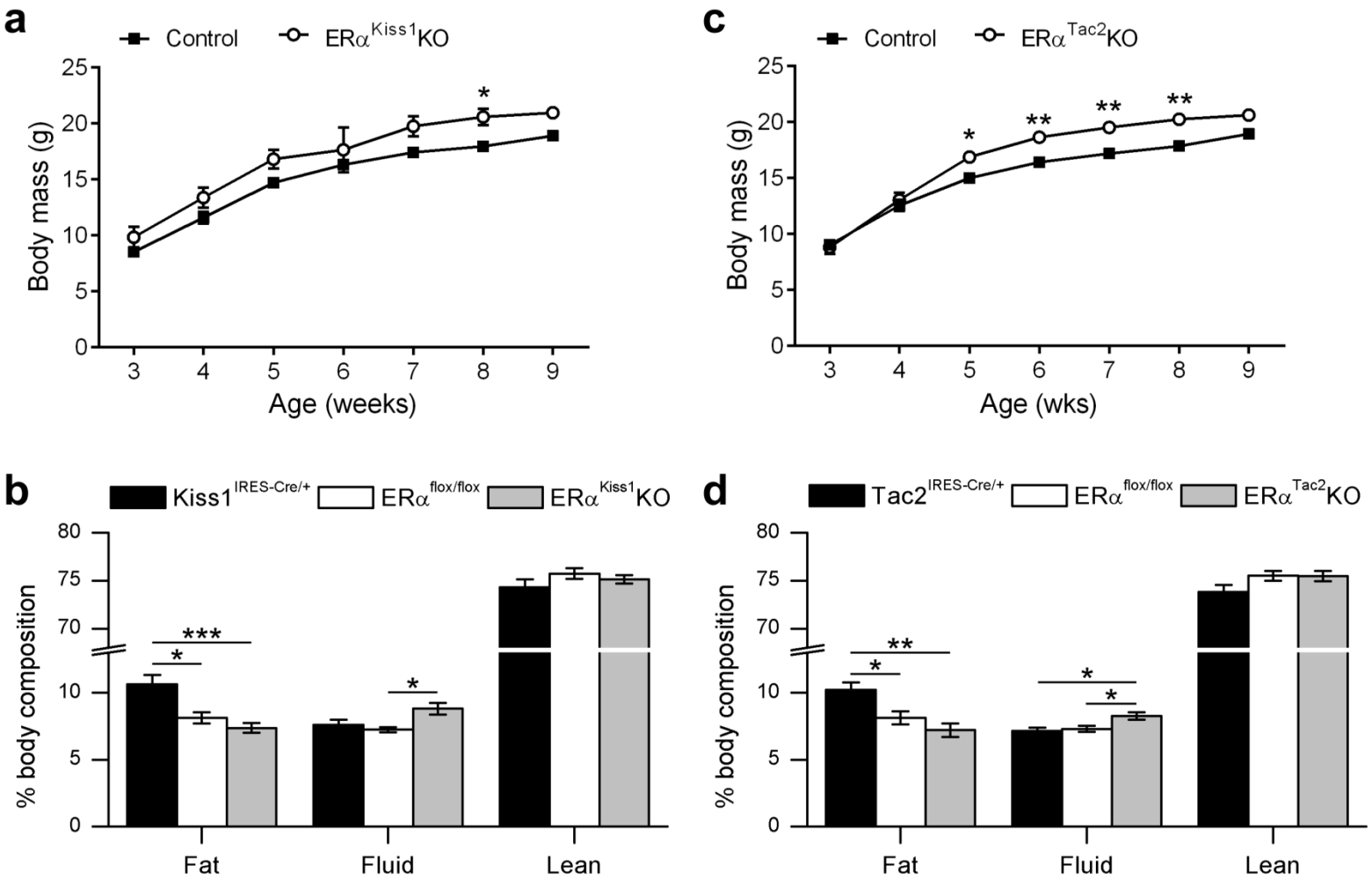
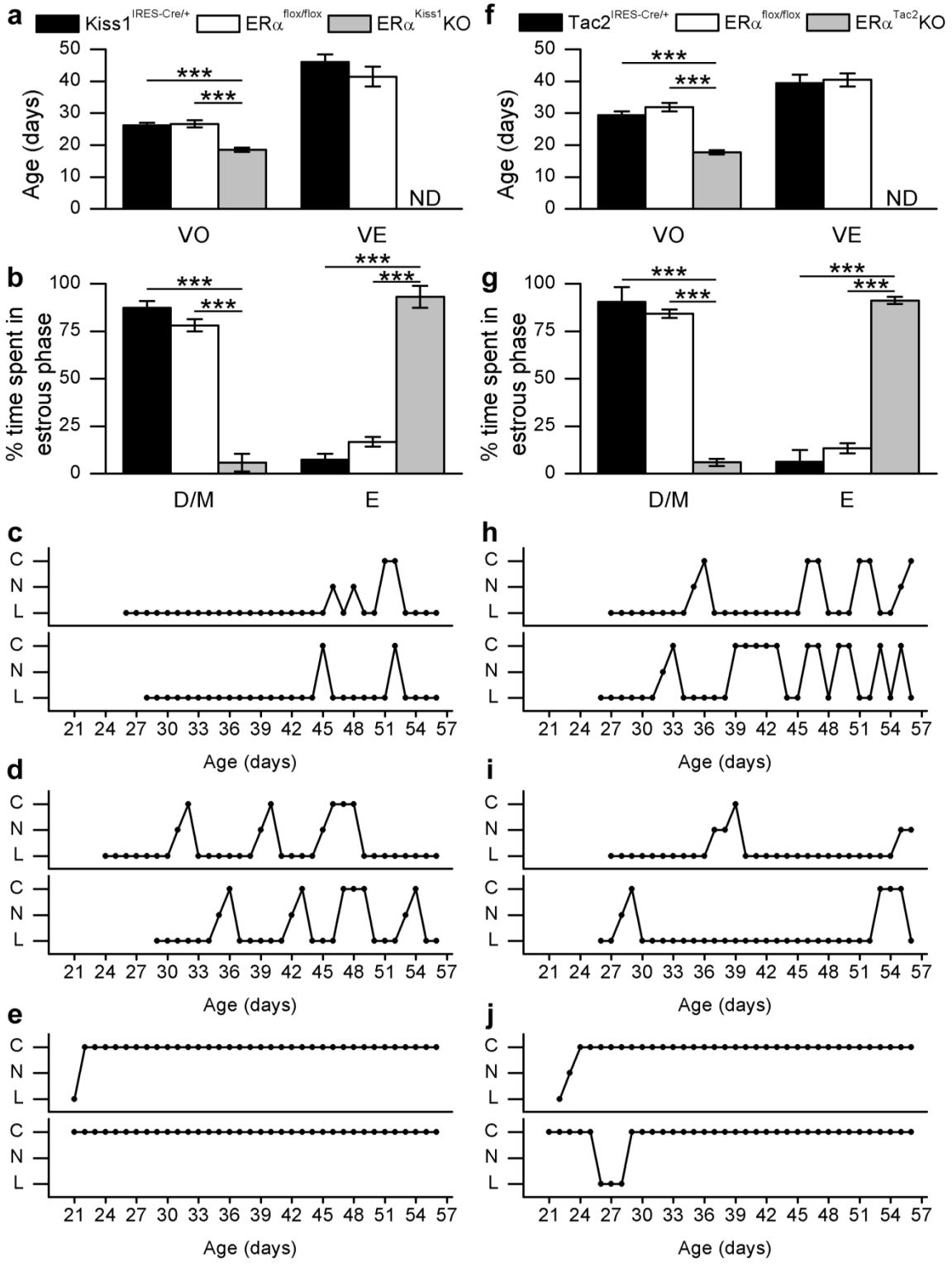

a
Control
ERα Kiss1 KO
Body mass (g)
Age (weeks)
c
Control
ERα Tac2 KO
Body mass (g)
Age (wks)
b
Kiss1 IRES-Cre/+
ERα flox/flox
ERα Kiss1 KO
% body composition
Fat
Fluid
Lean
d
Tac2 IRES-Cre/+
ERα flox/flox
ERα Tac2 KO
% body composition
Fat
Fluid
Lean

Figure 10 - Body mass and composition of female mice lacking ERα in either *Kiss1*- or *Tac2*-expressing neurons

(**a**) Body mass of littermate controls (Kiss1$^{IRES\text{-}cre/+}$ and ERα$^{flox/flox}$; "Control"; filled squares) and *Kiss1*-specific ERα knockout ("ERα^{Kiss1}KO"; open circles) mice from 3-9 weeks of age (n = 6-16). (**b**) Body composition of 8-week-old control and ERα^{Kiss1}KO mice (n = 10 each group). (**c**) Body mass of littermate controls (Tac2$^{IRES\text{-}Cre/+}$ and ERα$^{flox/flox}$; "Control"; filled squares) and *Tac2*-specific ERα knockout ("ERα^{Tac2}KO"; open circles) mice from 3-9 weeks of age (n = 10-18). (**d**) Body composition of 8-week-old control and ERα^{Tac2}KO mice (n = 8-9). (**a** & **c**) Repeated measures ANOVA, Bonferroni's multiple comparisons test: *, p<.05; **, p<.01. (**b** & **d**) One-way ANOVA, Bonferroni post-hoc: *, p<.05; **, p<.01; ***, p<.001.


a
Kiss1 IRES-Cre/+
ERα flox/flox
ERα Kiss1 KO
Age (days)
50
40
30
20
10
0
***
***
ND
VO
VE
f
Tac2 IRES-Cre/+
ERα flox/flox
ERα Tac2 KO
Age (days)
50
40
30
20
10
0
***
***
ND
VO
VE
b
% time spent in estrous phase
100
75
50
25
0
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***
***
D/M
E
g
% time spent in estrous phase
100
75
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D/M
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21 24 27 30 33 36 39 42 45 48 51 54 57
Age (days)
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i
C
N
L
21 24 27 30 33 36 39 42 45 48 51 54 57
Age (days)
e
j
C
N
L
21 24 27 30 33 36 39 42 45 48 51 54 57
Age (days)

Figure 11- Precocious but incomplete reproductive maturation in the absence of ERα from either *Kiss1*- or *Tac2*-expressing neurons

(**a**) Average age at vaginal opening and first vaginal estrus of littermate controls and ERα^{Kiss1}KO females (n = 9-10). (**b**) Percentage of time spent in diestrus/metestrus and estrus from the time of vaginal opening until 8 weeks of age (n = 5-8). Representative vaginal cytology of (**c**) Kiss1$^{IRES-Cre/+}$, (**d**) ERα$^{flox/flox}$ and (**e**) ERα^{Kiss1}KO females. (**f**) Average age at vaginal opening and first vaginal estrus of littermate controls and ERα^{Tac2}KO females (n = 7-11). (**g**) Percentage of time spent in diestrus/metestrus and estrus (based on vaginal cytology) from the time of vaginal opening until 8 weeks of age (n = 3-6). Representative vaginal cytology of 2 individual (**h**) Tac2$^{IRES-Cre/+}$, (**i**) ERα$^{flox/flox}$ and (**j**) ERα^{Tac2}KO females, beginning at the time of vaginal opening. One-way ANOVA, Bonferroni post-hoc: *, p<.05; **, p<.01; ***, p<.001. VO, vaginal opening; VE, first vaginal estrus; D/M, diestrus/metestrus; E, estrus; C, cornified epithelials; N, nucleated epithelials; L, leukocytes; ND, not determined.

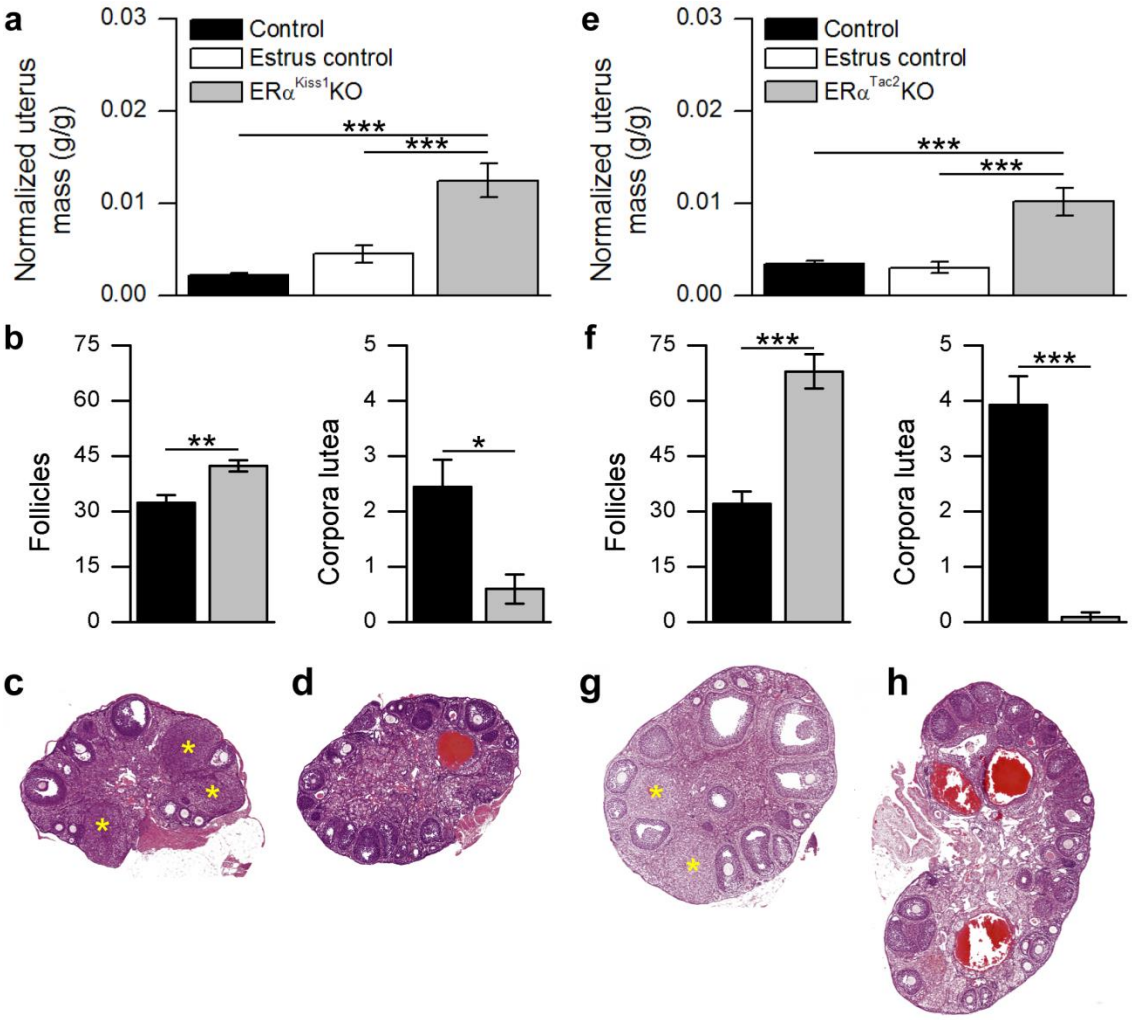

Figure 12 – Ovarian and uterine phenotype of adult female mice lacking ERα in *Kiss1*- or *Tac2*-expressing neurons

(**a**) Uterine mass [normalized to body mass (g/g)] of 9-week-old control (Kiss1$^{IRES\text{-}Cre/+}$ and ERα$^{flox/flox}$ females dissected in diestrus/metestrus), estrus control (littermate controls dissected in estrus) and ERα^{Kiss1}KO females (n = 7-18). (**b**) Number of follicles and corpora lutea per two representative sections from each mouse (n = 7-18). Representative H&E-stained ovary sections from (**c**) a littermate control and (**d**) an ERα^{Kiss1}KO. Yellow asterisks designate corpora lutea. (**e**) Uterine mass of control, estrus control and ERα^{Tac2}KO females (n = 8-16). (**f**) Quantification of the number of follicles and number of corpora lutea per two representative ovary sections from each mouse (n = 11-14). Representative H&E-stained ovary sections from (**g**) a littermate control and (**h**) an ERα^{Tac2}KO. One-way ANOVA, Bonferroni post-hoc: ***, p<.001. Student's *t* test: *, p<.05; **, p<.01; ***, p<.001.

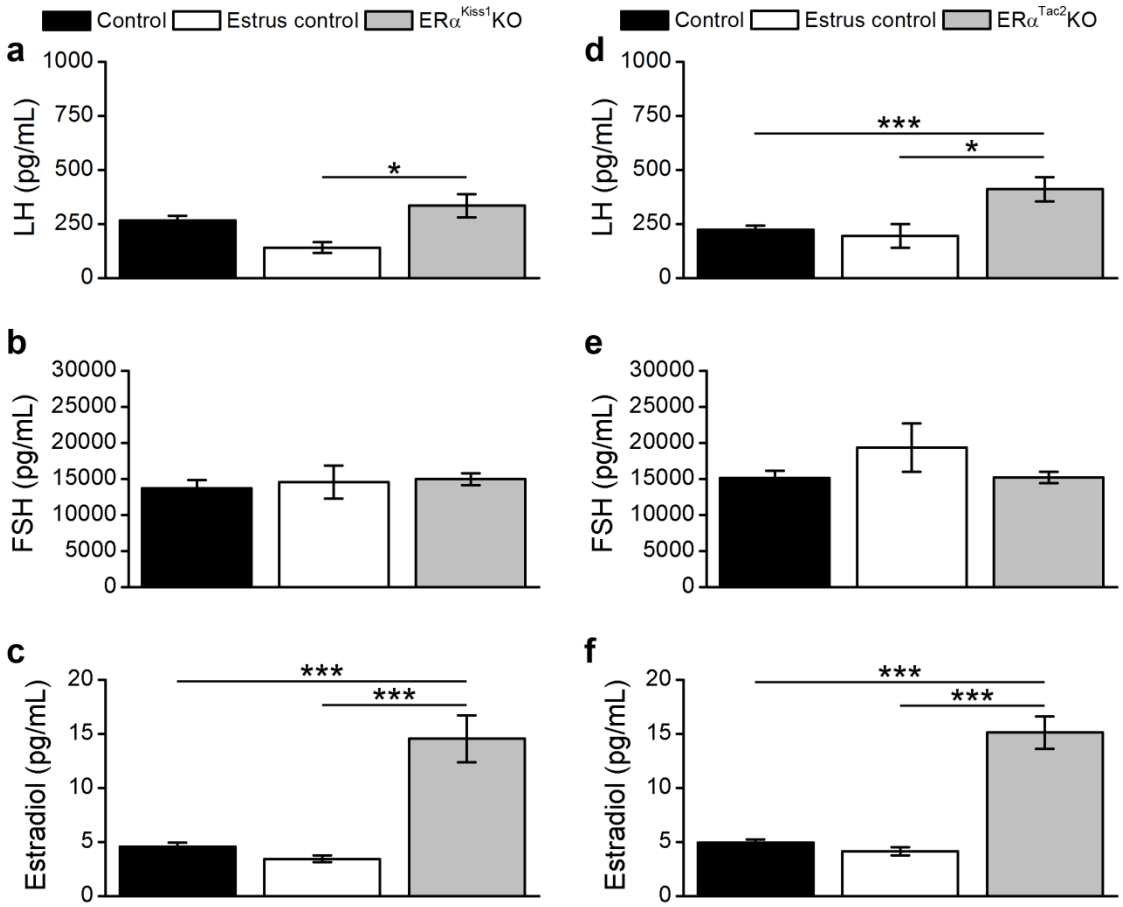

Figure 13 - Gonadotropin and gonadal hormone levels of intact adult females

Serum (**a**) LH (n = 7-29), (**b**) FSH (n = 7-30) and (**c**) estradiol (7-18) levels of 9-week-old control [littermate controls (Kiss1$^{IRES-Cre/+}$ and ERα$^{flox/flox}$) in diestrus at the time of blood collection], estrus control (littermate controls in estrus) and ERα^{Kiss1}KO females. (**d**) LH (n = 6-36), (**e**) FSH (n = 6-35) and (**f**) estradiol (n = 5-18) levels of 9-week-old control and ERα^{Tac2}KO females. One-way ANOVA, Bonferroni post-hoc: *, p<.05; **, p<.01; ***, p<.001. LH, luteinizing hormone; FSH, follicle-stimulating hormone.

