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The Role of Phosophoinositide 3-Kinase in Regulating Kisspeptin Neurons Within the Hypothalamic-Pituitary-Gonadal Axis

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Abstract of the Dissertation

The Role of Phosophoinositide 3-Kinase in Regulating Kisspeptin Neurons within the Hypothalamic-Pituitary-Gonadal Axis

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Kisspeptin neurons are potent regulators of gonadotropin-releasing hormone (GnRH) neurons, necessary for pubertal development and adult reproductive competence. Estradiol (E_2) and testosterone (T), along with peripheral metabolic signals, such as leptin and insulin, regulate GnRH release indirectly, in part through the activation or inhibition of kisspeptin neurons. The changes in GnRH release and consequently in gonadotropin levels caused by these peripheral signals are strongly linked to changes in hypothalamic kisspeptin (*Kiss1*) gene expression. However, little is known about the intracellular signaling pathways that regulate *Kiss1*. The phospholipid enzyme phosphoinositide 3-kinase (PI3K) is a major downstream effector of steroid hormones and metabolic cues. We hypothesize that PI3K in kisspeptin neurons integrates peripheral signals to regulate *Kiss1* expression, thus altering GnRH release. To test our hypothesis, we used transgenic mice and Cre-LoxP technology to delete PI3K catalytic subunits,

p110α and p110β, in kisspeptin cells (Kiss-p110α/β KO). Ablation of PI3K from kisspeptin neurons did not alter overall pubertal development in either male or female mice. In adult animals, LH levels were lower in Kiss-p110α/β KO male mice regardless of gonadal status when compared to wild type. However, basal LH levels in Kiss-p110α/β KO female mice were not significantly different from wild type. We also examined *Kiss1* mRNA expression among groups of gonad-intact, gonadectomized (GDX), and GDX + steroid-replaced mice. Compared to wild type, *Kiss1* cell number was lower in the anteroventral periventricular nucleus (AVPV) of intact Kiss-p110α/β KO males. In the arcuate (ARC) nucleus KO males showed lower *Kiss1* cell numbers regardless of gonadal status. A significant genotype effect was observed in the ARC of females from the GDX group, but not in the intact or in the E_2-replaced group. In addition, compared to wild type, a substantial reduction in kisspeptin immunoreactivity was observed in the ARC of intact adult Kiss-p110α/β KO males and females. Our data supports a key role of PI3K signaling in the regulation of *Kiss1* expression in both sexes and LH levels in males. These studies have the potential to identify novel therapeutic targets to treat reproductive disorders of unknown etiology.

Dedication Page

I would like to dedicate my dissertation work to my mother, Sheryl Yates, and father, John Beymer, who have always been supportive in my pursuit of higher education and have helped me through many of the rough times I've had in graduate school. I could not have accomplished this work without their continued love and support.

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List of Abbreviations

AgRP – Agouti Related Protein

AR – Androgen Receptor

ARC – Arcuate

ArKO – Aromatase Knockout

AVPV – Anteroventral Periventricular

ChIP – Chromatin Immunoprecipitation

cKO – Conditional Knockout

CUX1 – Cut-Like Homeobox 1

DNA – Deoxyribonucleic Acid

E – Estrogen

E_2 – Estradiol

EAP1 – Enhanced at Puberty 1

EED – Embryonic Ectoderm Development

ERα – Estrogen Receptor Alpha

ERE – Estrogen Responsive Element

EYFP – Enhanced Yellow Fluorescent Protein

FACS – Fluorescence-Associated Cell Sorting

FSH – Follicle-Stimulating Hormone

GABA – Gamma-Amino butyric Acid

GALP – Galanin-Like Peptide

GDX – Gonadectomy

GFP – Green Fluorescent Protein

GnRH – Gonadotropin-Releasing Hormone

HFD – High Fat Diet

HPG – Hypothalamic-Pituitary-Gonadal

IGF-1 – Insulin-Like Growth Factor 1

IR – Insulin Receptor

ISH – In Situ Hybridization

KERKO – Kisspeptin-Specific Estrogen Receptor Alpha Knockout

LH – Luteinizing Hormone

MBH – Mediobasal Hypothalamus

ME – Median Eminence

mRNA – Messenger Ribonucleic Acid

NPY – Neuropeptide Y

OVX – Ovariectomy

PcG – Polycomb Group

PH – Pleckstrin Homology

PI3K – Phosphoinositide 3-Kinase

PIP2 – Phosphatidylinositol 4,5-bisphosphate

PIP3 – Phosphatidylinositol 3,4,5-bisphosphate

PND – Post-natal Day

POA – Preoptic Area

POMC – Proopiomelanocortin

RIA – Radioimmunoassay

RTK – Receptor Tyrosine Kinase

SH2 – Src-Homology 2

T – Testosterone

TTF-1 – Thyroid Transcription Factor 1

WT – Wild type

YY1 – Ying Yang 1

Chapter 1

THE REPRODUCTIVE AXIS IN MAMMALS

REPRODUCTION IN MAMMALS:

In mammals, proper control of reproduction is essential for the successful propagation of the species. All mammals must reach sexual maturity and have adequate nutrients and energy stores in order to effectively generate and maintain offspring (Hill *et al.* 2008). The hypothalamic-pituitary-gonadal (HPG) axis is comprised of the hypothalamus, the pituitary gland, and the respective sex specific gonads, testes or ovaries (Clarke *et al.* 2011). Many peripheral and central factors are involved in both the inhibition and activation of the HPG axis (Clarke 2011). For example, the stress hormone cortisol suppresses the HPG axis leading to a decrease in luteinizing hormone (LH) release (Oakley *et al.* 2009; Breen *et al.* 2012; Chabbi & Ganesh 2014). While metabolic hormones such as insulin and leptin increase the expression of hypothalamic factors leading to activation of the HPG axis (Zieba *et al.* 2005; Smith *et al.* 2006a). Perturbations in detecting these hormones or deficiencies in the hormones themselves, such as in *ob/ob* mice, which are deficient for leptin, can lead to precocity, delay, or even absence of sexual maturity, which can disrupt the ability of the organism to mate and produce offspring (Smith *et al.* 2006a).

The master neuronal gatekeeper of the HPG axis is the gonadotropin-releasing hormone (GnRH) network (Tsutsumi & Webster 2009). These important cells reside in the rostral aspect

of the preoptic area in many mammalian species (Salvi *et al.* 2009). GnRH neurons send projections to the median eminence (ME), which is highly vascularized by the hypophysial portal system. These neurons release GnRH into this circulation and it is carried to the anterior lobe of the pituitary where it binds its cognate receptor on gonadotrope cells. The gonadotropes in turn, respond to pulses of GnRH by releasing luteinizing hormone (LH) and follicle-stimulating hormone (FSH) (Thompson & Kaiser 2014). LH and FSH act on cells in both the ovaries and testes to induce the production of the steroid hormones estrogen (E) and testosterone (T), respectively, as well as to regulate and control spermatogenesis in males as well as oogenesis and ovulation in females (Michael *et al.* 1980; Hunzicker-Dunn & Maizels 2006). Sex hormones, E and T, circulate throughout the periphery and regulate and control many developmental as well as normal physiological functions (Semaan & Kauffman 2010). Estradiol (E_2) and T also act on the central nervous system in a feedback loop regulating activation or inhibition of the GnRH network (Zhang *et al.* 2013).

The ease of hormonal and genetic manipulation as well as their rapid development has made rodents an extremely attractive model to use in the study of puberty and fertility. The rodent estrous cycle differs from that of non-human primates and humans in that in rodents the cycle consists of 4 stages: diestrus, metestrus, proestrus, and estrus, where as non-human primates have only two stages, the luteal and follicular phases. Diestrus and metestrus stages are characterized by low circulating levels of LH, FSH, and E_2 (Nelson *et al.* 1992). The proestrus stage consists of an E surge during the late morning, which signals the brain to activate GnRH neurons (Bronson & Saal 1979). These neurons then signal to the pituitary to cause the pre-ovulatory LH surge. This large amount of LH circulates through the periphery to the ovaries to

cause ovulation during the morning of estrous, the final stage of the cycle (Clifton & Sawyer 1979).

In order to determine the different stages of the estrous cycle, vaginal smears can be taken and the cytology of each smear indicates the current stage of the cycle of the animal (Allen 1922; Nelson *et al.* 1982). During diestrus and metestrus leukocytes are predominant in the smear. In the proestrus stage increasing E induces the vaginal epithelium to proliferate causing larger nucleated cells to predominate during this stage (Nelson *et al.* 1982). Finally, during the day of estrous the outermost epithelial layers cornify, losing their nuclei before being sloughed off (Nelson *et al.* 1982).

DEVELOPMENT OF THE HPG AXIS:

Sex differences in brain morphology and connectivity are essential for setting up the differences in sexual behavior and reproductive physiology observed in adulthood (McCarthy 2010). Steroid hormones released from the gonads during early development are potent mediators of how these sex differences are established in both the brain and throughout the periphery. The hypothalamus, a region often studied when looking at sexual differentiation, contains many populations of neurons including those important for the regulation of reproductive capacity. One of these, the kisspeptin neuron, is an upstream regulator of the gonadotropin-releasing hormone/luteinizing hormone (GnRH/LH) pulse system (Semaan *et al.* 2012).

During perinatal development two peaks of T occur in males (Wu *et al.* 2009; Mong & McCarthy 2012). The first occurs during the late gestational period around embryonic day 18

and the second occurs during the first four hours after parturition (Lenz & McCarthy 2010). These surges are thought to be the basis of T's organizational effects on sexual differentiation within the brain (Lenz & McCarthy 2010). The circulating T is locally converted to E_2 by the enzyme aromatase (McCarthy & Arnold 2011). One important action of E_2 is in promoting sex differences in cell number in certain brain regions. For example, the presence of T derived E_2 leads to reduced numbers of tyrosine hydroxylase expressing neurons, including neurons that express kisspeptin (Clarkson & Herbison 2011; McCarthy & Arnold 2011). However, mouse models in which deletions for estrogen receptor alpha (ERα) and androgen receptor (AR) have been made, show that T itself can exert certain effects on sexual differentiation in the male brain (Table 1).

Since, GnRH neurons are the gatekeepers of HPG axis function it would be tempting to assume that steroid hormones act directly on GnRH neurons themselves to regulate puberty and fertility in mammals. However, GnRH neurons do not express ERα or AR as well as other hormone receptors known to regulate the HPG axis (Radovick *et al.* 2012). This opens the possibility that afferent neurons expressing receptors for different peripheral signals, such as steroid hormones, transmit this information to GnRH neurons. Populations of neurons that express the decapeptide, kisspeptin, reside within the mediobasal hypothalamus and are in a prime position to relay information from the periphery to the population of GnRH neurons.