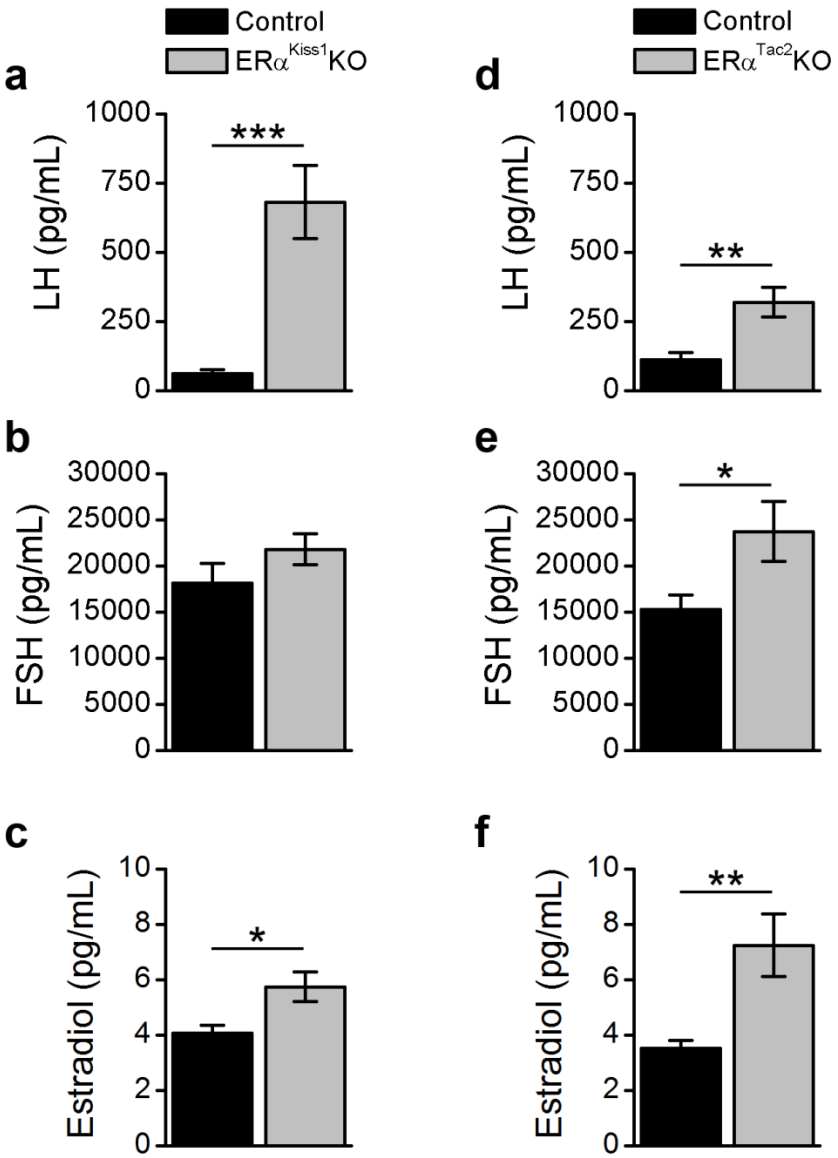

Figure 14 - Gonadotropin and gonadal hormone levels of intact juvenile females

Serum (**a**) LH (n = 9-10), (**b**) FSH (n = 9-10) and (**c**) estradiol (n = 9-10) levels of 3-week-old control (Kiss1$^{IRES\text{-}Cre/+}$ and ERα$^{flox/flox}$) and ERα^{Kiss1}KO females. (**d**) LH (n = 8-10), (**e**) FSH (n = 9-10) and (**f**) estradiol (n = 8-10) levels of 3-week-old control and ERα^{Tac2}KO females. Student's *t* test: *, p<.05; **, p<.01; **, p<.01; ***, p<.001. LH, luteinizing hormone; FSH, follicle-stimulating hormone.

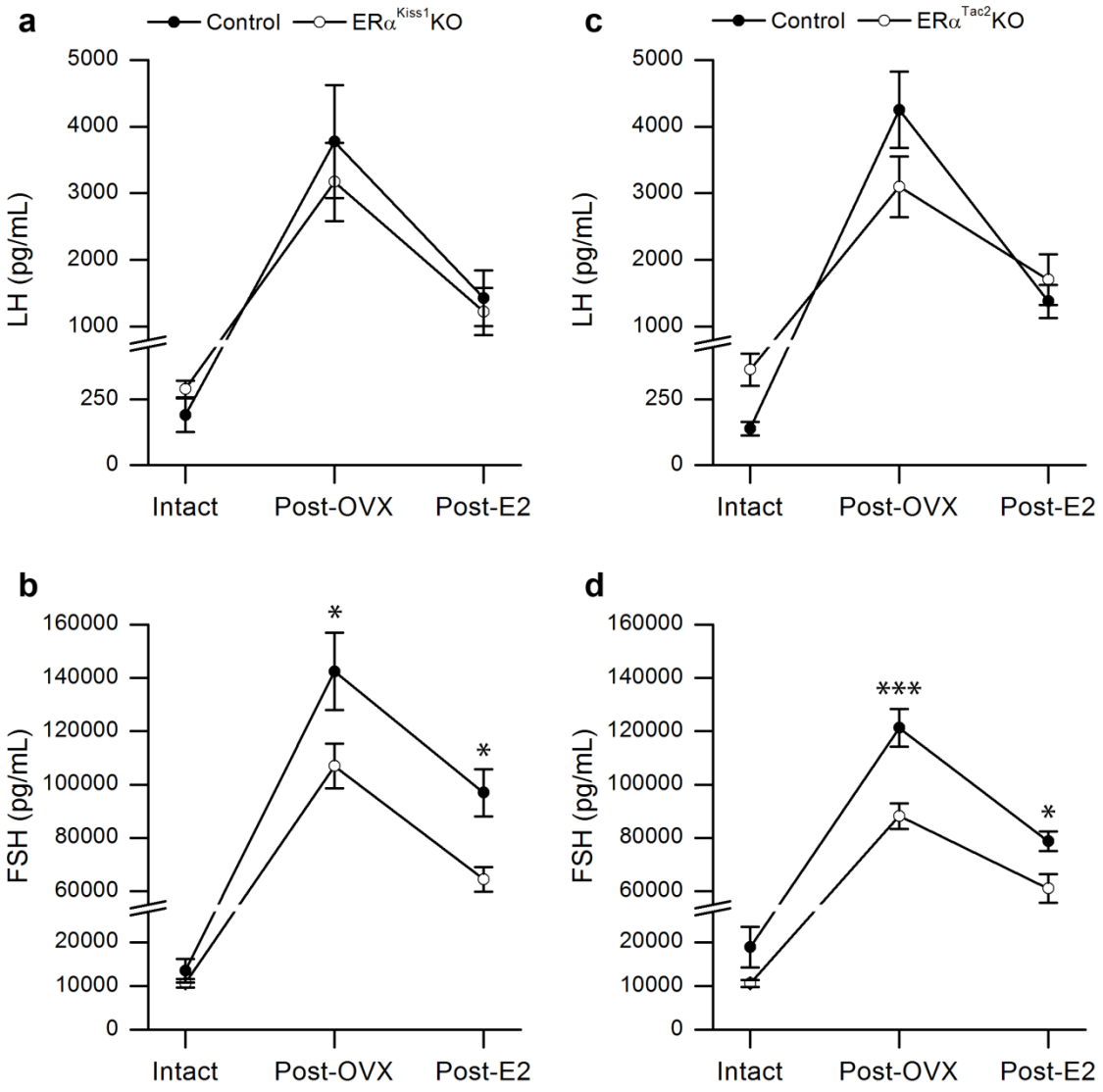

Figure 15 - Effect of ovariectomy and acute estradiol treatment on LH and FSH levels

Effect of OVX and subsequent acute estradiol treatment on serum (**a**) LH and (**b**) FSH levels in ERα^{Kiss1}KO adult females (open circles) and their littermate controls (filled circles) (n = 8-10). Effect of the same treatment on serum (**c**) LH and (**d**) FSH levels of ERα^{Tac2}KO adult females (open circles) and their littermate controls (filled circles) (n = 8-10). Repeated measures ANOVA, Bonferroni's multiple comparisons test: *, p<.05; ***, p<.001. LH, luteinizing hormone; FSH, follicle-stimulating hormone; OVX, ovariectomy.

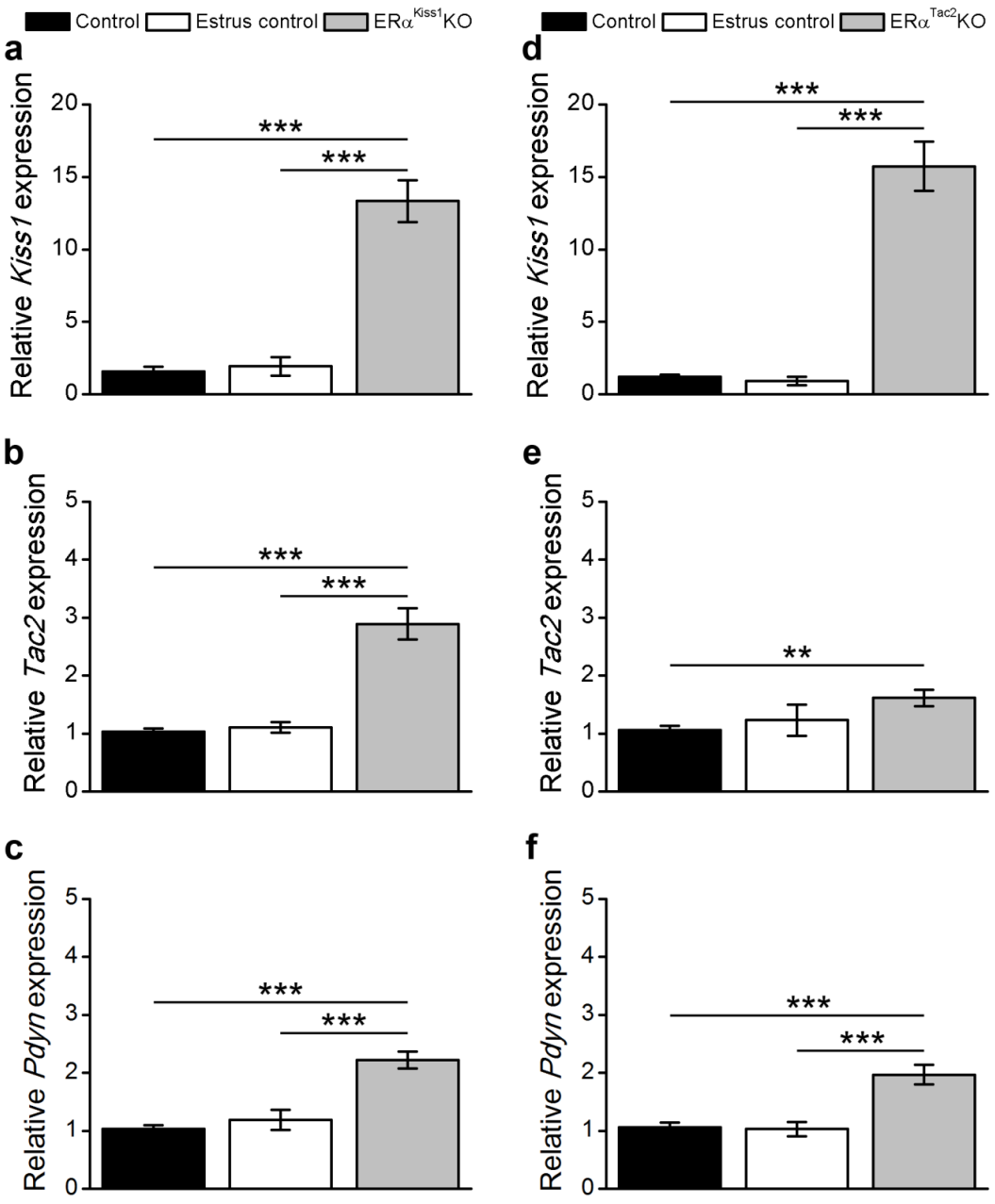

Figure 16 - Altered ARC gene expression in intact female mice lacking ERα in either *Kiss1*- or *Tac2*-expressing neurons

Relative expression of (**a**) *Kiss1*, (**b**) *Tac2* and (**c**) *Pdyn* in the ARC of adult female littermate controls [in either diestrus/metestrus ("Control") or estrus ("Estrus control") at the time of tissue collection] and ERα^{Kiss1}KOs (n = 7-19). Relative expression of (**d**) *Kiss1*, (**e**) *Tac2*, and (**f**) *Pdyn* in the ARC of adult ERα^{Tac2}KO females and littermate controls (n = 6-19). Expression levels in estrus controls and KO animals are normalized to control (diestrus/metestrus) levels. One-way ANOVA, Bonferroni post-hoc: **, p<.01; ***, p<.001. ARC, arcuate nucleus.

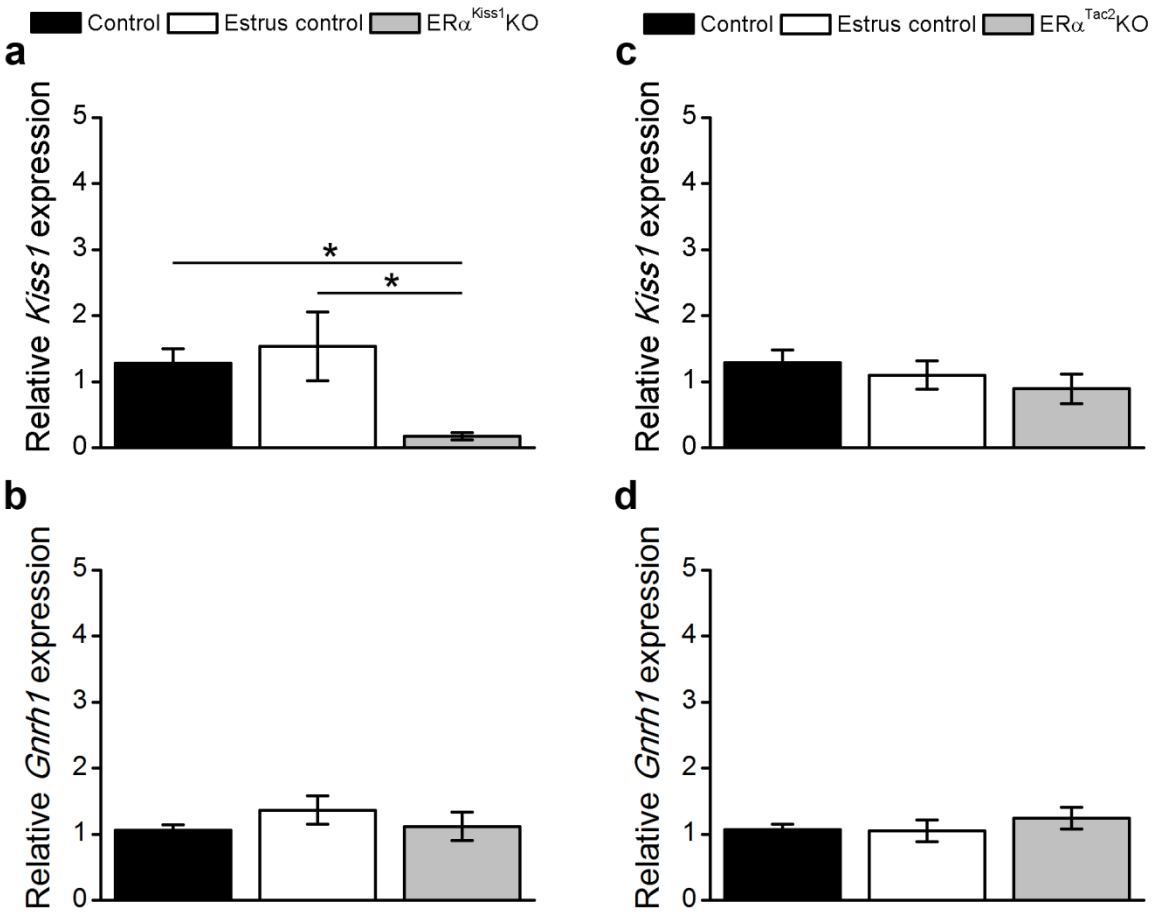

Figure 17 – *Kiss1* expression in the AVPV/PeN of intact females is diminished only in ERα^{Kiss1}KOs

Relative expression of (**a**) *Kiss1* and (**b**) *Gnrh1* in the rostral hypothalamus of control [littermate controls in either diestrus/metestrus ("Control") or in estrus ("Estrus control") at the time of tissue collection] and ERα^{Kiss1}KO adult females (n = 8-17). (**c**) *Kiss1* and (**d**) *Gnrh1* expression in the rostral hypothalamus of control and ERα^{Tac2}KO adult females (n = 8-19). Expression levels in estrus controls and KO animals are normalized to control (diestrus/metestrus) levels. One-way ANOVA, Bonferroni post-hoc: *, p<.05. AVPV/PeN, anteroventral periventricular nucleus/periventricular nucleus.

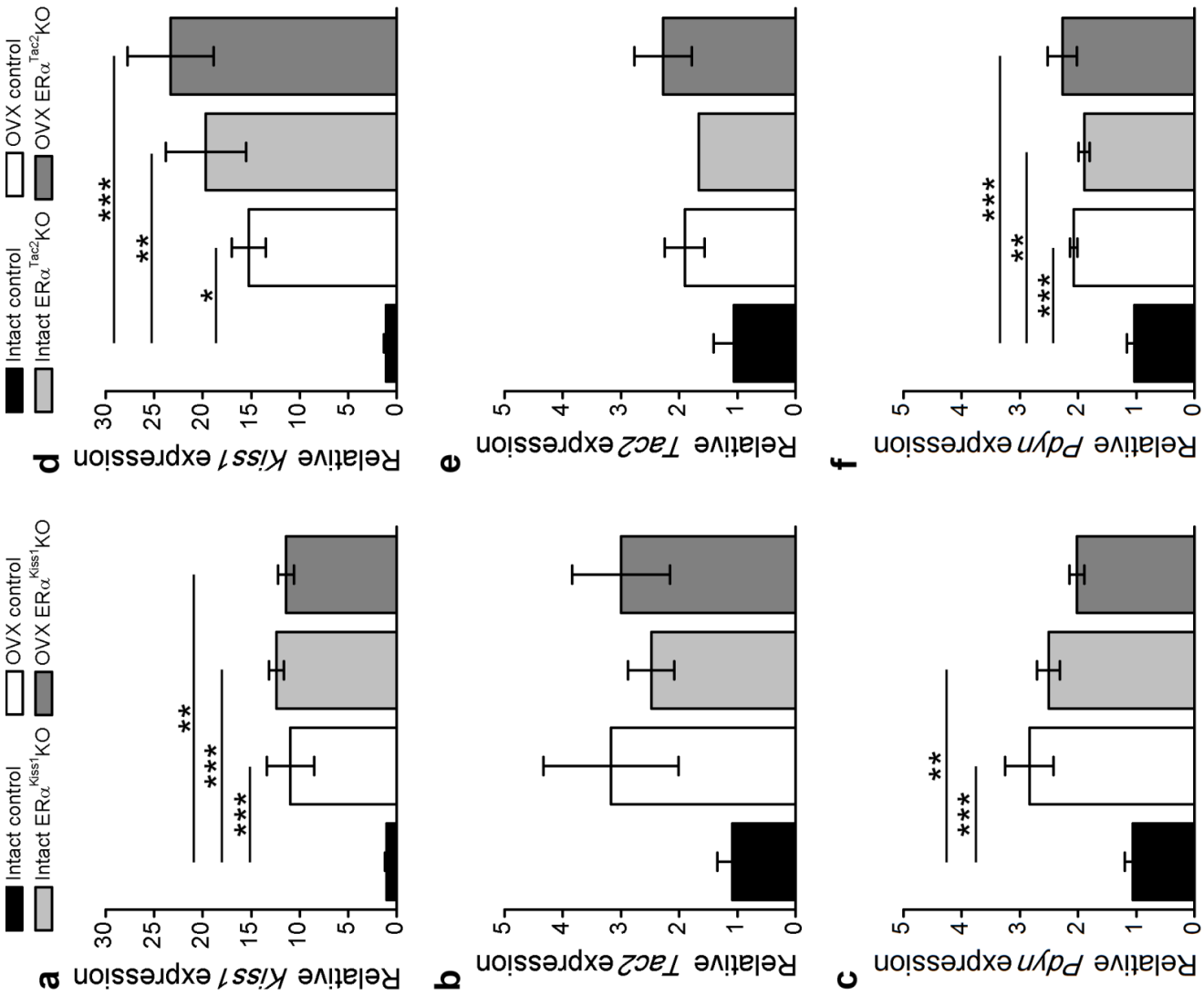

Figure 18 – Ovariectomy results in gene expression changes in the ARC that mimic loss of ERα from either *Kiss1*- or *Tac2*-expressing neurons

Relative expression of (**a**) *Kiss1* (n = 6-8), (**b**) *Tac2* (n = 4 per group), and (**c**) *Pdyn* (n = 7-8) in the ARC of adult female ERα^{Kiss1}KO and littermate controls (Kiss1$^{IRES-Cre/+}$ and ERα$^{flox/flox}$), either intact or ovariectomized. Relative expression of (**d**) *Kiss1* (n = 7-8), (**e**) *Tac2* (n = 1-4), and (**f**) *Pdyn* (n = 6-8) in the ARC of adult female ERα^{Tac2}KO and littermate controls, either intact or ovariectomized. One-way ANOVA, Bonferroni post-hoc: *, p<.05; **, p<.01; ***, p<.001. OVX, ovariectomized; ARC, Arcuate nucleus.

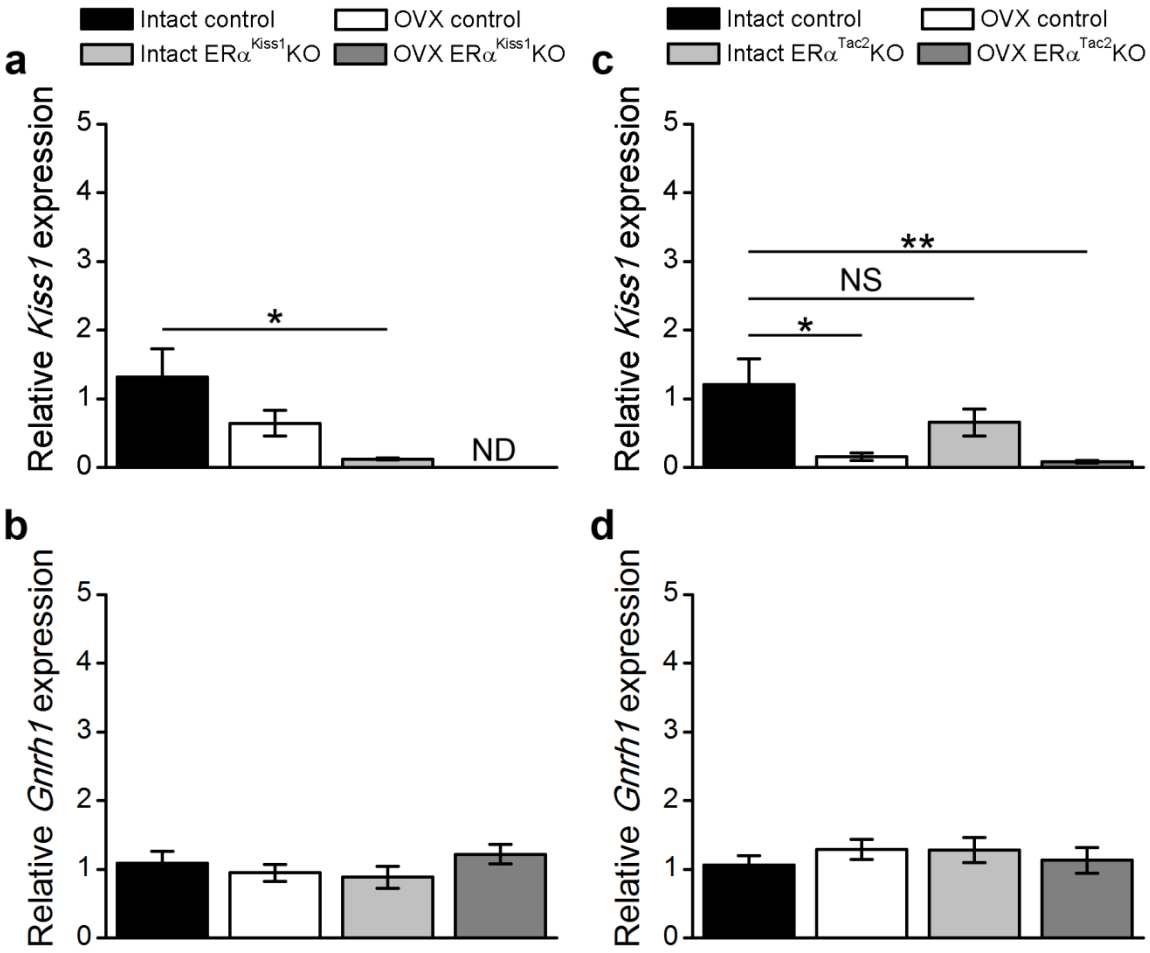

Figure 19 – AVPV/PeN *Kiss1* expression is diminished as a result of ovariectomy or ablation of ERα from all *Kiss1*-expressing neurons

Relative expression of (**a**) *Kiss1* (n = 5-7) and (**b**) *Gnrh1* (n = 8 per group) in the rostral hypothalamus of adult female ERα^{Kiss1}KO and littermate controls (Kiss1$^{IRES-Cre/+}$ and ERα$^{flox/flox}$), either intact or ovariectomized. Relative expression of (**c**) *Kiss1* (n = 5-7) and (**d**) *Gnrh1* (n = 7-8) in the rostral hypothalamus of adult female ERα^{Tac2}KO and littermate controls, either intact or ovariectomized. One-way ANOVA, Bonferroni post-hoc: *, p<.05; **, p<.01. AVPV/PeN, anteroventral periventricular/periventricular nucleus; OVX, ovariectomized; ND, not determined; NS, not significant.

Control
ERα^{Kiss1}KO
Control
ERα^{Tac2}KO
Juvenile
Adult
a
b
c
d
e
f
g
h

Figure 20 - ARC kisspeptin peptide levels are unchanged by ablation of ERα from either *Kiss1*- or *Tac2*-expressing neurons

Kisspeptin immunoreactivity in the ARC of a juvenile (21-day-old) (**a**) littermate control and a (**b**) ERα^{Kiss1}KO female. Kisspeptin immunoreactivity in the ARC of a juvenile (**c**) control and a (**d**) ERα^{Tac2}KO female. Kisspeptin immunoreactivity in the ARC of an adult (≈ 9 weeks old) (**e**) control and a (**f**) ERα^{Kiss1}KO female. Kisspeptin immunoreactivity in the ARC of an adult (**g**) control and a (**h**) ERα^{Tac2}KO female. ARC, Arcuate nucleus.

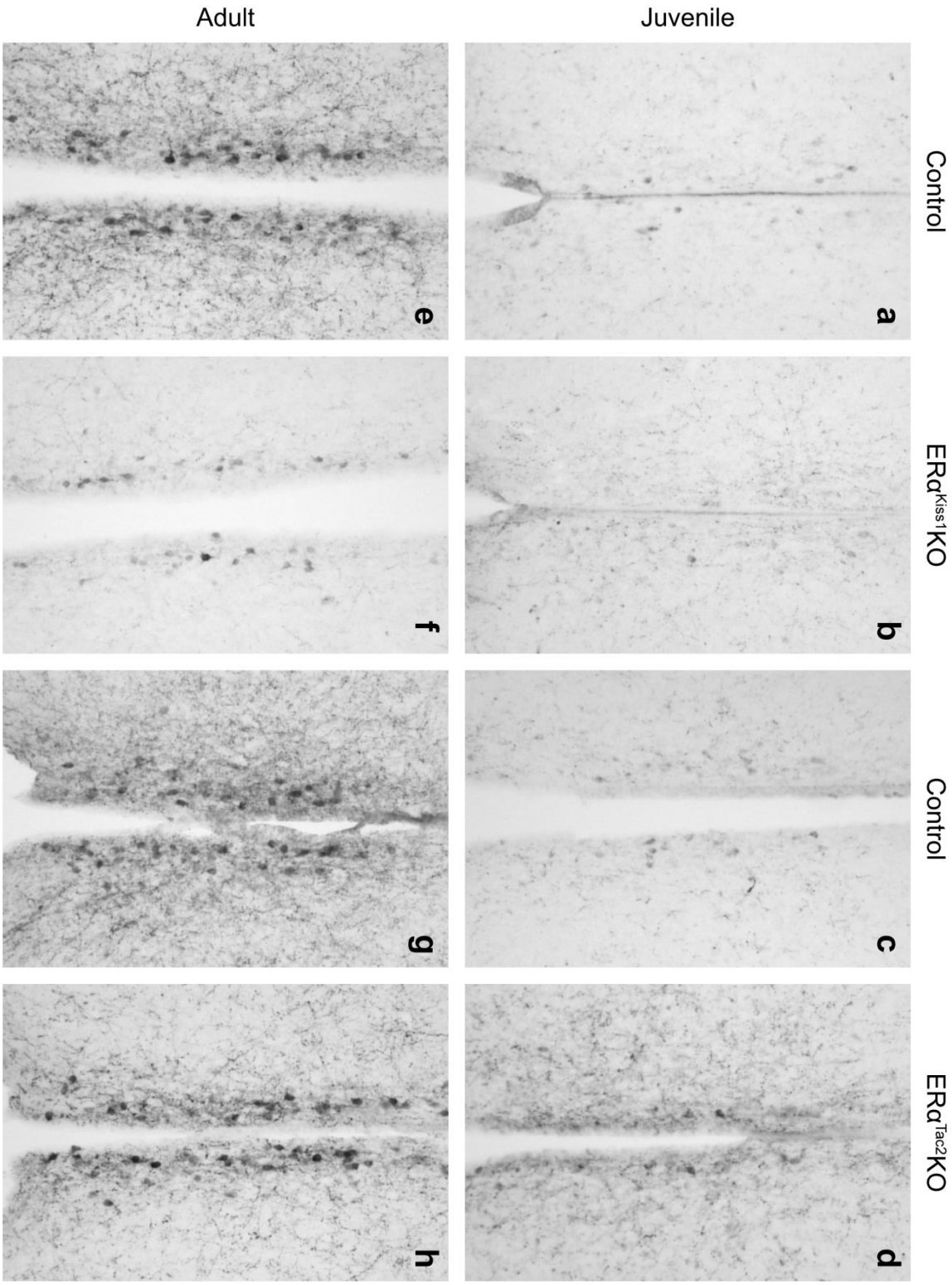
Juvenile
Adult
Control
ERα^Kiss1KO
Control
ERα^Tac2KO
a
b
c
d
e
f
g
h

Figure 21 – AVPV/PeN kisspeptin peptide levels are reduced when ERα is ablated from *Kiss1*-expressing neurons

Kisspeptin immunoreactivity in the AVPV of a juvenile (21-day-old) (**a**) control and a (**b**) ERα^{Kiss1}KO female. Kisspeptin immunoreactivity in the AVPV of a juvenile (**c**) control and a (**d**) ERα^{Tac2}KO female. Kisspeptin immunoreactivity in the AVPV of an adult (≈ 9 weeks old) (**e**) control and a (**f**) ERα^{Kiss1}KO female. Kisspeptin immunoreactivity in the AVPV of an adult (**g**) control and a (**h**) ERα^{Tac2}KO female. AVPV/PeN, anteroventral periventricular/periventricular nucleus.

Chapter 4 – Direct estrogen action on *Kiss1*/ERα neurons is required for negative feedback on the reproductive axis of male mice‡

In males, the testes exert negative feedback effects on the hypothalamo-pituitary system, modulating the release of gonadotropin-releasing hormone (GnRH) from the hypothalamus and the subsequent release of the gonadotropins luteinizing hormone (LH) and follicle-stimulating hormone (FSH) from the anterior pituitary (**Figure 1**). This effect is demonstrated by experiments in which removal of the testes (and thus circulating testosterone) results in substantial increases in LH and FSH levels; the increase in gonadotropin levels can be attenuated by administration of testosterone [152–154]. As testosterone can act either directly through the androgen receptor (AR) or be aromatized to estradiol and then act through either isoform of the estrogen receptor (ERα or ERβ), many studies have sought to determine which effects of testosterone require action via AR and which require one of the estrogen receptor isoforms.

The suppression of LH levels by testosterone treatment in a castrated

‡ In these studies, Dr. Courtney Marsh performed the quantification of the colocalization of ERα and eYFP in both mouse lines as well as assisting with the dissections at the end of the longitudinal study. Luhong Wang performed the gene expression analysis.

male is actually an estradiol-dependent phenomenon since it doesn't occur if dihydrotestosterone (DHT), a non-aromatizable androgen, is administered instead of testosterone [152]. The elevated LH levels in a castrated male can be suppressed using estradiol treatment in wild-type, but not in mice lacking ERα globally (αERKO mice) [48,152], implicating ERα in gonadal hormone negative feedback in the male. As fertility is not affected in male mice lacking ERβ globally, ERα appears to be the estrogen receptor isoform that plays a critical role in the male reproductive axis [13]. It's generally accepted that GnRH neurons themselves don't possess functional ERα, so the identity of the ERα-expressing neurons in the hypothalamus that relay sex steroid signals to the GnRH neurons has not yet been elucidated.

It has been proposed that neurons in the arcuate nucleus (ARC) that synthesize the neuropeptide kisspeptin may be the ERα-expressing neurons responsible for negative feedback regulation of GnRH neurons in male mice [45]. This hypothesis is based solely on the regulation of ARC *Kiss1* mRNA levels by testosterone; while castration leads to increased *Kiss1* expression, treatment with testosterone after the castration significantly reduces *Kiss1* levels [45]. While DHT treatment also reduces ARC *Kiss1* levels, the small magnitude of this reduction suggests that the majority of the negative regulation of ARC *Kiss1* expression requires an estrogen receptor. As is the case in females, the majority of ARC kisspeptin neurons in the male produce two other neuropeptides: neurokinin B (product of the gene *Tac2*) and dynorphin A (product of the gene *Pdyn*) [48]. As a result, these neurons have been termed KNDy neurons.

There is a second population of hypothalamic kisspeptin neurons in the anteroventral periventricular/periventricular nucleus (AVPV/PeN) which does not express either *Tac2* of *Pdyn*. Changing testosterone levels have the opposite effect on *Kiss1* mRNA levels in the AVPV/PeN; castration reduces *Kiss1* levels while testosterone or estrogen- but not DHT- treatment normalizes them [45]. The physiological relevance of this differential regulation of *Kiss1* expression in males is unknown at this time, although some have suggested that the ARC kisspeptin neurons may be involved in testosterone-mediated negative feedback control of gonadotropin secretion, whereas the AVPV/PeN kisspeptin neurons are simply a remnant of the estrogen positive feedback system that is present only in females for the purpose of generating the preovulatory LH surge [45].

In order to test whether ERα in ARC kisspeptin neurons is required for proper regulation of gonadotropin secretion and overall function of the male reproductive axis, we performed a conditional genetic deletion of ERα from all *Kiss1*-expressing neurons and compared the resulting phenotype to that of mice lacking ERα only in ARC KNDy neurons. Our results do not support the hypothesis that ERα in ARC KNDy neurons functions as part of the gonadal negative feedback system. Instead, it appears that a population of non-*Tac2*-expressing kisspeptin/ERα neurons is responsible for estrogen negative feedback in the male.

Methods

Animals

All animals were bred in our colony in the Unit for Laboratory Animal Medicine at the University of Michigan. All animals and procedures used were in accordance with the guidelines and approval of the University Committee on the Care and Use of Animals.

Generation of Kiss1$^{IRES\text{-}cre}$ and Tac2$^{IRES\text{-}cre}$ mice

The generation of mouse lines containing IRES-cre coding sequence knocked in to the 3' untranslated regions of the endogenous mouse *Kiss1* and *Tac2* alleles has been described previously (see **Chapter 3**).

Mouse model validation

Kiss1$^{IRES\text{-}cre/+}$ and Tac2$^{IRES\text{-}cre/+}$ male mice were bred to homozygous cre-inducible eYFP reporter females (Jax 006148) to generate Kiss1^{eYFP} and Tac2^{eYFP} animals respectively, allowing visualization and quantification of cre-expressing cells as eYFP expression is restricted to cells that express cre recombinase.

Perfusion and immunohistochemistry

Adult and juvenile (3-week-old) mice received an overdose of sodium pento-barbital and were then perfused transcardially with phosphate-buffered saline (PBS, pH 7.4) followed by 10% formalin. Testes and seminal vesicles

(from adult animals only) were isolated and weighed. The brain was removed and post-fixed in 10% formalin for 2-4 hours and then dehydrated in 30% sucrose in PBS until the time of sectioning. Brains were cut in 30 µm coronal sections on a sliding microtome, collected in four representative series, and either stored at -20° C in cryoprotectant.

For eYFP, kisspeptin and ERα immunostaining, sections were washed, blocked in normal donkey serum and then incubated in primary antibodies (chicken anti-GFP, Abcam, 1:1000; rabbit anti-ERα, Santa Cruz, 1:1000; rabbit anti-kisspeptin, Millipore, 1:2000) overnight. Brain sections were then thoroughly washed and incubated with Alexa Fluor-conjugated secondary antibodies (goat anti-chicken 488 and donkey anti-rabbit 568, Invitrogen, 1:250) for two hours. Sections were mounted onto gelatin-coated slides and coverslipped with ProLong Antifade mounting medium (Invitrogen).

Longitudinal study

Cre-positive males (either $Kiss1^{IRES\text{-}cre/+}$ or $Tac2^{IRES\text{-}cre/+}$) were bred to $ERα^{flox/flox}$ females. The resulting $Kiss1^{IRES\text{-}cre/+}$/ $ERα^{flox/+}$ or $Tac2^{IRES\text{-}cre/+}$/ $ERα^{flox/+}$ males and $ERα^{flox/+}$ females were bred to each other to generate *Kiss1*- or *Tac2*-specific ERα knockouts ($Kiss1^{IRES\text{-}cre/+}$/ $ERα^{flox/flox}$, "$ERα^{Kiss1}$KO" or $Tac2^{IRES\text{-}cre/+}$/ $ERα^{flox/flox}$, "$ERα^{Tac2}$KO") and their littermate controls $ERα^{flox/flox}$ and $Kiss1^{IRES\text{-}cre/+}$ or $Tac2^{IRES\text{-}cre/+}$. Genotyping was performed as previously described for the females (**Chapter 3**).

Male mice were individually housed beginning at PND21 and body weight

was recorded weekly. Body composition at 8 weeks of age was determined by a NMR-based Minispec LF90II (Bruker Optics) analyzer. At 9 weeks of age, mice were dissected between 13:00 and 16:00. Blood was collected after an overdose of sodium pentobarbital was given and allowed to clot for 30 minutes at room temperature. It was then centrifuged for 15 minutes at 2,000 x g and the serum was collected and stored at -20° C. Using a mouse brain matrix, the ARC and a block of tissue containing the AVPV/PeN as well as the preoptic area were dissected and individually snap-frozen on dry ice. Brain tissue was stored at -80° for later RNA extraction.