Species Specific Initiation of The HPG Axis During Development

In mammals, pubertal onset is marked by the activation of the pulsatile GnRH/LH system (Ojeda *et al.* 2006a). However, in recent years it has come to light that the underlying mechanisms involved in the activation of the GnRH system is species specific (Terasawa &

Kurian 2012). In rodents, a shift in the response to gonadal steroid hormones appears to trigger the initiation of the pulsatile GnRH system, which is the hallmark of pubertal onset (Ojeda *et al.* 2006b). In contrast, it appears that the initiation of puberty in non-human primates is due primarily to a release of tonic central inhibition independent of responses to gonadal derived steroid hormones (Terasawa & Kurian 2012). This central inhibition in primates is thought to be due to inhibition of Kisspeptin and GnRH by γ-amino butyric acid (GABA) producing afferents (Terasawa 2005). GABA is a key inhibitory neurotransmitter produced in the central nervous system of mammals (Varani *et al.* 2014). In non-human primates, GABA levels in the ME, where GnRH axonal terminals are found, of pre-pubertal monkeys are much higher than in mid-pubertal monkeys (Mitsushima *et al.* 1994). Furthermore, central infusion of GABA antagonist, bicuculline, advances the timing of pubertal onset in female monkeys (Keen *et al.* 1999). In pubertal female monkeys OVX increases pulsatile GnRH release, but OVX has no effect on pulsatile GnRH release in pre-pubertal females (Guerriero *et al.* 2012). These data support the hypothesis that pubertal onset in non-human primates is controlled by a central steroid hormone-independent mechanism.

THE HYPOTHALMIC KISSPEPTIN SYSTEM:

The hypothalamic kisspeptin system relays and integrates important central and peripheral information to GnRH neurons (Popa *et al.* 2008). GnRH neurons express the receptor for kisspeptin (Kiss1R) and kisspeptin can potently stimulate GnRH gene expression as well as GnRH release (Irwig *et al.* 2004). In humans, inactivating or loss-of-function mutations of the Kiss1R gene result in hypogonadotropic hypogonadism which blocks pubertal onset leading to

adult infertility (de Roux *et al.* 2003; de Roux 2006; Cerrato & Seminara 2007). Mouse models such as *Kiss1* KO and Kiss1R KO recapitulate the phenotypes that are seen in humans (Seminara *et al.* 2003; Topaloglu *et al.* 2012). For example, both male and female Kiss1R KO have significant decreases in circulating gonadotropin levels and gonadal weight as well as major delays or absence of pubertal onset, which are all hallmarks of hypogonadotropic hypogonadism in humans (Seminara *et al.* 2003; Mei *et al.* 2011; Topaloglu *et al.* 2012). On the other hand, in humans gain-of-function mutations in Kiss1R can cause significant advancement in the timing of pubertal onset as well as fertility problems in adulthood (Teles *et al.* 2008). During the juvenile period approximately 90% of GnRH neurons express Kiss1R, gradually peaking towards adult levels during the peripubertal period (Herbison *et al.* 2010). This suggests that GnRH neurons may be poised and ready for activation by kisspeptin well in advance of pubertal onset. However, in prepubertal male mice, approximately 44% of GnRH neurons are activated by central Kisspeptin exposure as compared to 90% in adult male mice(Han *et al.* 2005). Also kisspeptin-immunoreactive (kiss-ir) fiber apposition onto GnRH neurons increases during the peripubertal period (Clarkson & Herbison 2006). These data suggest that not only is there increasing contact of kisspeptin projections onto GnRH neurons but that Kiss1R in GnRH neurons may become increasingly more sensitive to kisspeptin activation throughout puberty.

Two principal hypothalamic kisspeptin populations, linked to the regulation of puberty and LH release, have been described in the rodent brain: one in the anteroventral periventricular (AVPV) nucleus of the preoptic area (POA) and a second in the arcuate (ARC) nuclei of the mediobasal hypothalamus (MBH) (Smith *et al.* 2005a, b; Clarkson *et al.* 2009a). Sex differences exist in these kisspeptin populations within the brain; in the AVPV, females have approximately ten times more kisspeptin neurons than males (Clarkson *et al.* 2009a). This difference is, in part,

due to cell death caused by the presence of T derived E_2 in the brains of males but not females during perinatal period of development (McCarthy 2010). During postnatal development AVPV *Kiss1* expression is detectable two weeks postnatal and increases gradually throughout the peripubertal period reaching peak levels in mid-to-late puberty (Poling *et al.* 2012).

The AVPV kisspeptin population is involved in the E_2-induced pre-ovulatory LH surge that occurs exclusively in females through an E positive feedback mechanism (Fig. 1) (Smith *et al.* 2006b; Clarkson *et al.* 2008; Dror *et al.* 2013). Using ovariectomized mice, exogenous levels of E_2 mimicking those of proestrus, potently increased AVPV *Kiss1* expression and kisspeptin neuronal activity, as measured by in situ hybridization and c-fos colocalization, respectively (Goodman 1978; Robertson *et al.* 2009). Mice that lack Kiss1R do not show a pre-ovulatory LH surge in a similar paradigm (Dror *et al.* 2013). Furthermore, mice containing a mutant ERα ($ERα^{-/AA}$) unable to bind E-responsive elements (ERE) in DNA, but can act through a non-classical ER pathway, are devoid of *Kiss1* expression in the AVPV (Table 1) (McDevitt *et al.* 2007). In this model AVPV Kisspeptin neurons are completely unresponsive to E_2 treatment, which suggests the classical ERα pathway is essential to the normal development and activation of AVPV *Kiss1* neurons in female mice (McDevitt *et al.* 2008).

The ARC kisspeptin population on the other hand, is proposed to be the site of steroid negative feedback in the brain (Fig. 1). Administration of T or E_2 to gonadectomized male or female mice significantly decreases ARC *Kiss1* expression (Smith *et al.* 2005a, b). However, a negative effect of steroid hormones on *Kiss1* mRNA levels does not prove a role of ARC Kisspeptin neurons in negative feedback. In mice, in which a non-classical ERα pathway is preserved ($ERα^{-/AA}$) a decrease of *Kiss1* expression in the ARC is observed. However, E_2 still exerts a suppressive effect on ARC *Kiss1* in these animals (Table 1) (McDevitt *et al.* 2007). This

suggests that ERα uses a non-classical signaling pathway to regulate *Kiss1* expression in the ARC (Fig. 1). Using electrophysiological recordings of brain slices from Kiss-GFP reporter mice, de Croft and colleagues found that approximately ninety percent of kisspeptin neurons in the ARC of males show irregular firing. In contrast, the ARC Kisspeptin neurons of females remain mostly silent (de Croft *et al.* 2012). It is clear that the specific role of the ARC kisspeptin population in both males and females has not yet been elucidated. In adult rodents there is no detectable difference in the ARC kisspeptin population based on both *Kiss1* mRNA levels and kiss-ir. However, based on radioactive in situ hybridization for *Kiss1* mRNA there are transient differences seen in kisspeptin cell numbers during early development, with females having more kisspeptin neurons than males (Poling & Kauffman 2013). However, in contrast to the changes of AVPV *Kiss1* during peripubertal development, there appear to be no significant changes in ARC *Kiss1* during pubertal progression. Interestingly, kiss-ir fibers in the ARC increase across pubertal development, some of which originate from the AVPV, which supports the gradual increase in AVPV *Kiss1* expression seen during puberty.

REGULATION OF KISSPEPTIN IN THE HYPOTHALAMUS:

In the past decade kisspeptin has gone from being a newly discovered metastasis suppressor gene to being one of the leading topics of research in the field of reproductive neuroendocrinology. Even with this tremendous increase in knowledge of the physiological functions of kisspeptin, both in regards to tumor progression and reproductive physiology, very little is know about the specific intracellular molecular mechanisms which regulate the transcriptional activity of *Kiss1*.

Steroid Hormone Regulation of Kisspeptin:

It is well known that kisspeptin expression in the brain is regulated by several hormonal factors, including E and T (Ojeda *et al.* 2006b). However, the exact mechanisms by which these factors regulate *Kiss1* transcription have remained elusive. Knockout studies have greatly advanced the knowledge of kisspeptin regulation (Table 1). For example, work done by Mayer and colleagues using Kisspeptin-specific ERα KO (KERKO) mice showed that ERα in *Kiss1*-expressing neurons is necessary for the proper timing of pubertal onset as well as normal ovarian cyclicity (Mayer *et al.* 2010). Female KERKO mice showed advanced puberty onset and adult females have aberrant ovarian cyclicity (Mayer *et al.* 2010). These results are similar to other models such as mice deficient for *CYP19*, the gene encoding aromatase (ArKO) as well as mice globally deficient for ERα (ERα$^{-/-}$) (Table 1). In ArKO female mice, kiss-ir increased in the AVPV of males, but decreased in the AVPV of females, while ERα$^{-/-}$ female mice have no *Kiss1* expression in the AVPV and decreased *Kiss1* expression in the ARC (Clarkson *et al.* 2009b; Bakker *et al.* 2010). In mice deficient for GnRH (*hpg*) no difference in the kiss-ir or *Kiss1* mRNA expression of the AVPV between males and females is observed (Gill *et al.* 2010). It is clear that steroid hormones, especially E, are essentially for the proper development and normal expression of *Kiss1*. The mechanisms by which E regulates *Kiss1* expression through ERα may be mechanistically different, as mentioned previously. Thus more investigations on specific molecular mechanisms of steroid hormone action need to be done.

Epigenetic Regulation of Kisspeptin:

In the past two decades epigenetic regulation has become increasingly important in investigating gene regulation throughout development and into adulthood. Epigenetics refers to a

variety of modifications of histone proteins as well as of DNA itself. These modifications can make the DNA more or less accessible to transcription and to co-factors that bind to the gene promoter. Modifications include DNA methylation on the cytosine of CpG dinucleotides as well as modifications of histone protein tails, including acetylation and methylation. These modifications are performed by sets of enzymes recruited to specific gene loci depending on the complement of transcription and DNA-binding factors that associate with that particular locus. (For review see Jaenisch & Bird 2003 and Goldberg *et al.* 2007)

Recent studies have begun to elucidate epigenetic factors regulating *Kiss1* gene expression. For example, E_2-responsive histone modifications coincide with changes in *Kiss1* gene expression in the AVPV (Tomikawa *et al.* 2012). Using chromatin immunoprecipitation (ChIP) an increase in histone H3 acetylation, a marker of increased gene expression, was observed in the *Kiss1* promoter in the AVPV (Tomikawa *et al.* 2012). In the ARC, there was a decrease in histone H3 acetylation of the *Kiss1* promoter upon E_2 treatment (Tomikawa *et al.* 2012). There is also increased association of ERα with the *Kiss1* promoter using ChIP analysis in the AVPV after E_2 treatment (Tomikawa *et al.* 2012). In contrast, in the absence of E_2 ERα was found to be associated with the *Kiss1* promoter in OVX female mice (Tomikawa *et al.* 2012). This suggests that unliganded DNA bound ERα may have a role in regulating *Kiss1* in the ARC. These in part support findings in $ER\alpha^{-/AA}$ mice (Table 1) in which the response to E_2 of *Kiss1* expression in the ARC is maintained, suggesting that the regulation of *Kiss1* expression in the ARC could be through non-classical ERα signaling.

In a recent study, Ojeda and colleagues investigated the role of polycomb group (PcG) proteins in the timing and initiation of puberty (Lomniczi *et al.* 2013). Polycomb group proteins are chromatin-remodeling complexes that aid in the epigenetic silencing of genes. Two PcG

genes were found, Eed, which interacts with histone deacetylases and aids in gene silencing, and Cbx7, which is a putative recruiter of histone methyltransferases, were decreased during puberty in female rats (Lomniczi *et al.* 2013). The decreases in both genes were blocked when a DNA metyltransferase inhibitor was administered before pubertal onset (Lomniczi *et al.* 2013). The inhibition of Eed and Cbx7 expression during puberty might be a key to understanding the increase in genes important for pubertal initiation, namely *Kiss1* (Lomniczi *et al.* 2013). Interestingly, Eed becomes dissociated from the *Kiss1* promoter during pubertal progression (Lomniczi *et al.* 2013).