RNA was extracted from microdissected brain tissue using TRIzol (Invitrogen) and then converted to cDNA with the iScript cDNA Synthesis Kit (Bio-Rad). cDNA was analyzed in triplicate by quantitative realtime-PCR using *Gapdh* (endogenous control)*, Kiss1, pDyn, Tac2* and *GnRH* Taqman assays (Invitrogen). Relative expression levels were calculated using the $2^{-\Delta\Delta Ct}$ method, with normalization of each sample's ΔCt value to the average ΔCt of the control samples.

Microscopy and image analysis

Microscopic images were obtained using an Olympus BX-51 microscope with a DP30BW camera (Olympus). Dual-channel images were pseudo-colored and merged using Adobe Photoshop.

Hormone analysis

Serum was analyzed by the Ligand Assay and Analysis Core of the University of Virginia Center for Research in Reproduction or the University of Michigan Chemistry Core. Testosterone levels were determined by RIA (catalog # TKTT2, Siemens) while LH and FSH levels were determined using the Millipore MILLIPLEX MAP rat pituitary panel.

Statistics

Student's *t*-test was used when only two groups were compared. One-way ANOVA with Bonferroni post-hoc analysis was used when comparing three or more groups. Repeated measures two-way ANOVA was used to analyze longitudinal body weight. Data was analyzed and graphs were generated using either OriginPro 8 or GraphPad Prism software. Differences were deemed significant if $p < .05$. Data are presented as mean±SEM.

Results

Generation of mouse models to study Kiss1- and Tac2-expressing neurons in the male mouse brain

To study the physiological role of hypothalamic ERα in the male mouse, we utilized the same mouse models described previously (**Chapter 3**). Briefly, the coding sequence for an IRES-cre cassette was knocked in to the endogenous *Tac2* (**Figure 22 a**) and *Kiss1* (**Figure 22 d**) loci. This results in the production of cre recombinase specifically in cells that express either of those genes. To visualize the pattern of cre expression, $Kiss1^{IRES\text{-}cre}$ and $Tac2^{IRES\text{-}cre}$ mice were bred to commercially available cre-mediated fluorescent reporter mice (**Figure 22 b**). Excision of the transcriptional blocker located between the loxP sites allows production of the fluorescent protein eYFP in all cre-expressing cells. The two cre-expressing mouse lines were also crossed to a mouse line that contains loxP sites flanking the third exon of the *Esr1* gene (**Figure 22 c**). Cre-mediated recombination around these sites results in excision of the coding sequence for the DNA binding domain of ERα, resulting in ablation of ERα in all cre-expressing neurons.

We assessed the pattern of cre expression in both $Tac2^{eYFP}$ and $Kiss1^{eYFP}$ male mice (**Figure 23**). As expected, we saw eYFP immunoreactivity in the ARC (**Figure 23 a**) and PeN (**Figure 23 b**) in a $Kiss1^{eYFP}$ male brain. In this mouse line, 96±0.2% (N = 5) of eYFP cells in the ARC and 20±2.8% (N = 5) of eYFP cells in the AVPV/PeN co-express ERα. In agreement with the known

expression of *Tac2*, the expression of eYFP in a Tac2^{eYFP} male brain was more extensive than what we observed in Kiss1^{eYFP} mice, but the colocalization of eYFP and ERα was mostly restricted to the ARC (**Figure 23 c**), where 95±0.5% (*N* = 3) of *Tac2* cells also express ERα. We did not identify any eYFP/ERα cells in the AVPV/PeN of the Tac2^{eYFP} male brain (**Figure 23 d**).

In the adult Kiss1^{eYFP} brain, not all eYFP cells contained detectable kisspeptin peptide, although the presence of eYFP suggests that expression of *Kiss1* is transient in these cells. Although staining in the ARC of a Kiss1^{eYFP} male reveals an abundance of kisspeptin-immunoreactive fibers (data not shown), staining in the vicinity of the AVPV/PeN revealed no kisspeptin-ir cells and few fibers but several eYFP-ir cells (**Figure 24 a**). The absence of kisspeptin immunoreactivity in males is not because of technical issues because we can readily detect kisspeptin cells and fiber in the AVPV/PeN of female Kiss1^{eYFP} mice using the same experimental method (**Figure 24 b**). We also detect some eYFP cells in the female that aren't currently producing detectable levels of kisspeptin peptide. These findings reflect the fact that there are neurons in the vicinity of the AVPV/PeN that only transiently express *Kiss1*, but may express ERα and thus still play a role in the regulation of the reproductive axis.

Generation of Kiss1- and Tac2-specific ERα knockout animals

To generate *Kiss1*-specific ERα knockout mice, we first crossed Kiss1$^{IRES\text{-}cre}$ heterozygotes to *Esr1*-flox homozygotes (ERα$^{flox/flox}$ mice) and then crossed the resultant Kiss1$^{IRES\text{-}cre/+}$/ERα$^{flox/+}$ males to ERα$^{flox/+}$ females. The F2 progeny

consisted of Kiss1$^{IRES\text{-}Cre/+}$/ERα$^{flox/flox}$ ("ERα^{Kiss1}KO") mice, ERα$^{flox/flox}$ mice (without the modified *Kiss1* allele) and Kiss1$^{IRES\text{-}Cre/+}$ mice (without a modified *Esr1* allele). The latter two genotypes were used as littermate controls to the ERα^{Kiss1}KO animals in all studies. When appropriate, the two groups of control genotypes were combined into one larger group. The same breeding strategy was used to generate *Tac2*-specific ERα knockout mice, eventually generating littermate Tac2$^{IRES\text{-}Cre/+}$/ERα$^{flox/flox}$ ("ERα^{Tac2}KO") mice, ERα$^{flox/flox}$ mice (without the modified *Tac2* allele) and Tac2$^{IRES\text{-}Cre/+}$ mice (without a modified *Esr1* allele).

Body mass and body composition

Global ERα knockout male mice have slightly decreased body mass but increased white adipose tissue mass [155,156]. We assessed body mass in our male mice from the time of weaning (3 weeks) until the time of dissection and body composition was determined in adult animals prior to dissection (**Figure 25**). We found absolutely no effect of ablation of ERα from *Kiss1*-expressing cells on body mass (**Figure 25 a**) or adult body composition (**Figure 25 b**). Similarly, ablation of ERα from all *Tac2*-expressing cells had no effect on body mass (**Figure 25 c**) or adult body composition (**Figure 25 d**). Thus estrogen action through ERα in *Kiss1* or *Tac2* cells is not necessary for the proper regulation of body mass or body composition in males.

Reproductive organ weights

The effect of global deletion of ERα on reproductive organ weights in

males has yielded mixed results [155,157,158]. We found testes weight to be unchanged in adult ERα^{Kiss1}KO males compared to their littermate controls (**Figure 26 a**). The seminal vesicles (accessory sex glands that produce the fluid that becomes the copulatory plug) are enlarged as a result of ablation of ERα from *Kiss1* neurons (**Figure 26 b**). This effect on seminal vesicle weight is not seen in global ERα knockouts [152,158]. Since we had observed early puberty onset in females lacking ERα in either *Kiss1* or *Tac2* neurons (**Figure 11**), we measured testes weight of 3 week old male mice as an indicator of sexual maturation. We found that the testes of young ERα^{Kiss1}KO males weighed more than those of their littermate controls (**Figure 26 c**). While adult male ERα^{Tac2}KO mice had unaffected testes (**Figure 26 d**) and seminal vesicle (**Figure 26 e**) weights, we saw increased testes weight in juvenile ERα^{Tac2}KOs compared to their littermate controls (**Figure 26 f**). These significant changes in juvenile testes weight indicate a potential acceleration in sexual maturation as a result of selective ERα ablation.

Gonadotropin and gonadal steroid hormone levels

Alterations in gonadotropin and gonadal hormone levels have been reported in male mice with decreased estrogen action, but the findings are inconsistent [152,158]. In adult ERα^{Kiss1}KO males, we found significantly elevated LH levels, decreased FSH levels and elevated testosterone levels (**Figure 27 a-c**). In contrast, we found no significant changes in any of these hormones in ERα^{Tac2}KO adults (**Figure 27 d-f**), suggesting the existence of a population of

Kiss1/ERα neurons which are critical for estrogen negative feedback on gonadotropin secretion but are separate from ARC *Kiss1*/*Tac2* neurons.

Gene expression

Expression of certain reproduction-related genes in the ARC and in the rostral hypothalamus are sensitive to gonadal steroid levels in the male mouse [45,48,152]. Since testosterone can act not only directly through the androgen receptor but also be aromatized to estrogen and act through ERα, we assessed relative expression levels of *Kiss1*, *Tac2* and *Pdyn* in the ARC and *Kiss1* and *Gnrh1* in the rostral hypothalamus in our mice. In mice lacking ERα in all *Kiss1*-expressing neurons, we found no effect on expression of *Kiss1* or *Gnrh1* in the rostral hypothalamus (**Figure 28 a**) or on expression of *Kiss1*, *Tac2* or *Pdyn* in the ARC (**Figure 28 b**). Although we observed a significant increase in AVPV/PeN *Kiss1* expression in ERα^{Tac2}KO males compared to littermate controls, the magnitude of this increase is very small (**Figure 28 c**). Similar to what we found in the ERα^{Kiss1}KO males, we observed no effect on *Gnrh1* expression (**Figure 28 c**) in ERα^{Tac2}KO males, or any of the three genes (*Kiss1*, *Tac2*, *Pdyn*) we investigated in the ARC (**Figure 28 d**). Thus, ablation of ERα from either all *Kiss1*-expressing neurons or just from *Tac2*-expressing neurons has no virtually no detectable effect on the expression of genes in the hypothalamus that are known to be important modulators of GnRH neuron function.

Conclusions & Discussion

Studies in a multitude of species have shown that the central actions of estradiol, the aromatization product of testosterone, play a critical role in the regulation of pulsatile gonadotropin release and thus the entire male reproductive axis. Yet the identity and location of the estrogen receptor-expressing neuronal population required for this effect has not yet been uncovered. As it doesn't appear that GnRH neurons themselves are responsible for this effect of estradiol on the system, we hypothesized that neurons producing the neuropeptide kisspeptin could be the critical population. To address the putative contribution of direct estrogen action on kisspeptin neurons to the regulation of the reproductive axis, we used cre-loxP technology to genetically ablate ERα from either all *Kiss1* neurons or from only the ARC kisspeptin neurons which co-express *Tac2*.

We saw almost no effect of deletion of ERα from only ARC KNDy neurons in males. While juvenile (3-week-old) ERα^{Tac2}KO males had slightly elevated testes weight, this difference was absent in adults. Although this may indicate accelerated puberty onset, the age at balano-preputial separation was not assessed in these studies and should be addressed in the future. The adult ERα^{Tac2}KO males exhibited no significant hormonal changes and only a very slight (but significant) increase in *Kiss1* expression in the AVPV/PeN.

In contrast with the underwhelming phenotype of the ERα^{Tac2}KO males, ERα^{Kiss1}KO males do show signs of impaired negative feedback on the

reproductive system. Adult ERα^{Kiss1}KO males have elevated LH and testosterone levels, indicating reduced restraint on the anterior pituitary. This was surprising since Mayer and colleagues previously found no changes in LH in their adult knockouts [46]. This discrepancy may be due to subtle differences in cre expression between the two different Kiss1$^{IRES\text{-}cre}$ lines used or slightly different study conditions. In agreement with previous studies of αERKO males, we did not observe any changes in adult testes weight but their seminal vesicles were significantly heavier than their littermate controls. The ERα^{Kiss1}KOs' elevated testosterone levels may be the cause of this effect since seminal vesicle weight is sensitive to circulating testosterone [152].

When we investigated the effect of conditional ERα deletion on hypothalamic gene expression we found a small but significant increase in AVPV/PeN *Kiss1* expression in ERα^{Tac2}KO males. This is likely due to the slightly (but not significantly [p = .08]) elevated testosterone levels in these mice, which stimulates *Kiss1* expression in this area in an aromatase-dependent manner [45]. There were no significant alterations in any of the other genes we analyzed in the hypothalamus of ERα^{Kiss1}KO males. If the loss of estrogen action through ERα on kisspeptin neurons resulted in a complete loss of negative feedback, we would expect effects on gene expression that mimic the effect of castration. This result was unlikely in our mouse models since negative feedback also occurs through the androgen receptor which we have not manipulated here.

While others have reported that there are more *Tac2*-expressing neurons

in the male ARC than there are *Tac2*/*Kiss1* neurons [48], we didn't observe any evidence of this in our comparison of $Tac2^{eYFP}$ and $Kiss1^{eYFP}$ mice. The neurons affected by our deletion of ERα from all *Kiss1* neurons include all those affected by the *Tac2*-specific deletion with the addition of any non-*Tac2*-expressing *Kiss1*/ERα neurons. We believe that the phenotypic differences between our two conditional ERα knockouts are a result of the non-*Tac2*-expressing *Kiss1*/ERα cells in the AVPV/PeN that expressed *Kiss1* at some point during development, but no longer produce detectable levels of the peptide. More work is needed to characterize these neurons and better determine their function in the regulation of the male reproductive system. Additionally, the development of an inducible $Kiss1^{IRES\text{-}cre}$ mouse line would allow researchers to further investigate this system without the potential confound of the neurons that only transiently express *Kiss1* at some point during development.

Using mouse models that allow genetic manipulation of either all *Kiss1*-expressing or only ARC *Kiss1*/*Tac2* neurons, we have uncovered a critical role of direct estrogen action on non-*Tac2*-expressing *Kiss1*/ERα neurons in negative feedback on the male reproductive system. Further study of these neurons is needed in order to determine the mechanism by which they affect GnRH output from the hypothalamus and thus regulate the functioning of the male reproductive axis.

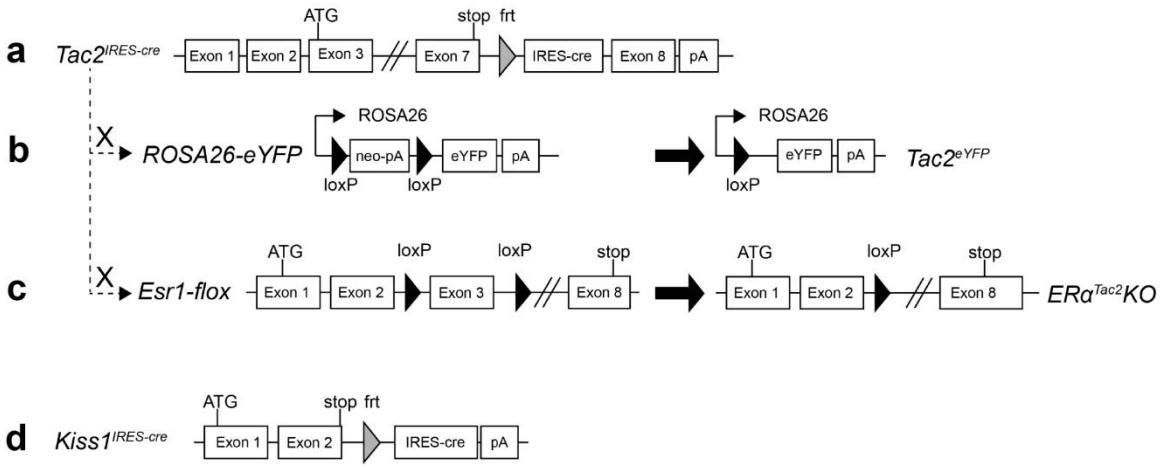

Figure 22 - Generation of mouse models to study *Kiss1*- and *Tac2*-expressing neurons in males

(**a**) Tac2$^{IRES\text{-}cre}$ mice carry an IRES-cre cassette in the 3' UTR of the endogenous murine *Tac2* gene. (**b**) Tac2^{eYFP} mice were generated by crossing the Tac2$^{IRES\text{-}cre}$ mouse to a commercially available ROSA26 fluorescent reporter strain (Jax 006148), which after recombination around the loxP sites results in eYFP production in all cre-expressing cells and thus fluorescent labeling of *Tac2*-expressing cells. (**c**) *Tac2*-specific ERα knockout animals ("ERα^{Tac2}KO") were generated by crossing the Tac2$^{IRES\text{-}cre}$ mice to *Esr1*-flox mice which have loxP sites flanking exon 3 which contains the sequence for the DNA binding domain of the receptor [142]. Cre-mediated recombination around the loxP sites results in a *Tac2* cell-specific ERα null mouse. (**d**) Kiss1$^{IRES\text{-}cre}$ mice carry the same IRES-cre cassette in the 3' UTR of the murine *Kiss1* gene. They were used to generate Kiss1^{eYFP} and ERα^{Kiss1}KO mice. IRES, internal ribosome entry site; pA, polyadenylation site; frt, flippase recognition target.

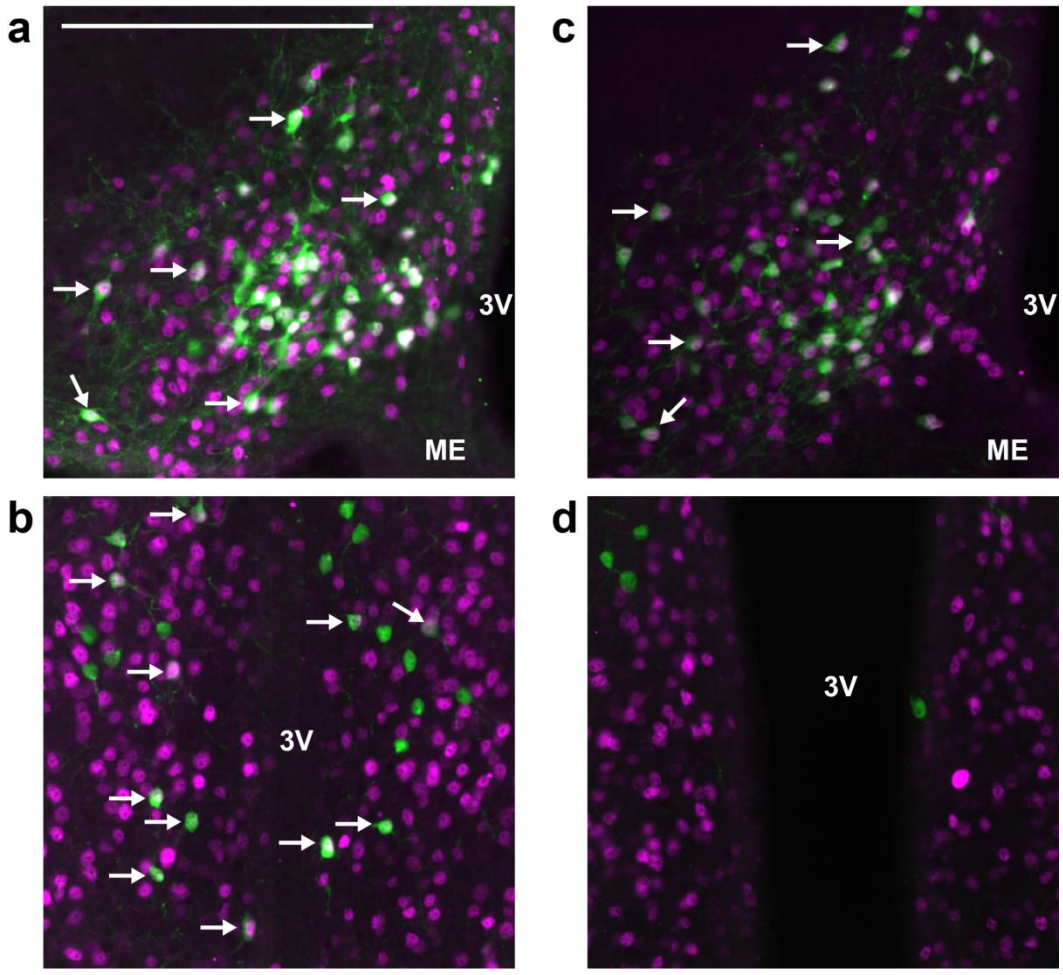

Figure 23 – Co-expression of eYFP and ERα in the hypothalamus of male Kiss1^{eYFP} and Tac2^{eYFP} mice

eYFP and ERα immunoreactivity (pseudocolored green and purple respectively) in the (**a**) ARC and the (**b**) PeN of a male Kiss1^{eYFP} mouse. eYFP and ERα immunoreactivity in the (**c**) ARC and (**d**) PeN of a male Tac2^{eYFP} mouse. Neurons expressing both eYFP and ERα appear white and some are indicated with a white arrow. 3V, third ventricle; ME, median eminence. Scale bar = 200 µm.