These studies show that epigenetic regulation and chromatin status are important factors for the proper pubertal and adult expression of *Kiss1* in the brain. However, which specific transcription factors regulate these epigenetic changes is still unknown. One recent study by Mueller and colleagues proposed that factors that are involved in controlling female puberty might regulate *Kiss1* expression (Mueller *et al.* 2011). For example several candidates were found to be recruited to the *Kiss1* promoter region including the transcription factors, CUX1, YY1, EAP1, and TTF1 (Mueller *et al.* 2011). Furthermore, using double-immunofluorescence in rat brain tissue, it was observed that all these transcription factors are co-expressed in *Kiss1* neurons in the rat hypothalamus (Mueller *et al.* 2011). Interestingly, many of these factors are typically seen in transcriptional repression. However, due to the fact that only *in vitro* cell lines were used, the functional relevance of these factors within kisspeptin neurons *in vivo* has yet to be determined.

It is clear that studies using *Kiss1* promoter constructs have begun to tease apart putative transcriptional regulators of kisspeptin, but further studies delineating the interactions between these and other transcription factors implicated in both pubertal onset as well as adult fertility

needs to be done. Many studies have used various cell lines of both human and non-human origin, however the results are difficult to resolve because of differing constituents of transcription factors in these various cell lines (Mueller *et al.* 2011; Kurian & Terasawa 2013; Semaan & Kauffman 2013). The technical difficulties faced when looking at the specific molecular mechanisms within kisspeptin neurons due to their intimate contact with other neurons in both the ARC and AVPV limit the breadth of acquirable knowledge that is needed to fully understand how physiological changes, such as alterations in sex steroid or metabolic signaling hormone availability, that are already known to regulate *Kiss1* and GnRH expression (Yeo 2013).

METABOLIC EFFECTS ON THE HPG AXIS:

Alterations in nutrient input are well known to effect the regulation of the HPG axis (Sullivan *et al.* 2003; Roa & Tena-Sempere 2010). Models that recapitulate parameters of under-nutrition, such as fasting, or over-nutrition, such as a high fat diet (HFD), are often used to investigate the interactions between metabolism and reproduction. For example, fasting has been shown to reduce serum LH levels and can prevent normal estrus cyclicity in the female mouse (Ahima *et al.* 1996; Smith *et al.* 2002). Fasting also decreases circulating leptin levels, and restoring leptin levels to normal by exogenous leptin administration can partially reverse the effects of fasting on LH levels and estrus cyclicity (Smith *et al.* 2002). Similar results of fasting on LH levels and cyclicity are seen across mammalian species (Smith *et al.* 2002; Jackson & Ahima 2006). Peripheral insulin increased serum LH levels. Female rats with streptozotocin-induced diabetes have a decrease in ovulation, LH surges, and reproductive behavior, namely

lordosis (Kovacs *et al.* 2003). Central insulin treatment can resolve the reproductive alterations seen in this model of diabetes. In the brain, GnRH neurons do not express the leptin receptor (LepR), however they do express both insulin receptor (IR) and insulin-like growth factor-1 receptor (IGF-1R) (DiVall *et al.* 2010).

As opposed to models of under-nutrition, models of over-nutrition can cause untimely overstimulation of the HPG axis. Compared to rats fed a lean diet, female rats fed a HFD after weaning have advanced pubertal onset as determined by vaginal opening. In rats that were fed a HFD at weaning advancement in increased LH pulse frequency associated with pubertal onset was observed (Li *et al.* 2012). *Kiss1* expression was increased at an earlier age in HFD fed rats as compared to rats fed the control diet (Quennell *et al.* 2011; Lie *et al.* 2013). HFD treatment also causes irregularities in ovarian cyclicity in female rats during adulthood, even after being returned to a control diet (Lie *et al.* 2013). These findings suggest that an increase in *Kiss1* expression may either contribute to, or coincide with, the advancement of pubertal onset seen in models of over-nutrition.

Based on the results from previous studies, kisspeptin neurons have been proposed as a relay conduit to convey metabolic information to the GnRH network. Approximately forty percent of ARC *Kiss1* neurons co-express leptin receptor (LepR), while no AVPV *Kiss1* neurons co-express LepR (Smith *et al.* 2006a). *Ob/ob* mice, which are leptin deficient, have decreased *Kiss1* expression in the ARC compared to WT (Smith *et al.* 2006a). Exogenous leptin administration partially rescues *Kiss1* expression in these mice suggesting leptin exerts a positive effect on kisspeptin expression (Donato *et al.* 2011). However, mice with kisspeptin-specific LepR KO show no significant effect on pubertal timing or ovarian cyclicity (Donato *et al.* 2011). These data suggest that although leptin may play a role in the regulation of *Kiss1* expression,

leptin signaling is not essential for the proper timing of pubertal onset. Furthermore, Cravo and colleagues recently showed that LepR null mice with kisspeptin-specific re-expression of LepR do not show significant *Kiss1* mRNA and LepR protein co-localization until sexual maturity has been reached (Cravo *et al.* 2013).

On the other hand, roughly twenty percent of ARC *Kiss1* neurons co-express insulin receptor (IR), while only three to five percent of AVPV *Kiss1*-expressing neurons co-express IR (Qiu *et al.* 2013). In contrast to mice with a Kisspeptin cell-specific deletion of LepR, Kisspeptin cell-specific IR KO female mice show a delay in vaginal opening and day of first estrus, both markers of pubertal onset. In addition, males with Kiss-IR KO have late sexual maturation measured by the age at which males were able to impregnate proven fertile female mice (Qiu *et al.* 2013). However, Kiss-IR KO mice remain fertile (Qiu *et al.* 2013).

Another peripheral signal known to regulate *Kiss1* is insulin-like growth factor-1 (IGF-1). Exogenous IGF-1 increased plasma LH levels in pre-pubertal female rats (Hiney *et al.* 2009). IGF-1 administration also increased *Kiss1* expression in the AVPV through activation of Akt. However, this activation was dependent on adequate E_2 levels (Hiney *et al.* 2010). Furthermore, treatment with exogenous IGF-1 receptor antagonist could suppress the E_2-induced LH surge (Quesada & Etgen 2002). These studies suggest that growth factors such as insulin, leptin, and IGF-1 may play an important role in the regulation of *Kiss1* expression and GnRH/LH secretion.

Additionally, ARC Kisspeptin neurons can regulate POMC and NPY activity. POMC neurons are directly excited by kisspeptin through the opening of non-selective cation and sodium/calcium exchange channels, while NPY/AgRP neurons are inhibited indirectly through enhancing GABA mediated inhibitory synaptic tone (Backholer *et al.* 2010; Fu & van den Pol

2010). These data support the hypothesis that metabolic factors, such as insulin, leptin, and IGF-1, may exert their influence on the reproductive axis through afferent projections, namely kisspeptin, to GnRH instead of directly on GnRH neurons themselves. Therefore, well-known downstream signaling cascades of these hormones, such as the PI3K pathway, in neurons that project to GnRH-expressing neurons should be the focus of future investigations. This is especially true for kisspeptin neurons, which are prime candidates for being major effectors of both metabolic and steroid hormone signaling in regulation of the HPG axis.

Another possible candidate for relaying metabolic information to the HPG axis are neurons that express galanin-like peptide (GALP). GALP neurons reside in the ARC and ME of the rodent brain, which are regions that are well known for their involvement in the control of both metabolic and reproductive homeostasis (Juréus *et al.* 2001; Takatsu *et al.* 2001; Takenoya *et al.* 2006). Like other hypothalamic neuropeptides implicated in both metabolism and reproduction, such as kisspeptin, GALP is a target for the metabolic hormones insulin and leptin (Gottsch *et al.* 2004). In models of altered nutrient intake such as nutrient deprivation a reduction in the expression of GALP is seen (Juréus *et al.* 2000). In these models exogenous leptin and insulin reverses the reduction of GALP mRNA levels. Models of obesity also show a decrease in *GALP* mRNA levels such as *ob/ob* mice with deficient leptin receptor signaling. In these mice, leptin treatment increases the number of GALP expressing cells in the ARC (Juresus 2001).

PI3K SIGNALING IN THE HYPOTHALAMUS:

Phosphoinositide 3-Kinase (PI3K) is a phospholipid enzyme that catalyzes the phosphorylation of phosphatidylinositol 4,5-biphosphate (PIP2) to generate phosphatidylinositol

3,4,5-triphosphate (PIP3) (Cantrell 2001; Cantley 2002; Vanhaesebroeck *et al.* 2005). Class IA PI3Ks are composed of a regulatory/adapter subunit (p85α, p85β, and p55γ) tightly associated with a catalytic subunit (p110α, p110β, and p110δ) (Cantley 2002). Upon binding external ligands, such as insulin, receptor tyrosine kinases (RTK), such as IR, autophosphorylate their tyrosine residues, producing binding sites for proteins containing Src-homology 2 (SH2) domains, such as the regulatory subunits of PI3K (Cantley 2002). This in turn brings the associated catalytic p110 subunits of PI3K to plasma membrane where it can catalyze the conversion of PIP2 into PIP3 (Vanhaesebroeck *et al.* 2005). PIP3 can then act as a second messenger lipid, recruiting a number of intracellular effectors to the plasma membrane (Carracedo & Pandolfi 2008). Effectors such as PDK1, Akt, Rho, and Ras, which all contain pleckstrin homology (PH) domains, that are able to bind to PIP3 (Cinar *et al.* 2007; Fan *et al.* 2012). This localizes these effectors to the plasma membrane where they subsequently become activated (Fan *et al.* 2012). Activation of PDK1 and Akt signaling within cells often leads to changes in cell survival, neuronal activity, and gene expression through interaction with such transcription factors as FoxO1 (Fabre *et al.* 2005; Belgardt *et al.* 2008) (Fig 2). Nutritional status, which is tightly coupled to reproductive maturation and fertility, is a known regulator of PI3K activity in discrete neuronal populations of the ARC (Acosta-Martinez 2011). PI3K is well known to be a major intracellular effector of leptin as well as insulin signaling (Al-Qassab *et al.* 2009). For example, both hormones can induce proopiomelanocortin (POMC) expression, a hypothalamic anorexigenic neuropeptide (Hill *et al.* 2009). Leptin can stimulate POMC neuron electrical activity through PI3K signaling because pharmacological inhibition of PI3K signaling prevents depolarization and increases POMC firing rate after leptin administration (Hill *et al.* 2008). Conversely, in neurons that express neuropeptide Y/agouti-related peptide (NPY/AgRP)

leptin inhibits expression of these transcripts through PI3K mediated actions (Morrison *et al.* 2005).