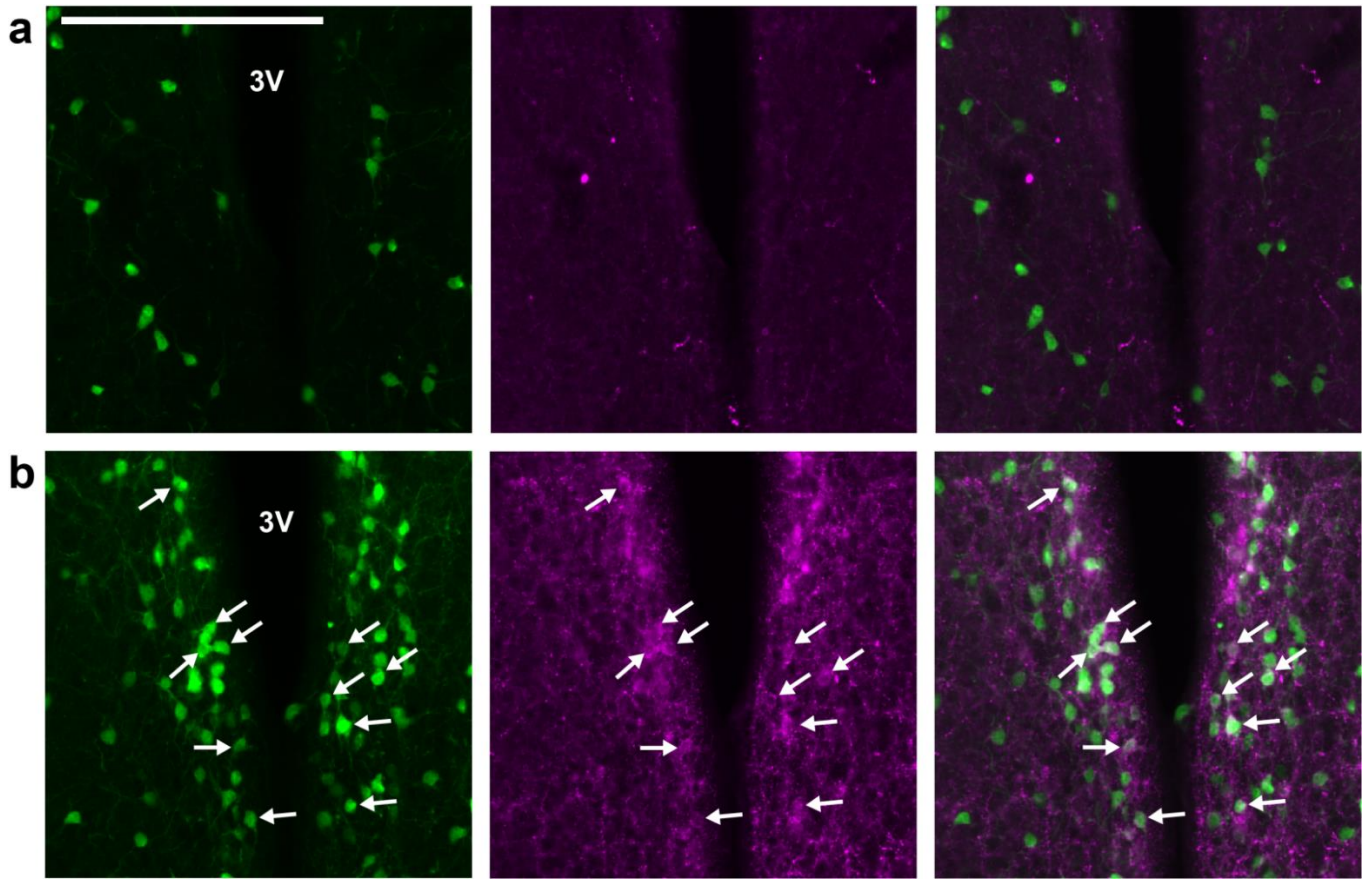

Figure 24 - Kisspeptin immunoreactivity in the rostral hypothalamus of Kiss1^{eYFP} male and female mice

eYFP (pseudo-colored green) and kisspeptin (pseudo-colored purple) immunoreactivity in the vicinity of the AVPV/PeN of a male (**a**) and a female (**b**) Kiss1^{eYFP} adult mouse. Neurons expressing both eYFP and kisspeptin appear white and some are denoted with a white arrow. 3V, third ventricle. Scale bar = 200 μm.

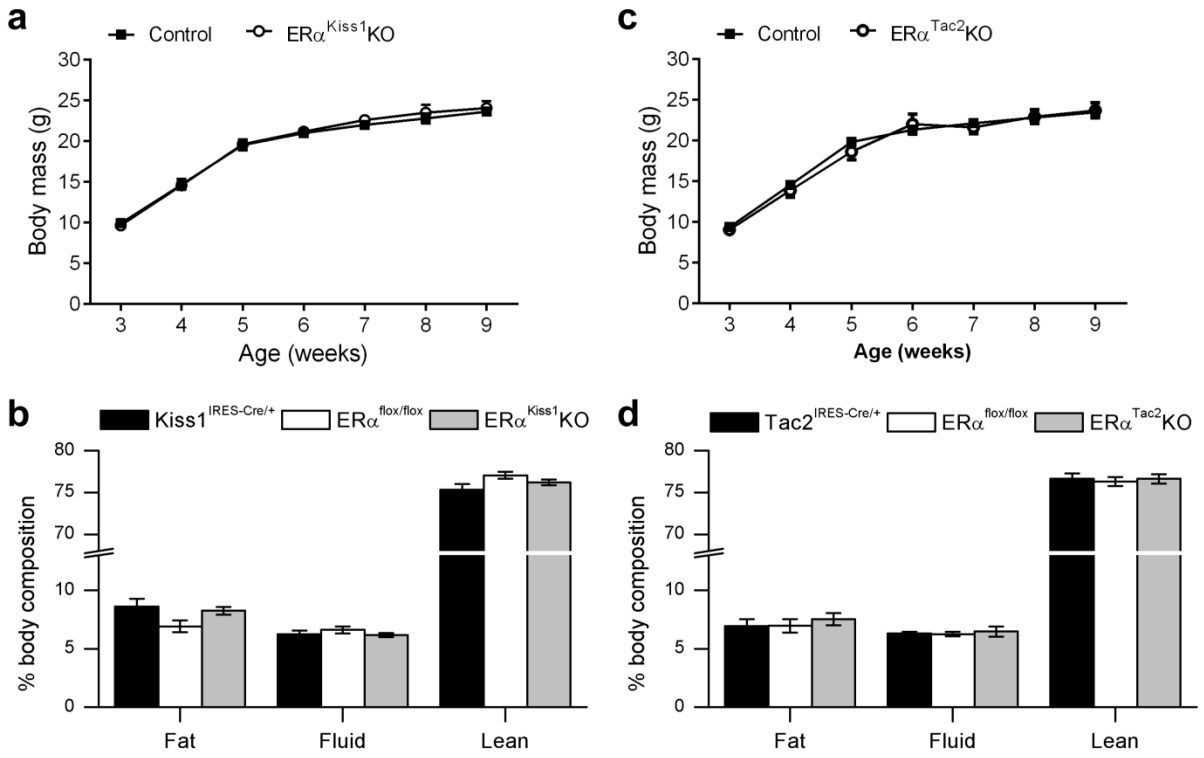

Figure 25 - Body mass and composition are unchanged in ERα^{Kiss1}KO and ERα^{Tac2} KO male mice

(**a**) Body mass of littermate controls (Kiss1$^{IRES\text{-}Cre/+}$ and ERα$^{flox/flox}$; "Control"; filled squares) and *Kiss1*-specific ERα knockout ("ERα^{Kiss1}KO"; open circles) male mice from 3-9 weeks of age (n = 10-11). (**b**) Body composition of 8-week-old control and ERα^{Kiss1}KO mice (n = 10-11). (**c**) Body mass of littermate controls (Tac2$^{IRES\text{-}Cre/+}$ and ERα$^{flox/flox}$; "Control"; filled squares) and *Tac2*-specific ERα knockout ("ERα^{Tac2}KO"; open circles) mice from 3-9 weeks of age (n = 8-20). (**d**) Body composition of 8-week-old control and ERα^{Tac2}KO mice (n = 8-9).

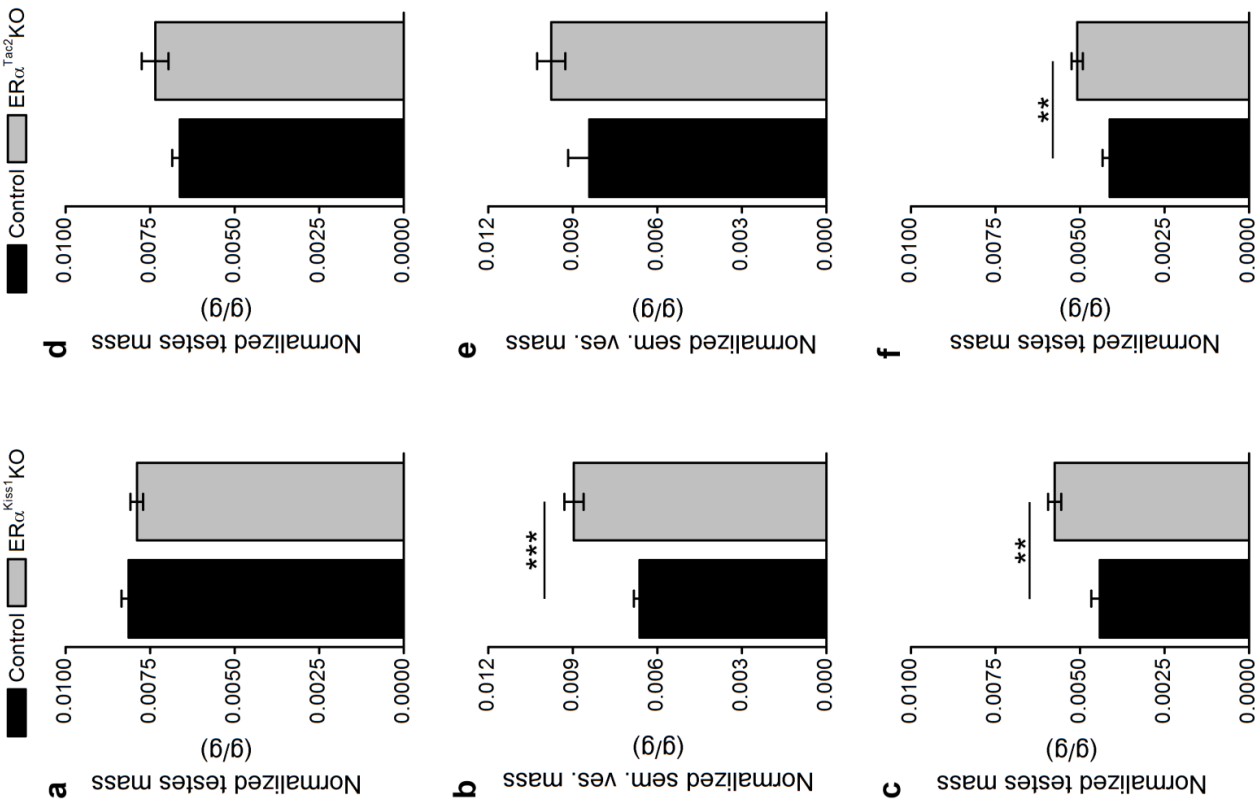

Figure 26 – Male reproductive organ weights are affected by ablation of ERα from either *Kiss1*- or *Tac2*-expressing neurons

(**a**) Testes and (**b**) seminal vesicle weights of adult (≈ 9 weeks old) ERα^{Kiss1}KO and littermate controls (Kiss1$^{IRES-Cre/+}$ and ERα$^{flox/flox}$) (n = 10-14). (**c**) Testes weight of juvenile (3-week-old) ERα^{Kiss1}KO and littermate controls (n = 8-13). (**d**) Testes and (**e**) seminal vesicle weights of adult (≈ 9 weeks old) ERα^{Tac2}KO and littermate controls (n = 8-12). (**f**) Testes weight of juvenile (3-week-old) ERα^{Tac2}KO and littermate controls (n = 7-8). All tissue weights are normalized to body weight (g/g). Student's *t* test: **, p<.01; ***, p<.001.

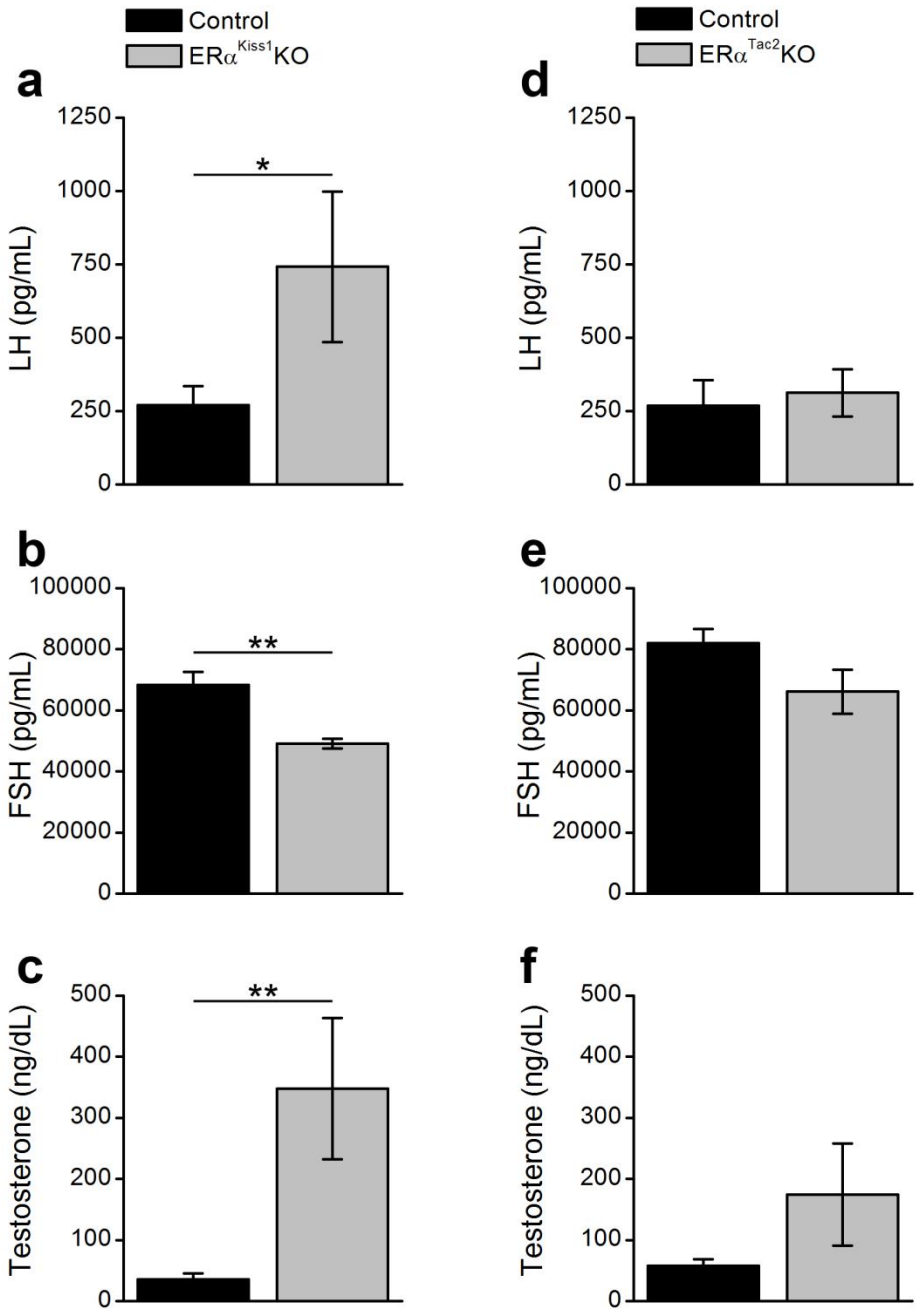

Figure 27 – Gonadotropin and gonadal hormone levels are altered by ablation of ERα from all *Kiss1*- but not *Tac2*-expressing neurons

Serum (**a**) LH (n = 11-17), (**b**) FSH (n = 10-20) and (**c**) testosterone (n = 11-15) levels in adult ERα^{Kiss1}KO and littermate control (Kiss1$^{IRES-Cre/+}$ and ERα$^{flox/flox}$) males. Serum (**d**) LH (n = 9-19), (**e**) FSH (n = 10-19) and (**f**) testosterone (n = 10-18) levels in adult ERα^{Tac2}KO and littermate control males. Student's *t* test: *, p<.05; **, p<.01. LH, luteinizing hormone; FSH, follicle-stimulating hormone.

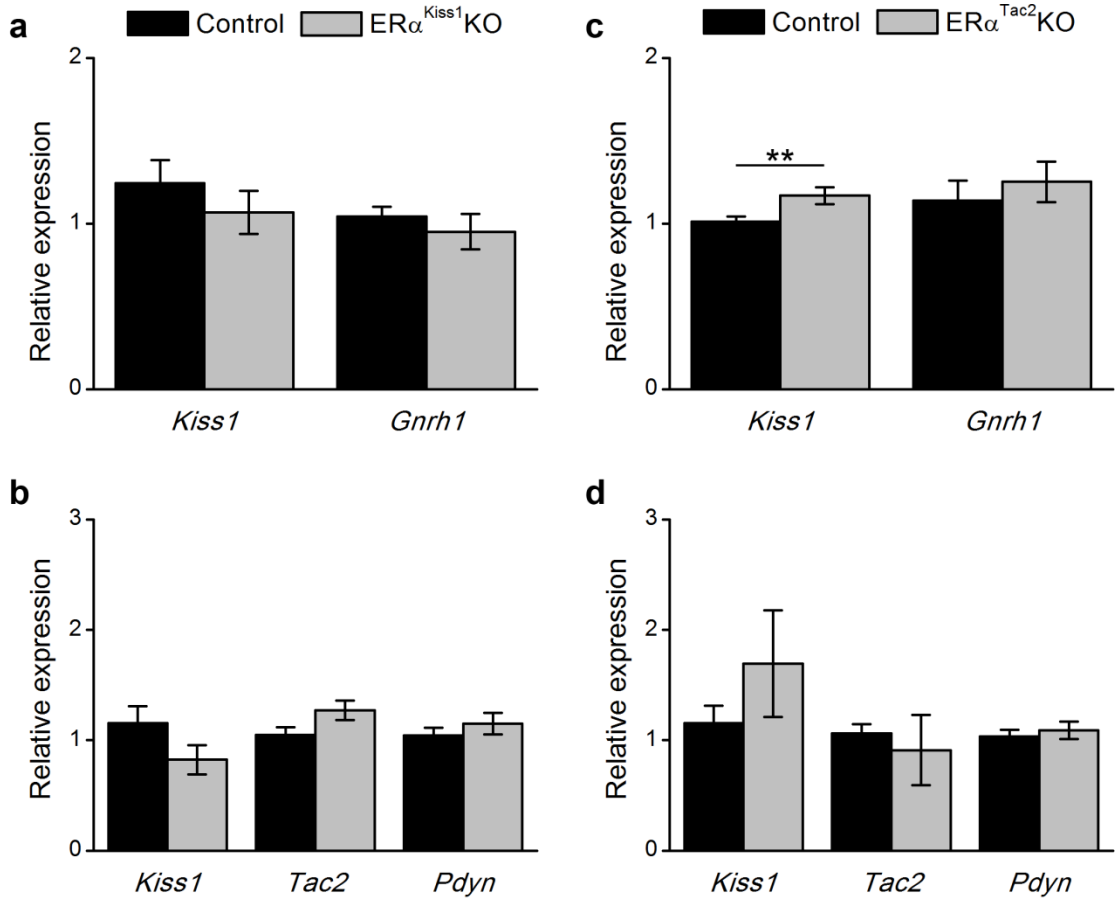

Figure 28 - Gene expression in the rostral hypothalamus and ARC

(**a**) *Kiss1* and *Gnrh1* expression in the rostral hypothalamus of ERα^{Kiss1}KO and littermate control males (n = 11-20). (**b**) *Kiss1*, *Tac2* and *Pdyn* expression in the ARC of ERα^{Kiss1}KO and littermate control (Tac2$^{IRES-Cre/+}$ and ERα$^{flox/flox}$) males (n = 10-20). (**c**) Relative expression of *Kiss1* and *GnRH* in the rostral hypothalamus of ERα^{Tac2}KO and littermate control males (n = 9-19). (**d**) *Kiss1*, *Tac2* and *Pdyn* expression in the ARC of ERα^{Tac2}KO and littermate control males (n = 8-19). Expression levels in KO animals are normalized to Control levels. Student's *t* test: **, p<.01. ARC, arcuate nucleus.

Chapter 5 – Summary & Conclusions

The mammalian hypothalamic-pituitary-gonadal (HPG) axis is a tightly regulated system responsible first for the transition from a child to a sexually mature adult and subsequently for maintenance of fertility throughout adulthood. The reproductive axis is modulated by a variety of signals, including but not limited to gonadal steroid hormones, stress hormones, circadian signals, and metabolic hormones. While the GnRH neurons are the final output from the hypothalamus, they are not capable of integrating the wide variety of signals known to act centrally and modulate the reproductive axis via GnRH neurons. We and others have sought to identify and characterize the neurons that are capable of responding to those signals and transmitting necessary information to GnRH neurons.

The role of direct leptin action on *Nos1*-expressing hypothalamic neurons

When an organism's energy stores are limited, physiological functions must be prioritized. This phenomenon has been described by researchers for many decades, most notably by Frisch and McArthur who are credited with the "critical weight hypothesis" [138]. They proposed that a certain threshold level of body mass is required in order for females to achieve menarche and maintain normal ovulatory menstrual cycles. While some progress has been made in

identifying and detailing the mechanisms that control the reproductive axis and those mechanisms that control energy balance, relatively little headway has been made in describing the intersection of these two processes.

The fat-derived hormone leptin controls a variety of physiological functions, including energy expenditure and food intake, but also reproduction. Mice lacking either leptin or its receptor (*ob/ob* or *db/db* mice respectively) are both infertile, but restoration of leptin signaling selectively in neurons of the CNS rescues the infertility, indicating a critical role for central leptin action [97]. While several leptin receptor isoforms exist, the isoform known as LepRb is the only known isoform with a long intracellular domain and the only isoform capable of intracellular signaling. LepRb-producing neurons are found throughout the brain, with a large concentration found in the hypothalamus. As there are a large number of LepRb neurons in the ventral premammilary nucleus (PMv), an area of the brain known to be involved in the control of the reproductive axis, we investigated the necessity of direct leptin action in the PMv (**Chapter 2**) for regulation of puberty onset, sexual maturation, and a variety of other measures.

Since most of the LepRb neurons in the PMv also express *Nos1*, we developed a mouse that expresses cre recombinase under the control of the endogenous *Nos1* promoter. We used cre recombinase activity in *Nos1*-expressing neurons to delete exon 17 of the *Lepr* gene and abolish direct leptin action via LepRb specifically in *Nos1*-expressing neurons. In females, the loss of direct leptin action in *Nos1* neurons had no effect on the age at vaginal opening, the first sign of puberty in the female mouse, but did substantially delay first

estrus, indicating delayed sexual maturation. Despite this delay, overall fertility is spared as evidenced by the ability of females to produce live litters with the same latency as control females with intact leptin action in *Nos1*-expressing neurons. In many physiological systems, including the reproductive system, multiple pathways exist that are capable of compensating for the loss of one pathway or in some systems, these pathways may be completely redundant and thus the loss of one has very little effect on the overall function of the system.

Since the expression of *Kiss1* (which encodes the reproductive neuropeptide kisspeptin) is diminished in the arcuate nucleus (ARC) of *ob/ob* mice [105], we hypothesized that changes in *Kiss1* expression in our mice could be responsible for the delay in sexual maturation. Interestingly, we saw no changes in ARC *Kiss1* expression, even in our global *Lepr* knockouts, although *Kiss1* levels in the AVPV/PeN were significantly reduced. The *Nos1*-specific *Lepr* knockouts did not exhibit any changes in AVPV/PeN *Kiss1* levels. Thus, changes in hypothalamic *Kiss1* gene expression are not likely to be the cause of the reproductive phenotype of $Lepr^{Nos1}$KO females, although changes to the kisspeptin system other than mRNA levels may. Further investigation is needed to determine whether the PMv *Lepr*/*Nos1* neurons directly modulate GnRH neurons instead of relying on the intermediary kisspeptin neurons or if the changes to the kisspeptin neurons are independent of mRNA changes.

While the reproductive phenotype in $Lepr^{Nos1}$KO females was not especially striking, both male and female knockouts exhibited a dramatic disruption of energy balance. Mice of both sexes were profoundly obese; this

was surprising since the PMv has no known role in feeding or energy expenditure. Additionally, the PMv is a mostly glutamatergic hypothalamic nucleus and studies have shown that loss of leptin action in all glutamatergic neurons does not lead to obesity of the magnitude that we observed in the Lepr^{Nos1}KO mice. Thus, it's likely that non-PMv *Lepr*/*Nos1* neurons are responsible for this effect, perhaps in the dorsomedial hypothalamic nucleus (DMH), which contains some non-glutamatergic *Lepr*/*Nos1* neurons and has a known role in energy balance regulation [112,159–163].

The arcuate nucleus has long been considered an important site of energy balance regulation. It contains two populations of first-order, leptin-responsive neurons: orexigenic Agrp/Npy neurons and anorexigenic Pomc/Cart neurons [164]. Since expression of *Agrp* and *Npy* is increased and *Pomc* expression is decreased in mice with deficient leptin signaling [165–168], we investigated whether our mice had similar changes in gene expression. We saw absolutely no effect of our deletion on either *Agrp* or *Npy* mRNA levels, which was not surprising given that *Nos1* does not seem to be expressed in these neurons [112]. Interestingly, even though our genetic deletion did not target *Pomc*-expressing neurons directly, we still saw a significant decrease in *Pomc* expression in the ARC, such that expression levels in the Lepr^{Nos1}KO mice were not significantly different from mice lacking Lepr globally (LeprKO mice). This raises the question as to how much of the change in *Pomc* neuron function in *ob/ob* or *db/db* mice is a result of loss of direct leptin action versus indirect leptin action on these neurons specifically.

Lastly, we assessed function of the hypothalamic-pituitary-adrenal (HPA) and hypothalamic-pituitary-thyroid (HPT) axes in our *Nos1*-specific *Lepr* knockouts. While serum corticosterone levels were significantly elevated in male global *Lepr* knockouts (in agreement with previous studies [133–136]) our specific deletion of LepRb from only *Nos1*-expressing neurons did not affect corticosterone levels in either sex. Thus, direct leptin action in *Nos1* neurons is dispensable for proper regulation of the HPA axis. In agreement with previous studies, we found that serum thyroxine (T4) levels were significantly diminished in global *Lepr* knockouts of both sexes [91,137]. Our more specific deletion did not affect T4 levels in females, but it did result in significantly lower T4 levels in males, indicating a possible sexual dimorphism in the hypothalamic control of thyroid function. The T4 levels in the $Lepr^{Nos1}$KO males were not decreased to the same extent as they were in global Lepr knockouts, so *Nos1*/LepRb neurons must not be the only *Lepr*-expressing neurons that modulate the HPT axis.

In this study, genetic ablation of LepRb from *Nos1*-expressing neurons revealed multiple physiological functions of direct leptin action from nNos-producing neurons. The multitude of effects of this deletion is not unexpected given the somewhat diffuse anatomical distribution of *Nos1*/*Lepr* neurons in the brain. Although the majority of the neurons affected by the deletion are in the PMv, the contribution of LepRb loss from extra-PMv neurons can not be ignored and may in fact be responsible for much of the metabolic and neuroendocrine phenotype of the $Lepr^{Nos1}$KO mice. Future genetic dissection of specific

individual leptin-responsive neuronal populations will hopefully clarify the precise contribution of PMv LepRb neurons (and other hypothalamic nuclei) to overall leptin action in the brain.

Regulation of the reproductive axis by estrogen action via ERα on kisspeptin neurons

Since the reports of delayed or absent puberty and infertility in humans and mice with loss-of-function mutations in the genes for kisspeptin or its receptor (KISS1 and KISS1R respectively in humans) [28,29], there has been a monumental effort to characterize and understand the kisspeptin system. One of the more interesting findings regarding these neurons is the manner in which *Kiss1* gene expression is differentially affected by circulating gonadal steroid hormones in the two hypothalamic populations, one in the ARC and the other in the AVPV/PeN. In both males and females, gonadal steroid hormones act in an inhibitory manner on *Kiss1* gene expression in the ARC, while acting in a stimulatory manner in the AVPV/PeN [43,45].

This effect, along with other evidence, has led to the hypothesis that the AVPV/PeN kisspeptin neurons are critical for the female pre-ovulatory LH surge via their regulation of GnRH-producing neurons. And more specifically that the elevated pre-ovulatory estradiol levels act through ERα on these AVPV/PeN neurons to elicit this effect. The physiological role of the second population- the ARC kisspeptin neurons that have been nicknamed KNDy neurons due to the co-expression of neurokinin B and dynorphin A in addition to kisspeptin- is clear.

While some have assumed that since they are negatively regulated by gonadal steroid hormones they must be responsible for negative feedback on the reproductive axis, there are only a handful of studies that have attempted to actually test this hypothesis.

Building on those few studies that have investigated the necessity of direct estrogen action on ARC KNDy neurons for estrogen negative feedback, we employed a genetic strategy to ablate ERα from either all *Kiss1*-expressing neurons or selectively in ARC KNDy neurons in both female and male mice (**Chapters 3 & 4** respectively). While previous studies had developed and utilized mouse models to study and manipulate all kisspeptin neurons, we developed a novel mouse model in order to specifically manipulate the ARC KNDy neurons without affecting the AVPV/PeN population. Since the gene *Tac2* is expressed in ARC KNDy neurons but not in AVPV/PeN kisspeptin neurons, the development of a mouse that produces cre recombinase in all *Tac2* neurons allowed us to ablate ERα from the ARC KNDy neurons but leave the *Esr1* allele unperturbed in the other population of kisspeptin neurons.

Females

In females lacking ERα in either all *Kiss1*- or only *Tac2*-(KNDy) neurons, we observed a dramatic advancement in age at vaginal opening, indicating a loss of prepubertal restraint on the reproductive axis. Both knockout genotypes also showed a complete absence of estrous cyclicity; although their vaginal cytology indicated that they were constantly in estrus, the ERα^{Kiss1}KO females appeared

to have reduced ovulatory function (few corpora lutea in the ovaries) and the ERα^{Tac2}KO females were completely anovulatory. Since the early puberty onset and lack of complete sexual maturation are identical whether ERα was ablated from only one or from both kisspeptin populations, we concluded that these effects are due to loss of ERα from ARC KNDy neurons.

The early puberty onset and impaired ovulation led us to investigate the hormonal milieu in ERα^{Kiss1}KO and ERα^{Tac2}KO females both as adults and also as juveniles, around the time of puberty onset. In agreement with previous studies, we did find elevated LH levels in not only juvenile female ERα^{Kiss1}KO mice, but also in ERα^{Tac2}KO mice. At this same age, estradiol levels were significantly elevated in both mouse lines, explaining their early vaginal opening, but in contrast with their elevated LH. Elevated LH levels in the face of elevated estradiol indicate impaired estrogen negative feedback in the juvenile females.

In adult ERα^{Tac2}KO female mice, we also found substantially and significantly elevated LH levels, confirming a substantial loss of estrogen negative feedback. In contrast (but in agreement with previous studies [44,46]), LH levels in adult ERα^{Kiss1}KO females were unchanged compared to their littermate controls. The knockouts' LH levels are actually inappropriately normal, considering their significantly elevated estradiol levels and reflect slightly impaired estrogen negative feedback. If estrogen negative feedback was functioning normally in these mice, their LH levels should be suppressed by their elevated estradiol levels. The impairment in estrogen negative feedback appears to be common to both conditional knockouts that we studied, but the magnitude

of the effect is larger in ERα^{Tac2}KO females.

Surgical removal of the ovaries (ovariectomy) results in a complete loss of gonadal hormone restraint on the hypothalamic-pituitary system. In animals completely lacking estrogen feedback, such as the αERKO females, intact LH levels are indistinguishable from those of ovariectomized wild-type females, and no further rise in LH as a result of ovariectomy is observed [20]. While the intact ERα^{Tac2}KO females had significantly elevated LH levels compared to their littermate controls, these levels were lower than what we observed in ovariectomized wild-type females. Additionally, ovariectomy of ERα^{Tac2}KO females led to a further elevation in circulating LH that could be significantly reduced by acute estradiol treatment. Taken together, this suggests that another estrogen negative feedback system (or perhaps several) is still intact and functioning in these animals. This is surprising given their lack of estrous cyclicity, anovulation and severely elevated estradiol levels. Determining whether the remaining negative feedback system is in the hypothalamus or in the pituitary of the ERα^{Tac2}KO females should be a goal of future research.

Although their circulating LH levels were not as high as what we observed in ovariectomized controls, the expression of *Kiss1* and *Pdyn* in the ARC of both knockout genotypes was elevated to levels similar to what we found in ovariectomized controls. Unfortunately, technical issues with the *Tac2* expression assay prevented us from performing the same analysis on the expression of that gene in ovariectomized animals, although *Tac2* expression was significantly elevated in the ARC of intact females of both knockout

genotypes. Thus, deletion of ERα from ARC KNDy neurons causes a complete loss of estrogen feedback on the expression of *Kiss1* and *Pdyn* (and potentially *Tac2*) in the ARC.

Surprisingly, we saw no change in *Gnrh1* expression in the rostral hypothalamus as a result of either ovariectomy or ablation of ERα from either all *Kiss1*-expressing neurons or all KNDy neurons. This is in contrast to what has been reported previously by Dorling et al., who saw an increase in *Gnrh1* mRNA per cell after ovariectomy [20]. Methodological differences in the quantification of *Gnrh1* expression may be the cause of this inconsistency.

As expected, deletion of ERα from *Tac2*-expressing neurons had no effect on *Kiss1* expression in the AVPV/PeN, while ovariectomy significantly decreased *Kiss1* expression in that area. This is in stark contrast with the extremely diminished *Kiss1* levels that we observed in the AVPV/PeN of ERα^{Kiss1}KO females. Direct estrogen action through ERα on AVPV/PeN kisspeptin neurons appears critical for maintenance of appropriate *Kiss1* expression in intact animals such that without it, AVPV/PeN *Kiss1* expression levels drop to ovariectomized levels.

In contrast with the elevated *Kiss1* expression in the ARC of both knockout genotypes, we found no overt changes in peptide expression in either ERα^{Kiss1}KO or ERα^{Tac2}KO females. This indicates that either the regulation of *Kiss1* translation compensates for the increased gene expression or that there are limitations in our method for quantifying peptide levels. We did, however, observe the expected decrease in kisspeptin peptide in the AVPV/PeN of

ERα^{Kiss1}KO adults, confirming our previous gene expression result. While we saw increased kisspeptin staining in the AVPV/PeN of 3-week-old ERα^{Tac2}KO females, the same change was not apparent in ERα^{Kiss1}KO females of the same age, likely due to the loss of ERα from these neurons. The early production of kisspeptin peptide in the AVPV/PeN in ERα^{Tac2}KO females can't be the direct cause of the precocious vaginal opening since ERα^{Kiss1}KOs exhibit the same vaginal opening phenotype without the change in peptide levels. Both the early vaginal opening and the early kisspeptin production in the AVPV/PeN may be due to the elevated estradiol levels in the 3-week-old females but the peptide change is only apparent in ERα^{Tac2}KOs because ERα is intact in the AVPV/PeN kisspeptin neurons.

In conclusion, we have shown that on a gross level, female mice lacking ERα either in all kisspeptin neurons or just in the ARC kisspeptin (KNDy) neurons have a very similar phenotype. In both cases, we saw precocious puberty onset, lack of estrous cyclicity, and evidence of estrogen excess. Importantly, only the mice lacking ERα in ARC KNDy neurons had significantly elevated adult LH levels although both knockout genotypes exhibited impaired feedback on LH secretion as juveniles. This data supports our hypothesis that direct estrogen action on ARC KNDy neurons via ERα is a critical component of estrogen negative feedback on the female reproductive axis. While it's not the only negative feedback system, the severity of the phenotypes presented here does indicate that it is an extremely important component of the overall regulation of the HPG axis under physiological conditions.

Males

Similar to what we observed in females, male mice lacking ERα in all *Kiss1*-expressing neurons exhibit evidence of impaired negative feedback on the reproductive system. Juvenile ERα^{Kiss1}KO males had enlarged testes which may indicate precocious sexual maturation. While we did not observe this same effect on testes weight in adults, they did have enlarged seminal vesicles, an accessory sex organ which is sensitive to circulating testosterone levels [152]. This is in contrast with the males lacking ERα only in *Tac2*-expressing neurons and thus only in ARC KNDy neurons. While the young ERα^{Tac2}KO males showed the same small increase in testes weight, seminal vesicle weights in the adults were unchanged as a result of the deletion. Since we did not directly assess puberty onset in either mouse line, future studies should determine whether the males enter puberty at an earlier age than controls, consistent with what we observed in the female knockouts.

In order to determine whether negative feedback on the anterior pituitary was affected by our genetic ablation of ERα from all kisspeptin neurons, we analyzed serum gonadotropin and gonadal steroid hormone levels in the adult males. Consistent with our hypothesis that direct estrogen action through ERα in kisspeptin neurons is required for negative feedback, we found elevated LH and testosterone levels in the ERα^{Kiss1}KO males. In mice with an intact negative feedback system, high levels of testosterone should function to reduce LH secretion. In the ERα^{Kiss1}KO males, we found reduced FSH levels, perhaps due to increased inhibin production by the testes in response to elevated testosterone

although circulating inhibin levels were not determined in this study. In contrast with these findings, we found that ERα^{Tac2}KO adult males have no significant changes in LH, FSH or testosterone compared to their littermate controls, although a trend toward elevated testosterone was observed. Thus, the loss of negative feedback that we observed in ERα^{Kiss1}KO males must be the result of deletion of ERα from non-*Tac2*-expressing kisspeptin neurons.