In addition to metabolic signals PI3K signaling also interacts with ERα and AR signaling in various systems. For example, the E_2-mediated organization of dendritic spine synapses of the developing rat hypothalamus requires a transcription-independent activation of PI3K (Lenz & McCarthy 2010). E_2 induces activation of PI3K through interaction with ERα. The increased PI3K activity causes a release of glutamate, which triggers the formation of dendritic spines in local neurons (Schwarz *et al.* 2008). Pharmacological inhibition of PI3K activity blocks this regional increase in dendritic spine density (Schwarz *et al.* 2008). This PI3K-dependent mechanism contributes to sex-specific sexual behavior and neuroendocrine function (Matsuda *et al.* 2012). Like ERα, AR can physically interact with the p85α regulatory subunit of PI3K up regulating the activity of Akt (Carver *et al.* 2011). Conversely, Akt activation increases expression of AR as well as AR cellular stability (Wang *et al.* 2007). AR directly interacts with Akt, albeit in *in vitro* models (Kang *et al.* 2004). Conversely, some studies have also shown repressive effects between the interactions of these two pathways. For example Lin and colleagues demonstrate that Akt can phosphorylate AR, which can lead to phosphorylation-dependent ubiquitination and degradation (Lin *et al.* 2001). It is clear from these studies that strong evidence exists for the interaction between the ERα, AR, and PI3K pathways. However, the exact stimulatory or repressive nature of these interactions as well as at to what degree these interactions might be taking place in Kisspeptin neurons is unknown and warrants future investigation.

CONCLUSIONS:

There is a major lack of data exploring the specific intracellular signaling pathways through which factors such as leptin and insulin, as well as steroid hormones, affect the reproductive axis, specifically within kisspeptin neurons. Previous work in which the specific receptors for upstream regulators of the PI3K signaling in both kisspeptin and GnRH neurons have been knocked out, which has yielded mixed results (Table 1) (Quennell *et al.* 2009; Donato *et al.* 2011; Qiu *et al.* 2013). This might suggest that neither kisspeptin nor GnRH neurons are integral for the reproductive axis's response to peripheral cues. However it is possible that compensatory pathways can be activated in absence of the primary receptor. This makes it important that the signaling pathways within both Kisspeptin and GnRH neurons be clarified in order to properly compare the results from cell-specific receptor knock out studies.

Determining the molecular mechanisms by which such pathways, like PI3K signaling, are involved in regulating *Kiss1* expression and kisspeptin neuronal function during development and adulthood will open the door to better understanding the full role of these neurons in regards to reproduction. The study of the role of PI3K signaling pathway in the regulation of kisspeptin expression will be essential in identifying new targets and new treatments for reproductive disorders affecting both men and women.

The main purpose of this study was to determine the signaling mechanisms within kisspeptin neurons that are important for relaying information from the body to GnRH neurons. Specifically, we will determine what role phosophoinositide 3-Kinase (PI3K) signaling has in regulating kisspeptin expression or neuronal activity with respect to peripheral metabolic cues. Also, we ask how alterations in PI3K signaling within kisspeptin neurons might affect the timing

and initiation of puberty. *The overall goal of these studies will be to determine how PI3K signaling within kisspeptin neurons in the brain plays a role in the development and function of the reproductive axis.*

Genetic Model	Phenotypes Observed	Reference
Kiss-Insulin Receptor KO ($IR^{\Delta Kiss}$)	Delayed sexual maturity in males Delayed VO in females	Qiu et al. 2013
$ER\alpha^{AA/-}$ (Global)	Absent AVPV *Kiss1* expression No response to E_2 in AVPV Decreased ARC *Kiss1* expression Preserved E2 response Increased LH levels in females	Gottsch et al. 2009 McDevitt et al. 2007 Glidewell-Kinney et al. 2007
$ER\alpha^{-/-}$ (Global)	Absent AVPV *Kiss1* expression Decreased ARC *Kiss1* expression No response to E_2 in AVPV or ARC Feminized genitalia in males	Gottsch et al. 2009 McDevitt et al. 2007 Glidewell-Kinney et al. 2007
Kiss-ERα KO (KERKO)	Advanced VO in females Abnormal Estrous cycle Increased ARC *Kiss1* Decreased AVPV *Kiss1*	Mayer et al. 2010
Aromatase KO (ArKO; Global)	Decreased *Kiss1* cell count in female AVPV Increased *Kiss1* cell count in male AVPV	Bakker et al. 2010 Clarkson et al. 2009
Hypogonadal (*hpg*)	AVPV *Kiss1* Sex difference abolished Response to positive E_2 feedback abolished	Gill et al. 2010
Kiss-LepR KO (KLERKO)	No effects on sexual maturation No effects on fertility	Donato Jr. et al. 2011
Androgen Receptor KO (ARKO; Global)	Decreased *Kiss1* in AVPV during LH surge Absent OVX-induced LH increase Female subfertility Greater LH suppressibility by E_2	Walters et al. 2013 Handelsman et al. 2009
$GABA_{B1}$ Receptor KO	Unaltered *Kiss1* levels in ARC or AVPV Increased *Kiss1* levels in BNST, MeA, and Lateral septum Increased GnRH pulse frequency in Females	Di Giorgio et al. 2013

Table 1. *Transgenic Mouse Model.* Transgenic rodent models used in the past decade in Reproductive physiological investigation. Specific focus on kisspeptin neuronal phenotype and *Kiss1* gene expression for each model listed.

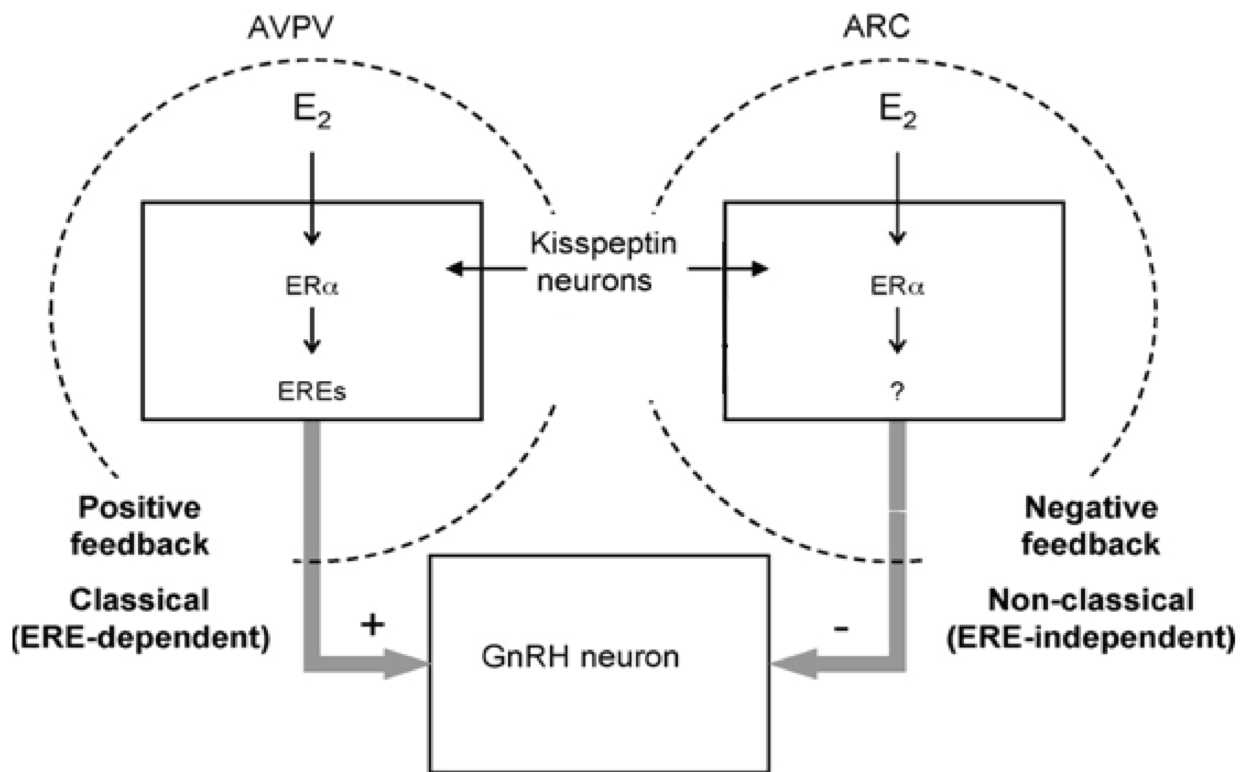


Figure 1. *Kisspeptin Regulation of the GnRH Neuron.* A schematic view of the two main neuronal populations of Kiss1 neurons showing their proposed roles in regulating GnRH neurons. Modified from McDevitt et al. 2008

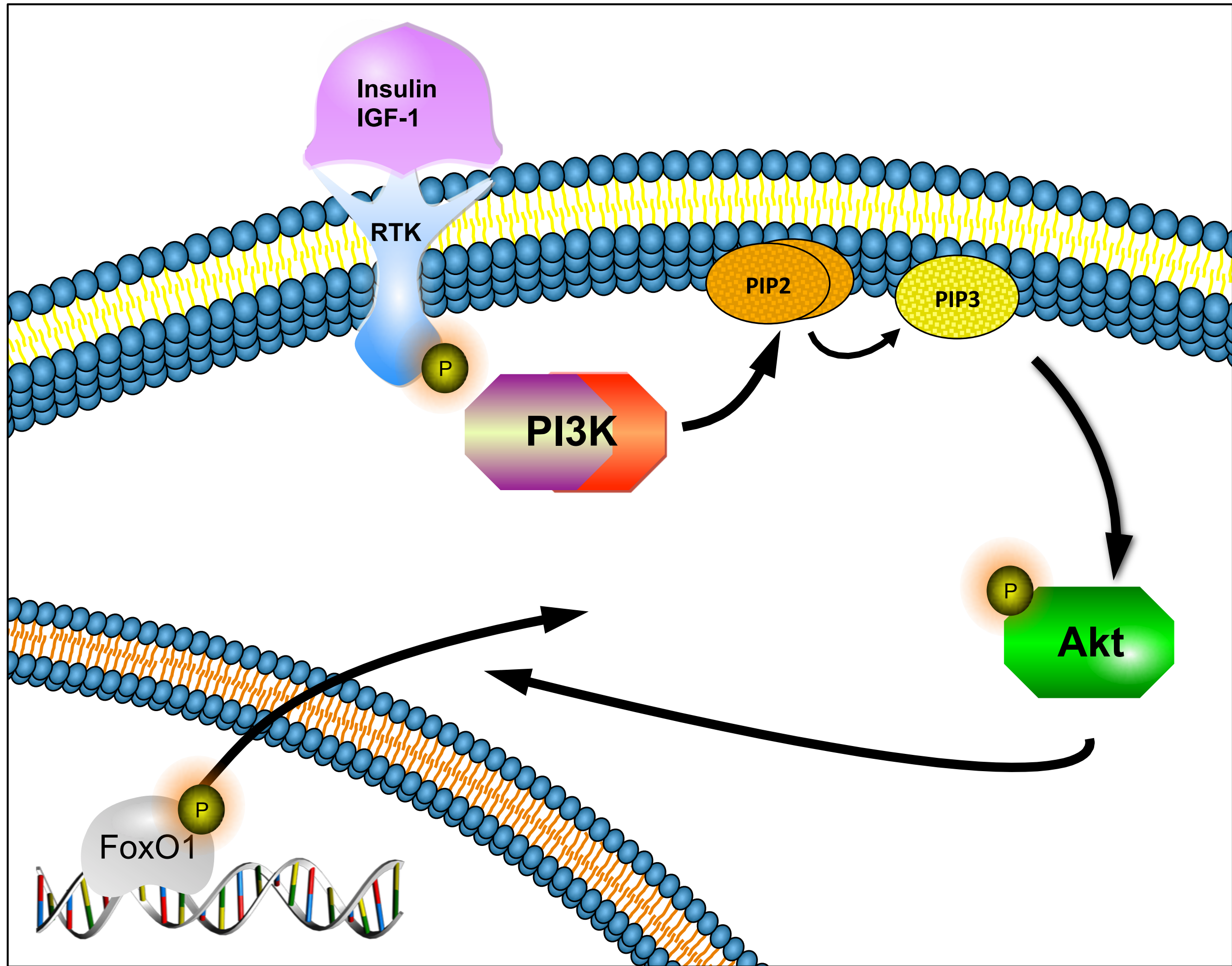


Fig 2. *Phosphoinositide 3-Kinase (PI3K) Signaling Pathway.* A basic schematic of PI3K signaling pathway including Akt/FoxO1.