Since the expression of many hypothalamic genes is regulated by circulating gonadal hormone levels, we hypothesized that some of them would be significantly altered by ablation of ERα from either *Kiss1*- or *Tac2*-expressing neurons. Surprisingly, we saw no effect of our deletion on *Kiss1* or *Gnrh1* expression in the rostral hypothalamus of ERα^{Kiss1}KO males. Since estrogen treatment affects *Kiss1* expression in this area of castrated males [30], this phenomenon is either specific to castrated males, or isn't a result of direct estrogen action through ERα on these neurons. The additional confound of elevated testosterone levels and intact AR in these males may also be masking any effects of the loss of ERα from the kisspeptin neurons in the rostral hypothalamus.

In the ARC, expression of the three genes co-expressed within the KNDy neurons is completely unchanged as well. This was surprising as well, because in castrated animals, estrogen treatment significantly reduces the expression of *Kiss1*, *Tac2* and *Pdyn* in the ARC [48]. Without estrogen negative feedback on gene expression within ARC KNDy neurons, we would expect to see elevated expression of all three genes. Again, either the increased expression that others

have found is 1) specific to castrated males, 2) doesn't require direct estrogen action in ARC kisspeptin neurons via ERα or 3) can be compensated for by the remaining androgen receptor in these neurons.

Overall, we saw the same lack of gene expression changes in the ERα^{Tac2}KO males with one exception. In the rostral hypothalamus, we saw a very slight but significant increase in the expression of *Kiss1* in mice lacking ERα in all *Tac2*-expressing neurons. We attribute this effect to the trend toward elevated testosterone levels and intact ERα in the AVPV/PeN kisspeptin neurons in these mice. This is consistent with previous reports showing an increase in AVPV/PeN *Kiss1* levels after either testosterone or estradiol treatment [45].

In summary, in this study we have shown that while loss of ERα from the ARC KNDy neurons has very little effect on the male reproductive system, the loss of ERα from all kisspeptin neurons, including non-*Tac2*-expressing kisspeptin cells, results in a significant loss of gonadal steroid negative feedback. These critical *Kiss1*/ERα neurons may be located in the AVPV/PeN; while they do not produce detectable levels of kisspeptin peptide, they appear necessary for proper regulation of gonadotropin secretion, as well as testosterone production.

Despite the evolutionary necessity of reproduction, there are still many gaps in our understanding of the mammalian reproductive system- especially at the level of the hypothalamus. While it is clear that the secretion of GnRH is the critical final output of the central nervous system with regard to reproduction, the

mechanisms by which the multitude of central signals that modulate the function of the axis are integrated in the hypothalamus remain unclear. The studies described here have identified neuronal populations in the hypothalamus that are important and necessary components of this system. While some hypothalamic neurons relay information to the GnRH neurons about how much energy is stored in the body, others function to restrain the axis prior to puberty and also in adulthood in an estrogen-dependent manner. As our understanding of how the HPG axis functions improves, we will be better able to treat and potentially prevent the numerous disorders of the reproductive system, ranging from precocious or delayed puberty to adult infertility.

References

1. Wennink, J. M. B., Delemarre Van De Waal, H. A., Schoemaker, R., Schoemaker, H. & Schoemaker, J. Luteinizing Hormone and Follicle Stimulating Hormone Secretion Patterns in Boys Throughout Puberty Measured Using Highly Sensitive Immunoradiometric Assays. *Clin. Endocrinol. (Oxf.)* **31,** 551–564 (1989).
2. Wennink, J. M. B., Delemarre Van De Waal, H. A., Schoemaker, R., Schoemaker, H. & Schoemaker, J. Luteinizing Hormone and Follicle Stimulating Hormone Secretion Patterns in Girls Throughout Puberty Measured Using Highly Sensitive Immunoradiometric Assays. *Clin. Endocrinol. (Oxf.)* **33,** 333–344 (1990).
3. Jakacki, R. I. *et al.* Pulsatile secretion of luteinizing hormone in children. *J. Clin. Endocrinol. Metab.* **55,** 453–458 (1982).
4. Dunkel, L. *et al.* Developmental changes in 24-hour profiles of luteinizing hormone and follicle-stimulating hormone from prepuberty to midstages of puberty in boys. *J. Clin. Endocrinol. Metab.* **74,** 890–897 (1992).
5. Apter, D., Bützow, T. L., Laughlin, G. A. & Yen, S. S. Gonadotropin-releasing hormone pulse generator activity during pubertal transition in girls: pulsatile and diurnal patterns of circulating gonadotropins. *J. Clin. Endocrinol. Metab.* **76,** 940–949 (1993).
6. Reiter, E. O. & Grumbach, M. M. Neuroendocrine control mechanisms and the onset of puberty. *Annu. Rev. Physiol.* **44,** 595–613 (1982).
7. Conte, F. A., Grumbach, M. M. & Kaplan, S. L. A diphasic pattern of gonadotropin secretion in patients with the syndrome of gonadal dysgenesis. *J. Clin. Endocrinol. Metab.* **40,** 670–674 (1975).
8. Winter, J. S. & Faiman, C. Serum gonadotropin in concentrations in agonadal children and adults. *J. Clin. Endocrinol. Metab.* **35,** 561–564 (1972).
9. Ojeda, S. & Skinner, M. in *Knobil Neills Physiol. Reprod.* (Neill, J.) 2061–2126 (Academic Press, 2006).
10. Parker, C. R. & Mahesh, V. B. Hormonal Events Surrounding the Natural Onset of Puberty in Female Rats. *Biol. Reprod.* **14,** 347–353 (1976).
11. Couse, J. F. & Korach, K. S. Estrogen Receptor Null Mice: What Have We Learned and Where Will They Lead Us? *Endocr. Rev.* **20,** 358–417 (1999).

12. Kuiper, G. G. J. M. *et al.* Comparison of the Ligand Binding Specificity and Transcript Tissue Distribution of Estrogen Receptors α and β. *Endocrinology* **138,** 863–870 (1997).

13. Krege, J. H. *et al.* Generation and reproductive phenotypes of mice lacking estrogen receptor beta. *Proc. Natl. Acad. Sci. U. S. A.* **95,** 15677–15682 (1998).

14. Lubahn, D. B. *et al.* Alteration of reproductive function but not prenatal sexual development after insertional disruption of the mouse estrogen receptor gene. *Proc. Natl. Acad. Sci. U. S. A.* **90,** 11162–11166 (1993).

15. Korach, K. S. *et al.* Estrogen receptor gene disruption: molecular characterization and experimental and clinical phenotypes. *Recent Prog. Horm. Res.* **51,** 159–186; discussion 186–188 (1996).

16. Dupont, S. *et al.* Effect of single and compound knockouts of estrogen receptors alpha (ERalpha) and beta (ERbeta) on mouse reproductive phenotypes. *Dev. Camb. Engl.* **127,** 4277–4291 (2000).

17. Couse, J. F., Bunch, D. O., Lindzey, J., Schomberg, D. W. & Korach, K. S. Prevention of the Polycystic Ovarian Phenotype and Characterization of Ovulatory Capacity in the Estrogen Receptor-α Knockout Mouse. *Endocrinology* **140,** 5855–5865 (1999).

18. Rosenfeld, C. S. *et al.* Gonadotropin Induction of Ovulation and Corpus Luteum Formation in Young Estrogen Receptor-α Knockout Mice. *Biol. Reprod.* **62,** 599–605 (2000).

19. Glidewell-Kenney, C. *et al.* Nonclassical estrogen receptor signaling mediates negative feedback in the female mouse reproductive axis. *Proc. Natl. Acad. Sci.* **104,** 8173–8177 (2007).

20. Dorling, A. A., Todman, M. G., Korach, K. S. & Herbison, A. E. Critical Role for Estrogen Receptor alpha in Negative Feedback Regulation of Gonadotropin-Releasing Hormone mRNA Expression in the Female Mouse. *Neuroendocrinology* **78,** 204–209 (2003).

21. Couse, J. F., Yates, M. M., Walker, V. R. & Korach, K. S. Characterization of the hypothalamic-pituitary-gonadal axis in estrogen receptor (ER) Null mice reveals hypergonadism and endocrine sex reversal in females lacking ERalpha but not ERbeta. *Mol. Endocrinol. Baltim. Md* **17,** 1039–1053 (2003).

22. Risma, K. A. *et al.* Targeted overexpression of luteinizing hormone in transgenic mice leads to infertility, polycystic ovaries, and ovarian tumors. *Proc. Natl. Acad. Sci. U. S. A.* **92,** 1322–1326 (1995).

23. Risma, K. A., Hirshfield, A. N. & Nilson, J. H. Elevated luteinizing hormone in prepubertal transgenic mice causes hyperandrogenemia, precocious puberty, and substantial ovarian pathology. *Endocrinology* **138,** 3540–3547 (1997).

24. Bocchinfuso, W. P. & Korach, K. S. Mammary gland development and tumorigenesis in estrogen receptor knockout mice. *J. Mammary Gland Biol. Neoplasia* **2,** 323–334 (1997).

25. Couse, J. F., Lindzey, J., Grandien, K., Gustafsson, J.-Å. & Korach, K. S. Tissue Distribution and Quantitative Analysis of Estrogen Receptor-α (ERα) and Estrogen Receptor-β (ERβ) Messenger Ribonucleic Acid in the Wild-Type and ERα-Knockout Mouse. *Endocrinology* **138,** 4613–4621 (1997).

26. Wintermantel, T. M. *et al.* Definition of estrogen receptor pathway critical for estrogen positive feedback to gonadotropin-releasing hormone neurons and fertility. *Neuron* **52,** 271–280 (2006).

27. Herbison, A. E. & Pape, J.-R. New Evidence for Estrogen Receptors in Gonadotropin-Releasing Hormone Neurons. *Front. Neuroendocrinol.* **22,** 292–308 (2001).

28. Seminara, S. B. *et al.* The GPR54 gene as a regulator of puberty. *N. Engl. J. Med.* **349,** 1614–1627 (2003).

29. De Roux, N. *et al.* Hypogonadotropic hypogonadism due to loss of function of the KiSS1-derived peptide receptor GPR54. *Proc. Natl. Acad. Sci. U. S. A.* **100,** 10972–10976 (2003).

30. Topaloglu, A. K. *et al.* Inactivating KISS1 mutation and hypogonadotropic hypogonadism. *N. Engl. J. Med.* **366,** 629–635 (2012).

31. Colledge, W. H. Transgenic mouse models to study Gpr54/kisspeptin physiology. *Peptides* **30,** 34–41 (2009).

32. Silveira, L. G. *et al.* Mutations of the KISS1 gene in disorders of puberty. *J. Clin. Endocrinol. Metab.* **95,** 2276–2280 (2010).

33. Teles, M. G. *et al.* A GPR54-activating mutation in a patient with central precocious puberty. *N. Engl. J. Med.* **358,** 709–715 (2008).

34. Clarkson, J., d' Anglemont de Tassigny, X., Moreno, A. S., Colledge, W. H. & Herbison, A. E. Kisspeptin-GPR54 Signaling Is Essential for Preovulatory Gonadotropin-Releasing Hormone Neuron Activation and the Luteinizing Hormone Surge. *J. Neurosci.* **28,** 8691–8697 (2008).

35. Roseweir, A. K. *et al.* Discovery of Potent Kisspeptin Antagonists Delineate Physiological Mechanisms of Gonadotropin Regulation. *J. Neurosci.* **29,** 3920–3929 (2009).

36. Kinoshita, M. *et al.* Involvement of Central Metastin in the Regulation of Preovulatory Luteinizing Hormone Surge and Estrous Cyclicity in Female Rats. *Endocrinology* **146,** 4431–4436 (2005).

37. Smith, J. T. *et al.* Kisspeptin Is Essential for the Full Preovulatory LH Surge and Stimulates GnRH Release from the Isolated Ovine Median Eminence. *Endocrinology* **152,** 1001–1012 (2011).

38. Navarro, V. M. *et al.* Regulation of gonadotropin-releasing hormone

secretion by kisspeptin/dynorphin/neurokinin B neurons in the arcuate nucleus of the mouse. *J. Neurosci. Off. J. Soc. Neurosci.* **29,** 11859–11866 (2009).

39. Goodman, R. L. *et al.* Kisspeptin Neurons in the Arcuate Nucleus of the Ewe Express Both Dynorphin A and Neurokinin B. *Endocrinology* **148,** 5752–5760 (2007).
40. Burke, M. C., Letts, P. A., Krajewski, S. J. & Rance, N. E. Coexpression of dynorphin and neurokinin B immunoreactivity in the rat hypothalamus: Morphologic evidence of interrelated function within the arcuate nucleus. *J. Comp. Neurol.* **498,** 712–726 (2006).
41. Smith, J. T., Li, Q., Pereira, A. & Clarke, I. J. Kisspeptin Neurons in the Ovine Arcuate Nucleus and Preoptic Area Are Involved in the Preovulatory Luteinizing Hormone Surge. *Endocrinology* **150,** 5530–5538 (2009).
42. Krajewski, S. J., Burke, M. C., Anderson, M. J., McMullen, N. T. & Rance, N. E. Forebrain projections of arcuate neurokinin B neurons demonstrated by anterograde tract-tracing and monosodium glutamate lesions in the rat. *Neuroscience* **166,** 680–697 (2010).
43. Smith, J. T., Cunningham, M. J., Rissman, E. F., Clifton, D. K. & Steiner, R. A. Regulation of Kiss1 gene expression in the brain of the female mouse. *Endocrinology* **146,** 3686–3692 (2005).
44. Frazão, R. *et al.* Shift in Kiss1 Cell Activity Requires Estrogen Receptor α. *J. Neurosci. Off. J. Soc. Neurosci.* **33,** 2807–2820 (2013).
45. Smith, J. T. *et al.* Differential regulation of KiSS-1 mRNA expression by sex steroids in the brain of the male mouse. *Endocrinology* **146,** 2976–2984 (2005).
46. Mayer, C. *et al.* Timing and completion of puberty in female mice depend on estrogen receptor -signaling in kisspeptin neurons. *Proc. Natl. Acad. Sci.* **107,** 22693–22698 (2010).
47. Mittelman-Smith, M. A. *et al.* Arcuate kisspeptin/neurokinin B/dynorphin (KNDy) neurons mediate the estrogen suppression of gonadotropin secretion and body weight. *Endocrinology* **153,** 2800–2812 (2012).
48. Navarro, V. M. *et al.* Regulation of NKB pathways and their roles in the control of Kiss1 neurons in the arcuate nucleus of the male mouse. *Endocrinology* **152,** 4265–4275 (2011).
49. Ingalls, A. M., Dickie, M. M. & Snell, G. D. Obese, a New Mutation in the House Mouse. *J. Hered.* **41,** 317–318 (1950).
50. Zhang, Y. *et al.* Positional cloning of the mouse obese gene and its human homologue. *Nature* **372,** 425–432 (1994).
51. Halaas, J. L. *et al.* Weight-reducing effects of the plasma protein encoded by the obese gene. *Science* **269,** 543–546 (1995).

52. Mounzih, K., Lu, R. & Chehab, F. F. Leptin Treatment Rescues the Sterility of Genetically Obese ob/ob Males. *Endocrinology* **138,** 1190–1193 (1997).

53. Swerdloff, R. S., Batt, R. A. & Bray, G. A. Reproductive hormonal function in the genetically obese (ob/ob) mouse. *Endocrinology* **98,** 1359–1364 (1976).

54. Jones, N. & Harrison, G. A. Genetically determined obesity and sterility in the mouse. *Stud. Fertil.* **9,** 51–64 (1957).

55. Barash, I. A. *et al.* Leptin is a metabolic signal to the reproductive system. *Endocrinology* **137,** 3144–3147 (1996).

56. Lane, P. W. & Dickie, M. M. Fertile, Obese Male Mice: Relative Sterility in Obese Males Corrected by Dietary Restriction. *J. Hered.* **45,** 56–58 (1954).

57. Chehab, F. F., Lim, M. E. & Lu, R. Correction of the sterility defect in homozygous obese female mice by treatment with the human recombinant leptin. *Nat. Genet.* **12,** 318–320 (1996).

58. Malik, N. M. *et al.* Leptin Requirement for Conception, Implantation, and Gestation in the Mouse. *Endocrinology* **142,** 5198–5202 (2001).

59. Batt, R. A. The response of the reproductive system in the female mutant mouse, obese (genotype obob) to gonadotrophin-releasing hormones. *J. Reprod. Fertil.* **31,** 496–497 (1972).

60. Runner, M. N. & Gates, A. Sterile, Obese Mothers. *J. Hered.* **45,** 51–55 (1954).

61. Hummel, K. Transplantation of ovaries of the obese mouse. *Anat. Rec.* **128,** 69 (1957).

62. Ahima, R. S., Dushay, J., Flier, S. N., Prabakaran, D. & Flier, J. S. Leptin accelerates the onset of puberty in normal female mice. *J. Clin. Invest.* **99,** 391–395 (1997).

63. Chehab, F. F., Mounzih, K., Lu, R. & Lim, M. E. Early Onset of Reproductive Function in Normal Female Mice Treated with Leptin. *Science* **275,** 88–90 (1997).

64. Yura, S. *et al.* Accelerated puberty and late-onset hypothalamic hypogonadism in female transgenic skinny mice overexpressing leptin. *J. Clin. Invest.* **105,** 749–755 (2000).

65. Considine, R. V. *et al.* Evidence against either a premature stop codon or the absence of obese gene mRNA in human obesity. *J. Clin. Invest.* **95,** 2986–2988 (1995).

66. Maffei, M. *et al.* Absence of mutations in the human OB gene in obese/diabetic subjects. *Diabetes* **45,** 679–682 (1996).

67. Montague, C. T. *et al.* Congenital leptin deficiency is associated with severe early-onset obesity in humans. *Nature* **387,** 903–908 (1997).

68. Farooqi, I. S. *et al.* Effects of recombinant leptin therapy in a child with congenital leptin deficiency. *N. Engl. J. Med.* **341,** 879–884 (1999).

69. Farooqi, I. S. *et al.* Beneficial effects of leptin on obesity, T cell hyporesponsiveness, and neuroendocrine/metabolic dysfunction of human congenital leptin deficiency. *J. Clin. Invest.* **110,** 1093–1103 (2002).

70. Von Schnurbein, J. *et al.* Leptin substitution results in the induction of menstrual cycles in an adolescent with leptin deficiency and hypogonadotropic hypogonadism. *Horm. Res. Pædiatrics* **77,** 127–133 (2012).

71. Clément, K. *et al.* A mutation in the human leptin receptor gene causes obesity and pituitary dysfunction. *Nature* **392,** 398–401 (1998).

72. Strobel, A., Issad, T., Camoin, L., Ozata, M. & Strosberg, A. D. A leptin missense mutation associated with hypogonadism and morbid obesity. *Nat. Genet.* **18,** 213–215 (1998).

73. Farooqi, I. S. *et al.* Clinical and Molecular Genetic Spectrum of Congenital Deficiency of the Leptin Receptor. *N. Engl. J. Med.* **356,** 237–247 (2007).

74. Nizard, J., Dommergues, M. & Clément, K. Pregnancy in a Woman with a Leptin-Receptor Mutation. *N. Engl. J. Med.* **366,** 1064–1065 (2012).

75. Chung, W. K. *et al.* Heterozygosity forLepob orLeprdb affects body composition and leptin homeostasis in adult mice. *Am. J. Physiol. - Regul. Integr. Comp. Physiol.* **274,** R985–R990 (1998).

76. Ioffe, E., Moon, B., Connolly, E. & Friedman, J. M. Abnormal regulation of the leptin gene in the pathogenesis of obesity. *Proc. Natl. Acad. Sci. U. S. A.* **95,** 11852–11857 (1998).

77. Musso, C. *et al.* The long-term effect of recombinant methionyl human leptin therapy on hyperandrogenism and menstrual function in female and pituitary function in male and female hypoleptinemic lipodystrophic patients. *Metabolism* **54,** 255–263 (2005).

78. Oral, E. A. *et al.* Effect of Leptin Replacement on Pituitary Hormone Regulation in Patients with Severe Lipodystrophy. *J. Clin. Endocrinol. Metab.* **87,** 3110–3117 (2002).

79. Maguire, M., Lungu, A., Gorden, P., Cochran, E. & Stratton, P. Pregnancy in a Woman With Congenital Generalized Lipodystrophy. *Obstet. Gynecol.* **119,** 452–455 (2012).

80. Chan, J. L., Heist, K., DePaoli, A. M., Veldhuis, J. D. & Mantzoros, C. S. The role of falling leptin levels in the neuroendocrine and metabolic adaptation to short-term starvation in healthy men. *J. Clin. Invest.* **111,** 1409–1421 (2003).

81. Welt, C. K. *et al.* Recombinant Human Leptin in Women with Hypothalamic Amenorrhea. *N. Engl. J. Med.* **351,** 987–997 (2004).

82. Tartaglia, L. A. *et al.* Identification and expression cloning of a leptin receptor, OB-R. *Cell* **83,** 1263–1271 (1995).

83. Chua, S. C. *et al.* Transgenic complementation of leptin receptor deficiency.

II. Increased leptin receptor transgene dose effects on obesity/diabetes and fertility/lactation in lepr-db/db mice. *Am. J. Physiol. - Endocrinol. Metab.* **286,** E384–E392 (2004).

84. Robertson, S. *et al.* Insufficiency of Janus Kinase 2-Autonomous Leptin Receptor Signals for Most Physiologic Leptin Actions. *Diabetes* **59,** 782–790 (2010).
85. Banks, A. S., Davis, S. M., Bates, S. H. & Myers, M. G. Activation of Downstream Signals by the Long Form of the Leptin Receptor. *J. Biol. Chem.* **275,** 14563–14572 (2000).
86. Bjørbæk, C. *et al.* Divergent Roles of SHP-2 in ERK Activation by Leptin Receptors. *J. Biol. Chem.* **276,** 4747–4755 (2001).
87. Bjørbæk, C. *et al.* SOCS3 Mediates Feedback Inhibition of the Leptin Receptor via Tyr985. *J. Biol. Chem.* **275,** 40649–40657 (2000).
88. Björnholm, M. *et al.* Mice lacking inhibitory leptin receptor signals are lean with normal endocrine function. *J. Clin. Invest.* **117,** 1354–1360 (2007).
89. Patterson, C. M. *et al.* Leptin action via LepR-b Tyr1077 contributes to the control of energy balance and female reproduction. *Mol. Metab.* **1,** 61–69 (2012).
90. Bates, S. H. *et al.* STAT3 signalling is required for leptin regulation of energy balance but not reproduction. *Nature* **421,** 856–859 (2003).
91. Bates, S. H. *et al.* LRb-STAT3 Signaling Is Required for the Neuroendocrine Regulation of Energy Expenditure by Leptin. *Diabetes* **53,** 3067–3073 (2004).
92. Balthasar, N. *et al.* Leptin Receptor Signaling in POMC Neurons Is Required for Normal Body Weight Homeostasis. *Neuron* **42,** 983–991 (2004).
93. Bingham, N. C., Anderson, K. K., Reuter, A. L., Stallings, N. R. & Parker, K. L. Selective loss of leptin receptors in the ventromedial hypothalamic nucleus results in increased adiposity and a metabolic syndrome. *Endocrinology* **149,** 2138–2148 (2008).
94. Cohen, P. *et al.* Selective deletion of leptin receptor in neurons leads to obesity. *J. Clin. Invest.* **108,** 1113–1121 (2001).
95. Dhillon, H. *et al.* Leptin Directly Activates SF1 Neurons in the VMH, and This Action by Leptin Is Required for Normal Body-Weight Homeostasis. *Neuron* **49,** 191–203 (2006).
96. Kowalski, T. J., Liu, S.-M., Leibel, R. L. & Chua, S. C. Transgenic Complementation of Leptin-Receptor Deficiency I. Rescue of the Obesity/Diabetes Phenotype of LEPR-Null Mice Expressing a LEPR-B Transgene. *Diabetes* **50,** 425–435 (2001).
97. De Luca, C. *et al.* Complete rescue of obesity, diabetes, and infertility in db/db mice by neuron-specific LEPR-B transgenes. *J. Clin. Invest.* **115,**

3484–3493 (2005).

98. Elmquist, J. K., Bjørbæk, C., Ahima, R. S., Flier, J. S. & Saper, C. B. Distributions of leptin receptor mRNA isoforms in the rat brain. *J. Comp. Neurol.* **395,** 535–547 (1998).

99. Leshan, R. L., Björnholm, M., Münzberg, H. & Myers, M. G., Jr. Leptin receptor signaling and action in the central nervous system. *Obesity* **14,** 208S–212S (2006).

100. Scott, M. M. *et al.* Leptin targets in the mouse brain. *J. Comp. Neurol.* **514,** 518–532 (2009).

101. Patterson, C. M., Leshan, R. L., Jones, J. C. & Myers Jr., M. G. Molecular mapping of mouse brain regions innervated by leptin receptor-expressing cells. *Brain Res.* **1378,** 18–28 (2011).

102. Finn, P. D. *et al.* The Stimulatory Effect of Leptin on the Neuroendocrine Reproductive Axis of the Monkey. *Endocrinology* **139,** 4652–4662 (1998).

103. Louis, G. W. *et al.* Molecular Mapping of the Neural Pathways Linking Leptin to the Neuroendocrine Reproductive Axis. *Endocrinology* **152,** 2302–2310 (2011).

104. Quennell, J. H. *et al.* Leptin Indirectly Regulates Gonadotropin-Releasing Hormone Neuronal Function. *Endocrinology* **150,** 2805–2812 (2009).

105. Smith, J. T., Acohido, B. V., Clifton, D. K. & Steiner, R. A. KiSS-1 Neurones Are Direct Targets for Leptin in the ob/ob Mouse. *J. Neuroendocrinol.* **18,** 298–303 (2006).

106. Donato, J. *et al.* Leptin's effect on puberty in mice is relayed by the ventral premammillary nucleus and does not require signaling in Kiss1 neurons. *J. Clin. Invest.* **121,** 355–368 (2011).

107. Canteras, N. S., Simerly, R. B. & Swanson, L. W. Projections of the ventral premammillary nucleus. *J. Comp. Neurol.* **324,** 195–212 (1992).

108. Rondini, T., Baddini, S., Sousa, L., Bittencourt, J. & Elias, C. Hypothalamic cocaine- and amphetamine-regulated transcript neurons project to areas expressing gonadotropin releasing hormone immunoreactivity and to the anteroventral periventricular nucleus in male and female rats. *Neuroscience* **125,** 735–748 (2004).

109. Donato, J. *et al.* The Ventral Premammillary Nucleus Links Fasting-Induced Changes in Leptin Levels and Coordinated Luteinizing Hormone Secretion. *J. Neurosci.* **29,** 5240–5250 (2009).

110. Leshan, R. L. *et al.* Direct Innervation of GnRH Neurons by Metabolic- and Sexual Odorant-Sensing Leptin Receptor Neurons in the Hypothalamic Ventral Premammillary Nucleus. *J. Neurosci.* **29,** 3138–3147 (2009).

111. Donato Jr., J. *et al.* Lesions of the ventral premammillary nucleus disrupt the dynamic changes in Kiss1 and GnRH expression characteristic of the

proestrus–estrus transition. *Neuroscience* **241,** 67–79

112. Leshan, R. L., Greenwald-Yarnell, M., Patterson, C. M., Gonzalez, I. E. & Jr, M. G. M. Leptin action through hypothalamic nitric oxide synthase-1-expressing neurons controls energy balance. *Nat. Med.* **18,** 820–823 (2012).

113. Ahima, R. S., Saper, C. B., Flier, J. S. & Elmquist, J. K. Leptin Regulation of Neuroendocrine Systems. *Front. Neuroendocrinol.* **21,** 263–307 (2000).

114. Friedman, J. M. & Halaas, J. L. Leptin and the regulation of body weight in mammals. *Nature* **395,** 763–770 (1998).

115. Farooqi, I. S. & O'Rahilly, S. Leptin: a pivotal regulator of human energy homeostasis. *Am. J. Clin. Nutr.* **89,** 980S–984S (2009).

116. Ring, L. E. & Zeltser, L. M. Disruption of hypothalamic leptin signaling in mice leads to early-onset obesity, but physiological adaptations in mature animals stabilize adiposity levels. *J. Clin. Invest.* **120,** 2931–2941 (2010).

117. Hayes, M. R. *et al.* Endogenous Leptin Signaling in the Caudal Nucleus Tractus Solitarius and Area Postrema Is Required for Energy Balance Regulation. *Cell Metab.* **11,** 77–83 (2010).

118. Scott, M. M., Williams, K. W., Rossi, J., Lee, C. E. & Elmquist, J. K. Leptin receptor expression in hindbrain Glp-1 neurons regulates food intake and energy balance in mice. *J. Clin. Invest.* **121,** 2413–2421 (2011).

119. Fulton, S. *et al.* Leptin Regulation of the Mesoaccumbens Dopamine Pathway. *Neuron* **51,** 811–822 (2006).

120. Hommel, J. D. *et al.* Leptin Receptor Signaling in Midbrain Dopamine Neurons Regulates Feeding. *Neuron* **51,** 801–810 (2006).

121. Liu, J., Perez, S. M., Zhang, W., Lodge, D. J. & Lu, X.-Y. Selective deletion of the leptin receptor in dopamine neurons produces anxiogenic-like behavior and increases dopaminergic activity in amygdala. *Mol. Psychiatry* **16,** 1024–1038 (2011).

122. Leshan, R. L. *et al.* Ventral Tegmental Area Leptin Receptor Neurons Specifically Project to and Regulate Cocaine- and Amphetamine-Regulated Transcript Neurons of the Extended Central Amygdala. *J. Neurosci.* **30,** 5713–5723 (2010).

123. Coppari, R. *et al.* The hypothalamic arcuate nucleus: A key site for mediating leptin's effects on glucose homeostasis and locomotor activity. *Cell Metab.* **1,** 63–72 (2005).

124. Canteras, N. S., Simerly, R. B. & Swanson, L. W. Organization of projections from the medial nucleus of the amygdala: A PHAL study in the rat. *J. Comp. Neurol.* **360,** 213–245 (1995).

125. Cavalcante, J. C., Sita, L. V., Mascaro, M. B., Bittencourt, J. C. & Elias, C. F. Distribution of urocortin 3 neurons innervating the ventral premammillary nucleus in the rat brain. *Brain Res.* **1089,** 116–125 (2006).

126. Hahn, J. D. & Coen, C. W. Comparative study of the sources of neuronal projections to the site of gonadotrophin-releasing hormone perikarya and to the anteroventral periventricular nucleus in female rats. *J. Comp. Neurol.* **494,** 190–214 (2006).

127. McMinn, J. E. *et al.* An allelic series for the leptin receptor gene generated by CRE and FLP recombinase. *Mamm. Genome* **15,** 677–685 (2004).

128. Backholer, K. *et al.* Kisspeptin cells in the ewe brain respond to leptin and communicate with neuropeptide Y and proopiomelanocortin cells. *Endocrinology* **151,** 2233–2243 (2010).

129. Luque, R. M., Kineman, R. D. & Tena-Sempere, M. Regulation of hypothalamic expression of KiSS-1 and GPR54 genes by metabolic factors: analyses using mouse models and a cell line. *Endocrinology* **148,** 4601–4611 (2007).

130. Wahab, F., Ullah, F., Chan, Y.-M., Seminara, S. B. & Shahab, M. Decrease in hypothalamic Kiss1 and Kiss1r expression: a potential mechanism for fasting-induced suppression of the HPG axis in the adult male rhesus monkey (Macaca mulatta). *Horm. Metab. Res.* **43,** 81–85 (2011).

131. Thornton, J. E., Cheung, C. C., Clifton, D. K. & Steiner, R. A. Regulation of Hypothalamic Proopiomelanocortin mRNA by Leptin in ob/ob Mice. *Endocrinology* **138,** 5063–5066 (1997).

132. Vong, L. *et al.* Leptin Action on GABAergic Neurons Prevents Obesity and Reduces Inhibitory Tone to POMC Neurons. *Neuron* **71,** 142–154 (2011).

133. Bates, S. H., Kulkarni, R. N., Seifert, M. & Myers, M. G., Jr. Roles for leptin receptor/STAT3-dependent and -independent signals in the regulation of glucose homeostasis. *Cell Metab.* **1,** 169–178 (2005).

134. Ahima, R. S., Prabakaran, D. & Flier, J. S. Postnatal leptin surge and regulation of circadian rhythm of leptin by feeding. Implications for energy homeostasis and neuroendocrine function. *J. Clin. Invest.* **101,** 1020–1027 (1998).

135. Herberg, L. & Kley, H. K. Adrenal function and the effect of a high-fat diet on C57BL/6J and C57BL/6J-ob/ob mice. *Horm. Metab. Res.* **7,** 410–415 (1975).

136. Tokuyama, K. & Himms-Hagen, J. Increased sensitivity of the genetically obese mouse to corticosterone. *Am. J. Physiol.* **252,** E202–208 (1987).

137. York, D. A., Otto, W. & Taylor, T. G. Thyroid status of obese (ob/ob) mice and its relationship to adipose tissue metabolism. *Comp. Biochem. Physiol. B* **59,** 59–65 (1978).

138. Frisch, R. E. & McArthur, J. W. Menstrual cycles: fatness as a determinant of minimum weight for height necessary for their maintenance or onset. *Science* **185,** 949–951 (1974).

139. Ahima, R. S. *et al.* Role of leptin in the neuroendocrine response to fasting.

Nature **382,** 250–252 (1996).

140. Oakley, A. E., Clifton, D. K. & Steiner, R. A. Kisspeptin Signaling in the Brain. *Endocr. Rev.* **30,** 713–743 (2009).

141. De Croft, S. *et al.* Spontaneous Kisspeptin Neuron Firing in the Adult Mouse Reveals Marked Sex and Brain Region Differences but No Support for a Direct Role in Negative Feedback. *Endocrinology* **153,** 5384–5393 (2012).

142. Feng, Y., Manka, D., Wagner, K.-U. & Khan, S. A. Estrogen receptor-α expression in the mammary epithelium is required for ductal and alveolar morphogenesis in mice. *Proc. Natl. Acad. Sci.* **104,** 14718–14723 (2007).

143. Soliman, G. A. *et al.* A simple qPCR-based method to detect correct insertion of homologous targeting vectors in murine ES cells. *Transgenic Res.* **16,** 665–670 (2007).

144. Williams, K. W. *et al.* The Acute Effects of Leptin Require PI3K Signaling in the Hypothalamic Ventral Premammillary Nucleus. *J. Neurosci.* **31,** 13147–13156 (2011).

145. Caligioni, C. Assessing Reproductive Status/Stages in Mice. *Curr. Protoc. Neurosci.* **48,** A.4I.1–A.4I.8 (2009).

146. Lee, K., Porteous, R., Campbell, R. E., Lüscher, B. & Herbison, A. E. Knockdown of GABA(A) receptor signaling in GnRH neurons has minimal effects upon fertility. *Endocrinology* **151,** 4428–4436 (2010).

147. Navarro, V. M. *et al.* Advanced vaginal opening and precocious activation of the reproductive axis by KiSS-1 peptide, the endogenous ligand of GPR54. *J. Physiol.* **561,** 379–386 (2004).

148. Nikaido, Y. *et al.* Effects of prepubertal exposure to xenoestrogen on development of estrogen target organs in female CD-1 mice. *Vivo Athens Greece* **19,** 487–494 (2005).

149. Nah, W. H., Park, M. J. & Gye, M. C. Effects of early prepubertal exposure to bisphenol A on the onset of puberty, ovarian weights, and estrous cycle in female mice. *Clin. Exp. Reprod. Med.* **38,** 75–81 (2011).

150. Singh, S. P. *et al.* Impaired Estrogen Feedback and Infertility in Female Mice with Pituitary-Specific Deletion of Estrogen Receptor Alpha (ESR1). *Biol. Reprod.* **81,** 488–496 (2009).

151. Gieske, M. C. *et al.* Pituitary Gonadotroph Estrogen Receptor-α Is Necessary for Fertility in Females. *Endocrinology* **149,** 20–27 (2008).

152. Lindzey, J. *et al.* Effects of Castration and Chronic Steroid Treatments on Hypothalamic Gonadotropin-Releasing Hormone Content and Pituitary Gonadotropins in Male Wild-Type and Estrogen Receptor-α Knockout Mice. *Endocrinology* **139,** 4092–4101 (1998).

153. Ramirez, V. D. & McCann, S. M. Inhibitory Effect of Testosterone on Luteinizing Hormone Secretion in Immature and Adult Rats. *Endocrinology*

76, 412–417 (1965).

154. Kauffman, A. S., Navarro, V. M., Kim, J., Clifton, D. K. & Steiner, R. A. Sex differences in the regulation of Kiss1/NKB neurons in juvenile mice: implications for the timing of puberty. *Am. J. Physiol. Endocrinol. Metab.* **297,** E1212–1221 (2009).

155. Vidal, O. *et al.* Estrogen receptor specificity in the regulation of skeletal growth and maturation in male mice. *Proc. Natl. Acad. Sci.* **97,** 5474–5479 (2000).

156. Heine, P. A., Taylor, J. A., Iwamoto, G. A., Lubahn, D. B. & Cooke, P. S. Increased adipose tissue in male and female estrogen receptor-alpha knockout mice. *Proc. Natl. Acad. Sci. U. S. A.* **97,** 12729–12734 (2000).

157. Lee, K.-H. *et al.* Estrogen Receptor α Has a Functional Role in the Mouse Rete Testis and Efferent Ductules. *Biol. Reprod.* **63,** 1873–1880 (2000).

158. Eddy, E. M. *et al.* Targeted disruption of the estrogen receptor gene in male mice causes alteration of spermatogenesis and infertility. *Endocrinology* **137,** 4796–4805 (1996).

159. Yang, L. *et al.* Role of dorsomedial hypothalamic neuropeptide Y in modulating food intake and energy balance. *J. Neurosci. Off. J. Soc. Neurosci.* **29,** 179–190 (2009).

160. Bi, S., Kim, Y. J. & Zheng, F. Dorsomedial hypothalamic NPY and energy balance control. *Neuropeptides* **46,** 309–314 (2012).

161. Zhu, G., Yan, J., Smith, W. W., Moran, T. H. & Bi, S. Roles of dorsomedial hypothalamic cholecystokinin signaling in the controls of meal patterns and glucose homeostasis. *Physiol. Behav.* **105,** 234–241 (2012).

162. Bartness, T. J. A potential link between dorsomedial hypothalamic nucleus NPY and energy balance. *Cell Metab.* **13,** 493–494 (2011).

163. Chao, P.-T., Yang, L., Aja, S., Moran, T. H. & Bi, S. Knockdown of NPY Expression in the Dorsomedial Hypothalamus Promotes Development of Brown Adipocytes and Prevents Diet-Induced Obesity. *Cell Metab.* **13,** 573–583 (2011).

164. Schwartz, M. W., Woods, S. C., Porte, D., Seeley, R. J. & Baskin, D. G. Central nervous system control of food intake. *Nature* **404,** 661–671 (2000).

165. Schwartz, M. W. *et al.* Leptin increases hypothalamic pro-opiomelanocortin mRNA expression in the rostral arcuate nucleus. *Diabetes* **46,** 2119–2123 (1997).

166. Schwartz, M. W. *et al.* Specificity of leptin action on elevated blood glucose levels and hypothalamic neuropeptide Y gene expression in ob/ob mice. *Diabetes* **45,** 531–535 (1996).

167. Shutter, J. R. *et al.* Hypothalamic expression of ART, a novel gene related to agouti, is up-regulated in obese and diabetic mutant mice. *Genes Dev.* **11,**

593–602 (1997).

168. Ollmann, M. M. *et al.* Antagonism of central melanocortin receptors in vitro and in vivo by agouti-related protein. *Science* **278,** 135–138 (1997).