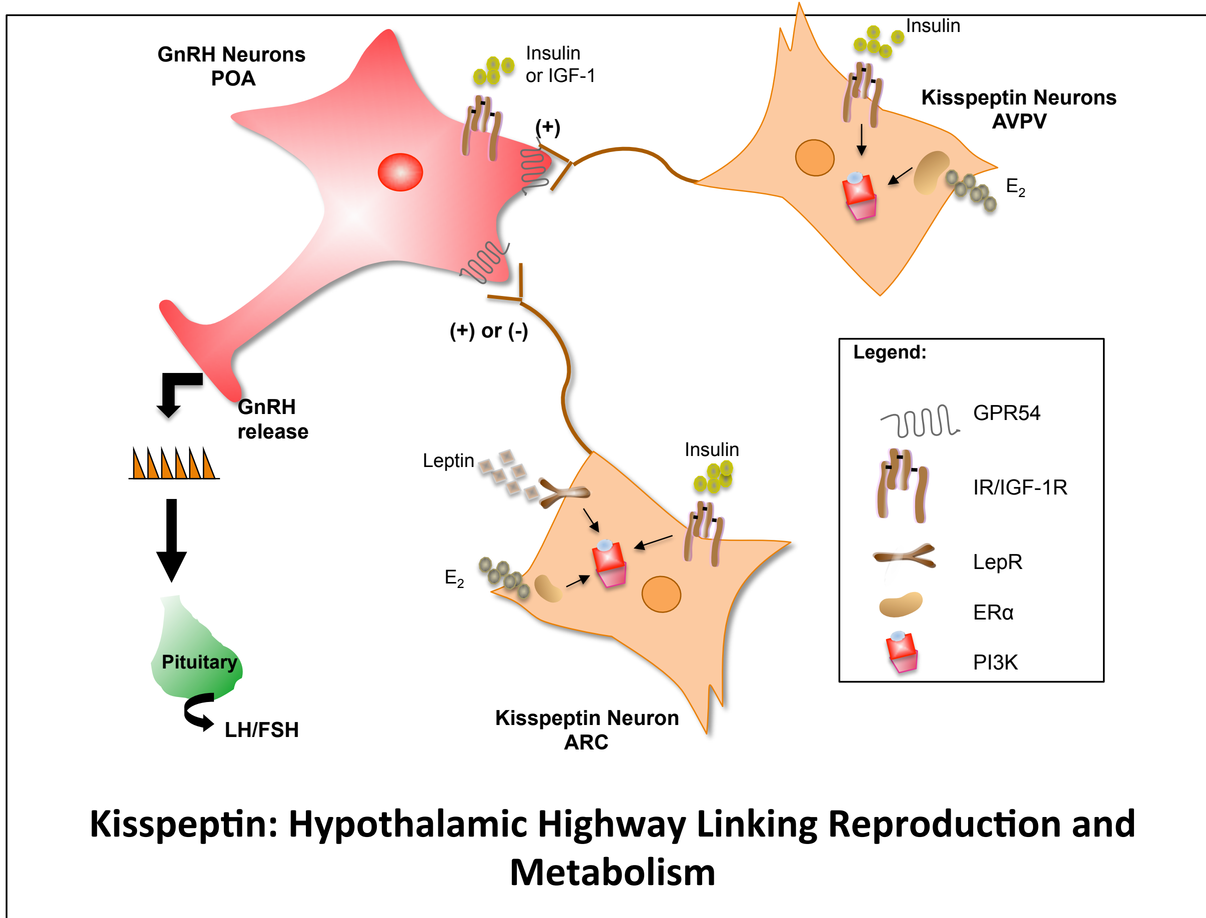


Fig. 3. *Kisspeptin: Hypothalamic Highway Linking Reproduction and Metabolism*. A schematic model of our proposed possible role of PI3K signaling in integrating peripheral signals within Kisspeptin neurons to relay the information to the GnRH/LH pulse system.

Chapter 2

PHOSPHOINOSITIDE 3-KINASE SIGNALING IN KISSPEPTIN NEURONS

INTRODUCTION:

The factors that regulate kisspeptin expression and activity on a physiological scale, such as E and insulin, are well characterized. However, how these factors effect signaling within kisspeptin neurons and whether the intracellular signaling pathways are involved in direct neuronal activation or regulating *Kiss1* expression is not known. We have taken a transgenic knockout approach to investigate the role of Kisspeptin-specific signaling of Phosphoinositide 3-Kinase. Using a Kiss-Cre mediated KO of PI3K catalytic subunits, p110α and p110β, we investigated the role of Kisspeptin-specific PI3K signaling with regards to pubertal onset, glucose and insulin homeostasis, and the effects of steroid hormone negative feedback on LH levels and *Kiss1* expression in both the AVPV and ARC. This study is the first, to our knowledge, to delineate investigate specific signaling pathways that may be important for the proper function of kisspeptin neurons within the HPG axis.

MATERIALS AND METHODS:

Puberty:

Pups were weaned postnatal day 21 (PND21) and visually assessed daily for vaginal opening (females) or balanopreputial separation (males) as well as anogenital distance using digital calipers. Upon vaginal opening daily vaginal lavages were taken to determine age of first estrus.

Adult reproductive phenotype:

Females – Estrous cycles were monitored for 20 consecutive days by daily vaginal lavage starting at two months of age. Serum was obtained from three month-old females bled at the morning of diestrus and serum LH levels were measured using RIA. Ovarian histology was done by H&E staining of 5 µm sections. Four month old female mice were placed into one of three groups: sham, ovariectomized (OVX), or OVX + estrodiol (E_2). Silastic capsules were used to replace E_2, while oil filled capsules were controls. Seven days later mice were euthanized using ketamine/xylazine and blood was collected via cardiac puncture. Serum LH and E_2 was measured by RIA (done at the University of Virginia Ligand Assay & Analysis Core of the Center for Research in Reproduction). Brains were frozen, cut in series of four at 20 µm, and thaw mounted onto SuperFrost Plus slides (Fischer Scientific) for in situ hybridization.

Males – Four month old mice were placed into one of three groups: sham, gonadectomized (GDX), or GDX + testosterone (T). Silastic capsules (Length-10mm, inner diameter-1.02mm, outer diameter-2.16mm) containing 100 µg of testosterone propionate were used to replace T, while empty capsules served as controls. Seven days later mice were euthanized using ketamine/xylazine and blood was collected via cardiac puncture. Serum LH was measured by RIA, while T levels were measure by ELISA. LH RIA was done at the University of Virginia Ligand Assay & Analysis Core of the Center for Research in Reproduction. Brains were frozen, cut in series of four at 20 µm, and thaw mounted onto SuperFrost Plus slides (Fischer Scientific) for in situ hybridization. Testicular histology was assessed by H&E stained 5 µm sections.

Quantitative PCR for Kisspeptin:

The MBH and POA regions were dissected from Kiss-p110α/β KO and WT males that were intact, GDX, or GDX + T. For the POA a cut just caudal of the olfactory lobe with a second just caudal of the optic chiasm was made. The lateral limits were parallel to the lateral ventricles and the dorsal limit was the anterior commissure. For the MBH a cut just caudal of the optic chiasm with a second at the start of the mammillary nuclei, while lateral limits were from optic tracts to the fornixes and a dorsal limit of just dorsal of fornix and third ventricle. Trizol Reagent was used to isolate RNA from MBH and POA dissections. Reverse transcription was performed on 2.5ug of RNA using SuperScript VILO (Invitrogen). 100ng of RT reaction was used in each qPCR reaction done in duplicate for each sample. *Kiss1* levels and Rn18S levels were measured using TaqMan probes Mm03058560_m1 and Mm03928990_g1, respectively. 96-well plates were run on Applied Biosystems 7300 Real-Time PCR System in the Stony Brook School of Medicine DNA Sequencing Facility.

Monitoring PI3K signaling in kisspeptin neurons:

Kisspeptin-FoxO1GFP mice (n=6) underwent stereotaxic surgery and implanted with a cannula into the left lateral ventricle (0.34mm caudal, 0.9mm lateral to bregma). To test for successful targeting, mice were infused with 20 μg of angiotensin six days after surgery. Mice were fasted overnight and then infused with saline (n=3) or insulin (15 mU; n=3). Fifteen minutes later, mice were anesthetized and transcardially perfused. The brains were removed and post-fixed 24 hours. After immersion in 30% sucrose, 25 μm sections were cut using a cryostat and stored in polyvinyl propanol cryoprotectant. Sections were subsequently washed with PBS to remove cryoprotectant and incubated in 3% hydrogen peroxide solution to quench endogenous

peroxidase activity. Sections were then incubated in 1:1000 goat anti-GFP antibody in 0.3% Triton X-100 in PBS overnight at 4°C. Sections were washed with PBS then incubated in 1:500 rabbit anti-goat Alexa Fluor 488. Images were obtained using a Zeiss Ax overt 200M and Apotome system.

In situ hybridization:

Radiolabeled probes were synthesized *in vitro* by inclusion of the following ingredients in a volume of 20 µL: 250 µCi ^{35}S-UTP (PerkinElmer Life Sciences, Boston, MA); 1.5 µg linearized DNA; 0.5 mM each ATP, CTP, GTP; 5 µM UTP; T7 RNA polymerase; 10mM DTT; RNase IN. Residual DNA was digested with RQ1 DNase. The riboprobes were separated from unincorporated nucleotides with ProQuant G-50 MicroColumns (Amersham). Slides were fixed in 4% paraformaldehyde then washed in PBS and dipped in water, then in 0.1M TEA before being incubated in 0.25% acetic anhydride in TEA. Next slides were washed with 2X saline sodium citrate (SSC) followed by dehydrating in graded alcohol. Slides were then delipidated in chloroform, rehydrated using decreasing graded alcohol, and air-dried for at least 1 hour. Radiolabeled antisense KiSS-1 riboprobe was denatured, added to hybridization solution (50% formamide, 10% dextran sulfate, 300mM NaCl, 10mM Tris pH 8.0, 1mM EDTA, 1X Denhardt's solution, 10mM DTT, tRNA) at a concentration of 5 x 10^6 cpm/ml and applied to slides. Slides were covered with glass coverslips, placed in a humid chamber, and incubated overnight at 55°C. The next day, slides were washed in 4X SSC then incubated in RNase A in buffer (10mM Tris pH 8.0, 500mM NaCl, 1mM EDTA) followed by washing with 2X SSC and 0.1X SSC at 65°C. Slides are washed with room temperature 0.1X SSC then dehydrated in 50% and 80% alcohol with ammonium acetate followed by 100% alcohol. Slides were air dried for at least an hour then

dipped in NTB-2 liquid emulsion (Eastman Kodak Co., Rochester, NY). Slides were developed two weeks later, counterstained with thionin, dehydrated, cleared with citrisolve (Fisher Scientific), and coverslipped. Images of slides were taken under dark-field illumination and 3 sections per animal per region were analyzed using MCIDAnalysis grain counting module. Cells counted were considered kisspeptin positive when grain count was more than three times background. Counts are expressed as the number of positive neurons per animal and mean number of grains per cell.

Immunohistochemistry:

Mice were anesthetized and transcardially perfused with 4% paraformaldehyde. Brains were postfixed for 24 hours then allowed to saturate with 30% sucrose. 25 μm sections were cut using a cryostat in series of three for GnRH and NKB. 30 μm sections were cut using a cryostat in series of two for Kisspeptin.

GnRH – Before staining, sections were washed PBS then incubated in 3% H_2O_2 in PBS to quench the endogenous peroxidase activity. After PBS washes, sections were washed with PBST (0.01M PBS with 0.3%Triton-X100). Sections were then incubated in PBST containing LR5 (rabbit anti-GnRH 1:20,000 a gift from Dr. Benoit) overnight at 4 C. After washing in PBST, sections were incubated in biotinylated goat anti-rabbit IgG (Vector Laboratories, Burlingame, CA) for 1 h, washed in PBST, and incubated with Vectastain ABC reagent (Vector Laboratories) for 1 h. Sections were subsequently washed and reacted with Vectastain DAB Peroxidase substrate kit (Vector Laboratories). Sections were washed, mounted and coverslipped.

Kisspeptin – Sections were treated with 3% hydrogen peroxide for 10 min to quench endogenous peroxidase activity. After rinses in Tris-buffered saline (TBS), the tissue was incubated for 48 h

at 4 °C in TBS Tween 20 (TBST; 0.3% Triton X-100, 0.25% BSA, and 2% normal goat serum) containing a polyclonal rabbit anti-kisspeptin-10 antiserum (1:10,000; a gift from A. Caraty, Institute National de la Recherche Agronomique, Paris). After washes in TBS, sections were incubated in TBST containing biotinylated goat anti-rabbit IgG (1:500; Vector Laboratories), and this was followed by incubation in Vectastain Elite ABC reagent (Vector Laboratories) for 90 min at room temperature. Sections were washed and the immunoreactivity was visualized using nickel-enhanced Vectastain DAB Peroxidase substrate kit (Vector Laboratories). Sections were washed, mounted on slides, dried overnight, and coverslipped.

NKB – Sections were treated with 3% hydrogen peroxide for 10 min to quench endogenous peroxidase activity. After rinses in Phosphate-buffered saline (PBS), the tissue was incubated for 1 hour at room temperature then 48 h at 4 °C in PBS with 0.4% Triton X-100 containing a polyclonal rabbit anti-NKB antiserum (1:10,000; gift from Dr. Coifi, France). After washes in PBS, sections were incubated in PBS + 0.4% Triton X-100 containing biotinylated goat anti-rabbit IgG (1:500; Vector Laboratories) for one hour followed by incubation in Vectastain Elite ABC reagent (Vector Laboratories) for 1 hour at room temperature. Sections were washed and the immunoreactivity was visualized using Vectastain DAB Peroxidase substrate kit (Vector Laboratories). Sections were rinsed, mounted on slides, dried overnight, and coverslipped before visualization.

Metabolic Phenotyping:

Glucose tolerance test – Mice (n=18-19; 7-11/genotype) were fasted overnight then post-fasting weights and blood glucose levels were recorded. Mice were then injected with 8 µL/g of a 25%

glucose solution i.p. Blood glucose measures were taken at 30, 60, and 120 minutes post injection.

Insulin tolerance test – Mice (n=18-21; 7-12/genotype) were fasted for four hours. Post-fasting weights and blood glucose measures were made. Mice were then injected with Humulin-R (Eli Lily, Indianapolis, IN). Females were injected i.p. with 0.5mU/g and males with 1mU/g. Blood glucose readings were taken 15, 30, 60, 90, and 120 minutes post injection.

Fasting Effect on LH levels:

Female mice (n=16, 8/genotype) were weighed, blood glucose was measured, and a blood sample was collected via submandibular bleeding, estrous stage was assessed by vaginal lavage. Mice were then fasted for 24 hours. Post-fasting blood glucose, weight, and estrous stage were recorded. Blood was collected via cardiac puncture and serum was separated. Serum LH was measured by RIA at the University of Virginia Ligand Assay & Analysis Core of the Center for Research in Reproduction. Ovaries were collected and fixed in 4% paraformaldehyde for histological assessment.

Male mice (n=22, 10-12/genotype) were weighed, blood glucose was measured, and blood sample was collected via submandibular bleeding, mice were fasted for 48 hours. Post-fasting blood glucose and weight were recorded. Blood was collected via cardiac puncture and serum was separated. Serum LH was measured with Millipore brand 'MILLIPLEX MAP Rat Pituitary Magnetic Bead Panel, RPTMAG-86K' plate by Andrew Wolfe at Johns Hopkins.

Fertility testing:

Fertility of Kiss-p110α/β KO female mice was examined by pairing Kiss-p110α/β KO adult

female mice with proven fertile adult WT males for seven days. After a week females were housed individually for approximately 28 days in order to allow time for pregnancy. Time to each litter and litter size will be recorded. To assess fertility in Kiss-p110α/β KO males, adult Kiss-p110α/β KO males were caged with adult WT females for seven days.

Fluorescence-associated cell sorting:

Kiss-EYFP mice were sacrificed by CO_2 inhalation and quick decapitation, and their brains excised and placed in ice cold 0.9% saline for 5 minutes. Using a brain matrix and razor blades 1mm slices were made from the rostral aspect of the pre-optic area to the caudal aspect of the mammillary bodies. In a small petri dish with cold PBS desired regions were dissected from the slice and combined into a clean tube. Dissections from three animals were pooled per sample. To each sample, 500 µL of 2.5mg/mL papain, 1X Trypsin in PBS was added to each tube which was subsequently incubated at 37°C for 30 minutes. Samples were triturated with fire polished glass Pasteur pipette until no clumps are seen. 500 µL of FACS Buffer was added and samples were mixed gently with glass Pasteur pipette. Gating with the FACSAria (BD Biosciences, San Jose, California) was set using EYFP negative (EYFP-) neurons. *Kiss1* (EYFP+) cells were isolated according to GFP wavelength (488nm laser). YFP+ cells were collected into 15 mL tubes containing 2 mL of RNAlater solution. Samples were centrifuged at 13,000 rpm for 30 minutes. RNA was extracted and isolated using the PicoPure RNA isolation kit. DNase treatment using DNA-free DNase was included. RNA was eluted with 12 µL of elution buffer. All 12 µL of elute was used for Reverse Transcription reaction following SuperScript VILO protocol.

RESULTS:

Validating Kiss-p110α/β KO:

FACS sorted Kiss-EYFP neurons from WT male mice showed expression of p110α, p110β, and *Kiss1* through RT-PCR (Fig 4A). FACS sorted Kiss-EYFP neurons from Kiss-p110α/β KO male mice had no detectable expression of p110α or p110β, but have detectable expression of *Kiss1* (Fig 4B).

Kiss-p110α/β KO lowers Kiss1 mRNA expression in males regardless of gonadal status:

In the AVPV, intact Kiss-p110α/β KO females and males had decreased *Kiss1* expression levels per cell compared to WT (Fig 5). In contrast, male Kiss-p110α/β KO mice showed a significant decrease in *Kiss1* expression per cell in the ARC compared to WT, while in females there was no significant genotype effect (Fig 7, Fig 6). Next, we examined the effects of T levels on *Kiss1* expression in males of both genotypes. In the ARC gonadectomy increased *Kiss1* expression and T replacement decreased *Kiss1* expression, however Kiss-p110α/β KO males had significantly lower levels than WT ($p<0.05$) in each treatment group. An interaction between treatment and genotype was observed as well ($F(2,23)=3.958$, $p=0.0333$, Two-way ANOVA, Fig 7). Given that males have10-fold fewer kisspeptin neurons within the AVPV than females and these few neurons express on average less kisspeptin it is technical difficult to precisely and accurately detect true positive kisspeptin neurons in males using radioactive in situ hybridization so we chose to measure this using quantitative PCR on dissected brain regions containing kisspeptin (MBH and POA). Kiss-p110α/β KO Males again had a significant decrease in *Kiss1* expression when testosterone was removed however although the Kiss-p110α/β KO intact and

testosterone replacement males had decreased relative expression this did not reach statistical significance ($F(2,29)=26.17$, $p<0.0001$, Fig 8).

Kiss-p110α/β KO decreases ARC kisspeptin-immunoreactivity (kiss-ir) in males and females:

To determine if the observed changes in *Kiss1* expression are accompanied by changes in protein levels, we performed immunohistochemistry for kisspeptin. In the AVPV, kisspeptin cell counts did not differ in either male or female Kiss-p110α/β KO mice compared to WT (Fig 9). Adult Kiss-p110α/β KO mice of both sexes had a reduction in fiber immunostaining in the ARC. In female Kiss-p110α/β KO mice, kiss-ir cell bodies in the ARC were observed, however, no kiss-ir cell bodies were detected in the ARC of Kiss-p110α/β KO males (Fig. 10). To investigate whether these changes in kisspeptin expression and immunoreactivity in males lead to observable changes in GnRH neurons, we used the LR-5 antibody to detect immunoreactive neurons. We observed no significant change in the distribution or cell counts of GnRH neurons between Kiss-p110α/β KO and WT males (160.0 ± 17.20 vs.147.2 ± 23.22 neurons; $p=0.6665$; $n=6$)

Kiss-p110α/β KO does not affect normal pubertal development or growth in males or females:

Upon characterizing the Kiss-p110α/β KO mice, we found no difference in post-weaning body weight in either sex (Fig11C, Fig 13B). The Kiss-p110α/β KO had no effect on pubertal onset, as determined by balanopreputial separation in males (30.50 ± 0.9916 vs. 30.50 ± 0.8118 days old, Fig 13A), or by day of vaginal opening (30.55 ± 1.073 vs. 31.47 ± 0.7887 days old, Fig 11A) in females. In female Kiss-p110α/β KO no difference in estrus cyclicity (Fig 12C), cycle

length (Fig 12B), or day of first estrus (49.40 ± 3.194 vs. 46.27 ± 3.565 days old, Fig 11A) was observed.

Next, we examined the effects of gonadectomy and E_2 replacement on the serum LH levels in WT and Kiss-p110α/β KO females. As expected gonadectomy increased serum LH levels, while replacement with E_2 suppressed serum LH levels. No genotype effect was seen on either serum LH or E_2 levels in females. Both Kiss-p110α/β KO and WT males responded as expected to gonadectomy, which increased serum LH, while T replacement suppressed LH levels. However, Kiss-p110α/β KO males had significantly lower serum LH levels compared to WT littermates in each of the treatment groups (Fig 14C). There was no observed effect of genotype on T levels (intact: 5.438 vs. 5.604 ng/mL; GDX: 3.508 vs. 3.512 ng/mL; GDX+T: 8.912 vs. 8.356 ng/mL, Fig 14C).

Kiss-p110α/β KO females have decreased fertility rates compared to WT:

In order to determine if the disruption of PI3K signaling altered the ability of female Kiss-p110α/β KO mice to become pregnant and carry a litter to term we bred WT and KO female mice with proven fertile WT male mice. Kiss-p110α/β KO female mice have a decreased fertility rate compared to WT females (43.75±18.75% vs. 85.5±7.22%, $p>0.05$) but this did not reach significance.

Peripheral Glucose and Insulin sensing unaltered in Kiss- p110α/β *KO mice:*

In order to determine whether disrupting PI3K signaling altered Glucose homeostasis we performed glucose and insulin tolerance tests on Kiss-p110α/β KO males and females. There was no significant difference in the ability to clear glucose or insulin sensitivity. Disrupting PI3K

signaling in kisspeptin cells did not alter peripheral glucose or insulin sensing (Fig 15). We also investigated whether peripheral tissues, such as the liver, which are known to express *Kiss1*, had altered response to insulin stimulation. Upon peripheral insulin stimulation no significant difference was seen in liver pAkt levels in Kiss-p110α/β KO males compared to WT male mice (Fig 16).

Ablation of PI3K signaling does not alter viability of kisspeptin neurons:

In order to address whether yellow fluorescent protein (EYFP) reporter mice were generated as both Kiss-Cre+ mice with and without the alleles for p110α and p110β flanked by lox P sites. Using immunofluorescence for EYFP there is no qualitative difference in the number of YFP+ cells in Kiss-p110α/β KO compared to WT (data not shown).

DISCUSSION:

Over recent years the importance of kisspeptin signaling within the HPG axis has been study in depth. It has come to light that kisspeptin is essential for proper activation and timing of the GnRH/LH pulse system that governs the reproductive capabilities in most higher organisms. Studies on loss-of-activation of the kisspeptin receptor or deficiency in the peptide itself result in hypogonadotropic hypogonadism and delay or absence of sexual maturation (de Roux *et al.* 2003; Seminara *et al.* 2003; de Roux 2006).

These are the first findings to our knowledge that implicate any specific intracellular signaling pathway in the involvement of *Kiss1* gene expression. PI3K signaling is important for both transcriptional regulation and neuronal activity in the mammalian hypothalamus. In particular both POMC and NYP neurons, which express p110α and p110β, use PI3K signaling to

regulate transcript levels and firing rates (Mirshamsi *et al.* 2004; Morrison *et al.* 2005; Hill *et al.* 2008). Given kisspeptin localization in the hypothalamus, namely in the ARC, it is not surprising that ablation of PI3K signaling has considerable effect on kisspeptin expression. However, based on our current results and other recent findings kisspeptin signaling may not be essential in relaying normal metabolic status to the GnRH system (Hill 2011, Qiu 2013). Furthermore, this Kiss-PI3K model needs to be further characterized in order to determine the precise mechanisms through which these effects on *Kiss1* expression are occurring. Of note is that both males and female knockouts show significant effects with removal of the respective sex steroid hormones, however the mechanism, which causes these changes, may differ. This may be due to the fact that E's actions on *Kiss1* expression are extremely potent and may partial occlude any effects due to the aberrant PI3K signaling within these cells. A possible reason for the difference in effects with physiological levels of circulating steroid hormones is due to the fact that *both E and T control Kiss1 expression in the hypothalamus of males*. This may further compound the difficulty in elucidating the specific effects of the deletion of PI3K from kisspeptin cells.

The alterations in fertility rate of Kiss-p110α/β KO females are unlikely due to aberrant mating behavior because males can plug both Kiss-p110α/β KO and WT females. Further investigation should to be done to determine if the females become pregnant but loose the fetuses during gestation or whether there are alterations that prevent the KO female of becoming pregnant to the degree of the WT females controls.

Overall, our data support the importance of PI3K signaling in the regulation of *Kiss1* expression in the hypothalamus of both males and females. However, the differences we see in the qualitative aspects of the phenotype would suggest that PI3K signaling maybe functioning differently within kisspeptin neurons of males compared to those of females. Further

investigation into the possibly sexually dimorphic role of PI3K signaling within kisspeptin neurons may help explain and support previous findings that suggest kisspeptin expression and neuronal activity in the ARC is not regulated in a similar fashion between males and females (de Croft *et al.* 2012; Poling & Kauffman 2012).

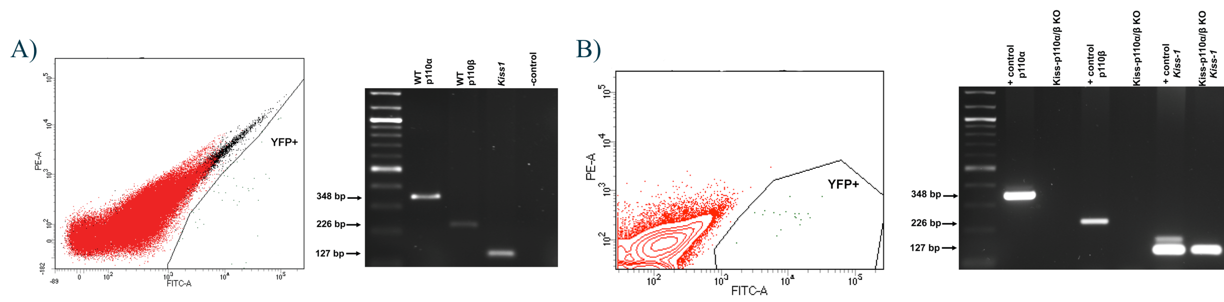


Fig. 4. *Validation of Kiss-p110α/β KO mice.* FACS sorted Kiss-EYFP neurons from WT (A) and Kiss-p110α/β KO (B) male mice. RT-PCR showing expression of p110α, p110β, and *Kiss1* in WT (A) and Kiss-p110α/β KO (B) male mice. Positive control = whole MBH mRNA.

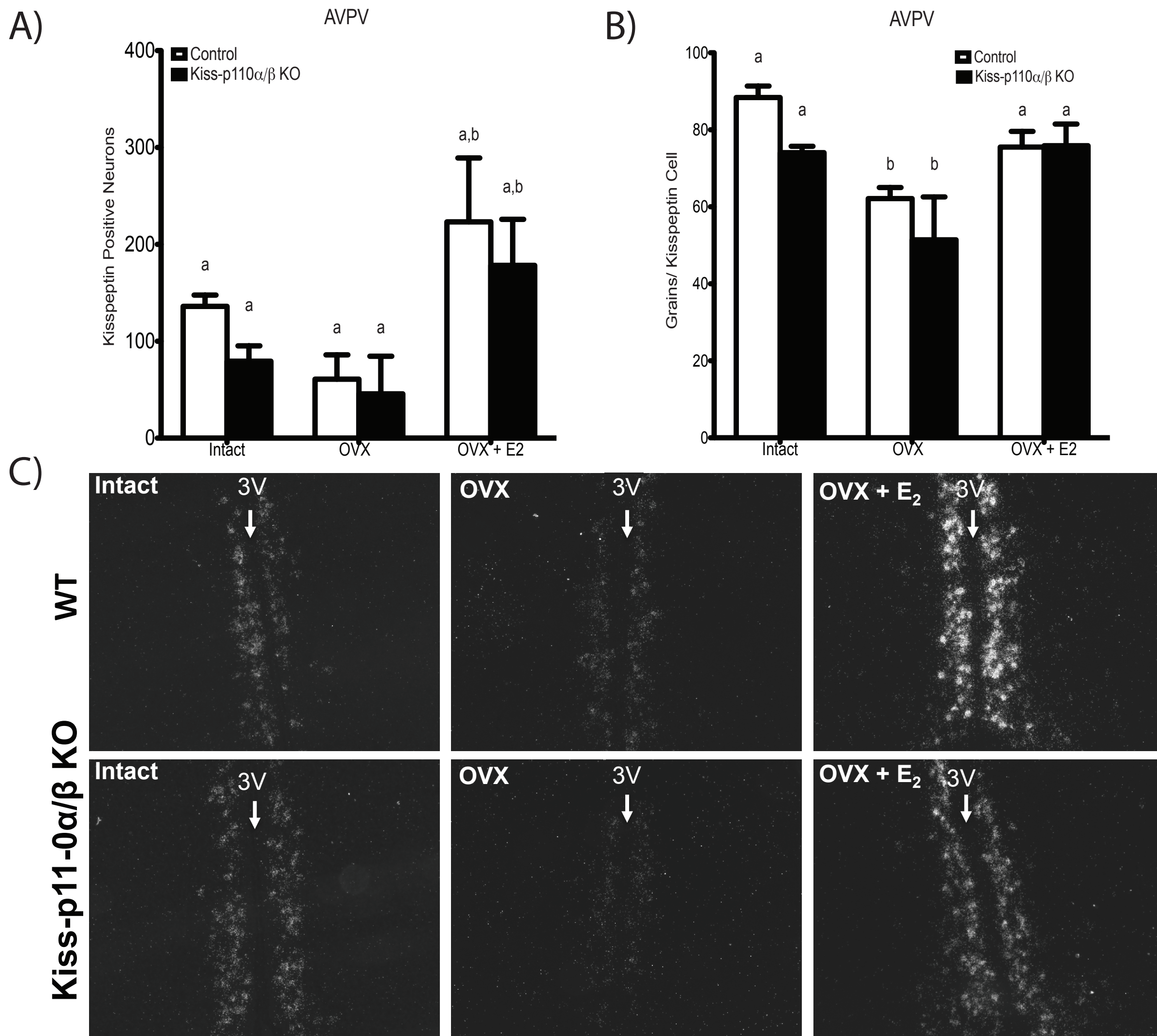


Fig. 5. *Kiss1 expression in the Female AVPV.* Number of detectable Kiss1 neurons (A) and grains per cell (B) in intact, OVX, and OVX + E_2 of WT and Kiss-p110α/β KO mice. Representative darkfield photomicrographs (C) showing *Kiss1* expressing cells (as reflected by the presence of white clusters of silver grains) in representative sections of the AVPV from intact, OVX, OVX + E2 WT and Kiss-p110α/β KO mice. 3V, Third ventricle.

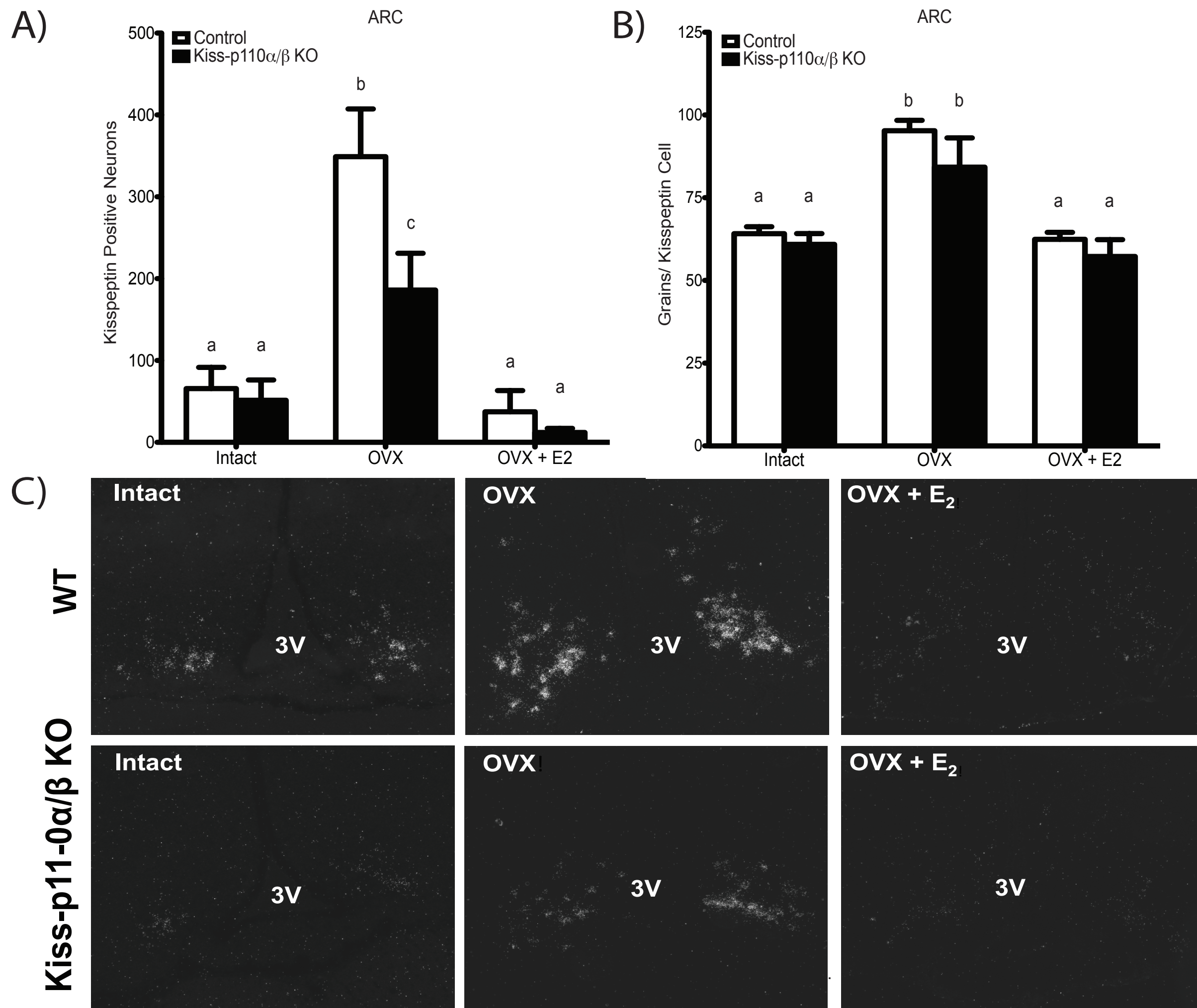


Fig. 6. *Kiss1 Expression in the Female ARC.* Number of detectable Kiss1 neurons (A) and grains per cell (B) in intact, OVX, and OVX + E_2 of WT and Kiss-p110α/β KO mice. Representative darkfield photomicrographs (C) showing *Kiss1* expressing cells (as reflected by the presence of white clusters of silver grains) in representative sections of the ARC from intact, OVX, OVX + E2 WT and Kiss-p110α/β KO mice. 3V, Third ventricle.

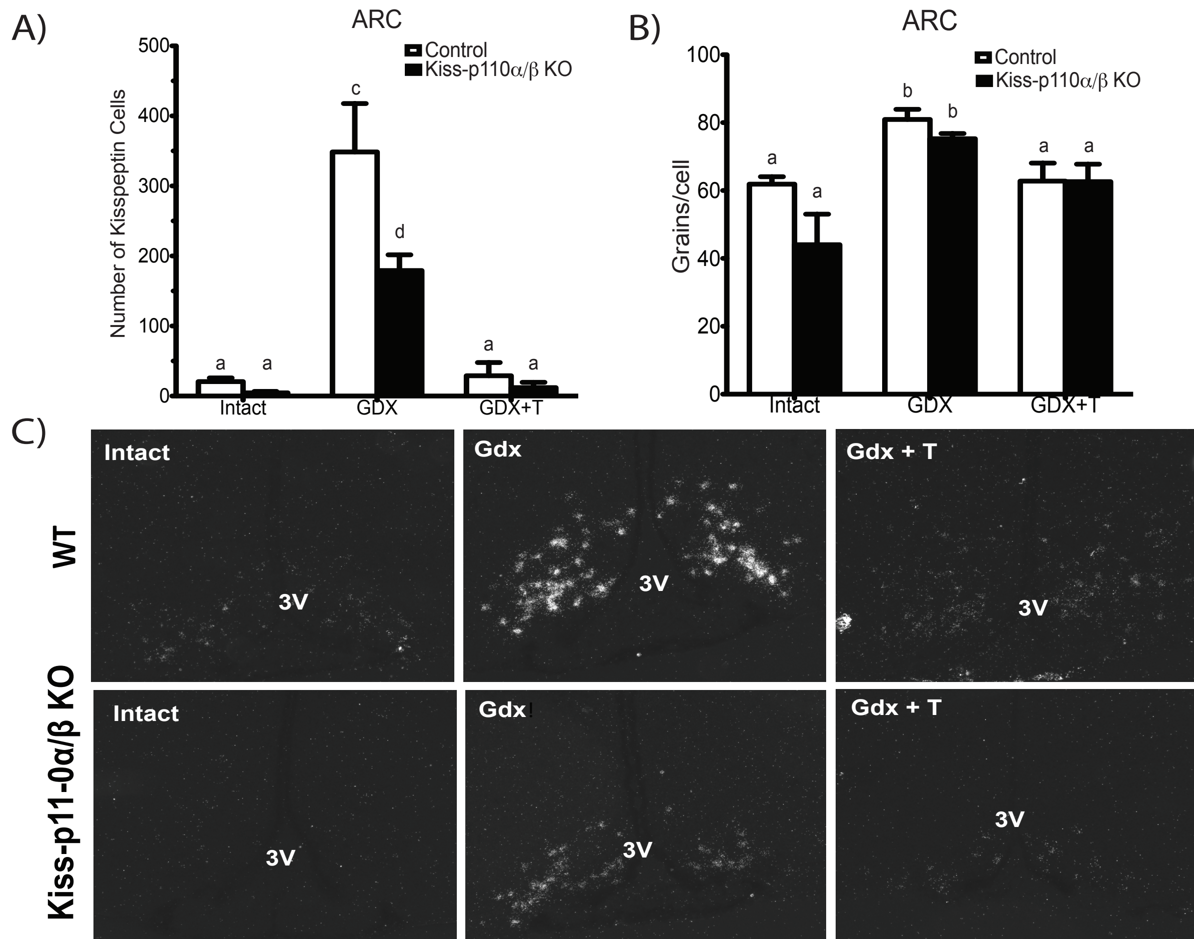


Fig. 7. *Kiss1 Expression in the Male ARC.* Number of detectable Kiss1 neurons (A) and grains per cell (B) in intact, GDX, and GDX + T of WT and Kiss-p110α/β KO male mice. Representative darkfield photomicrographs (C) showing *Kiss1* expressing cells (as reflected by the presence of white clusters of silver grains) in representative sections of the ARC from intact, GDX, GDX + T of WT and Kiss-p110α/β KO male mice. 3V, Third ventricle.

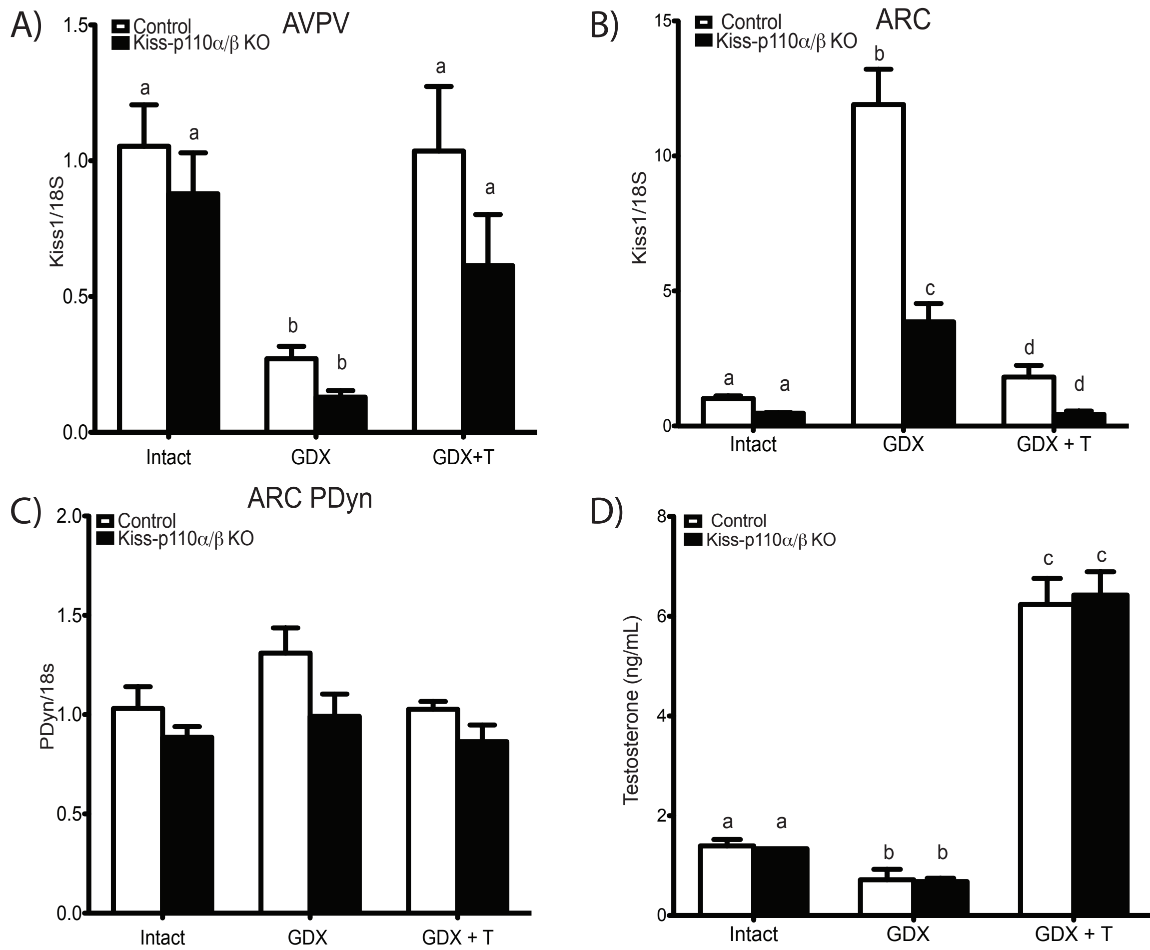


Fig. 8. *Kiss1 and PDyn Expression in WT and Kiss-p110α/β KO males.* Kiss1 Expression measured by qPCR of AVPV (A) and ARC (B) in intact, GDX, and GDX + T of WT and Kiss-p110α/β KO male mice. Prodynorphin expression in the ARC (C) in intact, GDX, and GDX + T of WT and Kiss-p110α/β KO male mice. T levels (D) in WT and Kiss-p110α/β KO mice of each treatment.

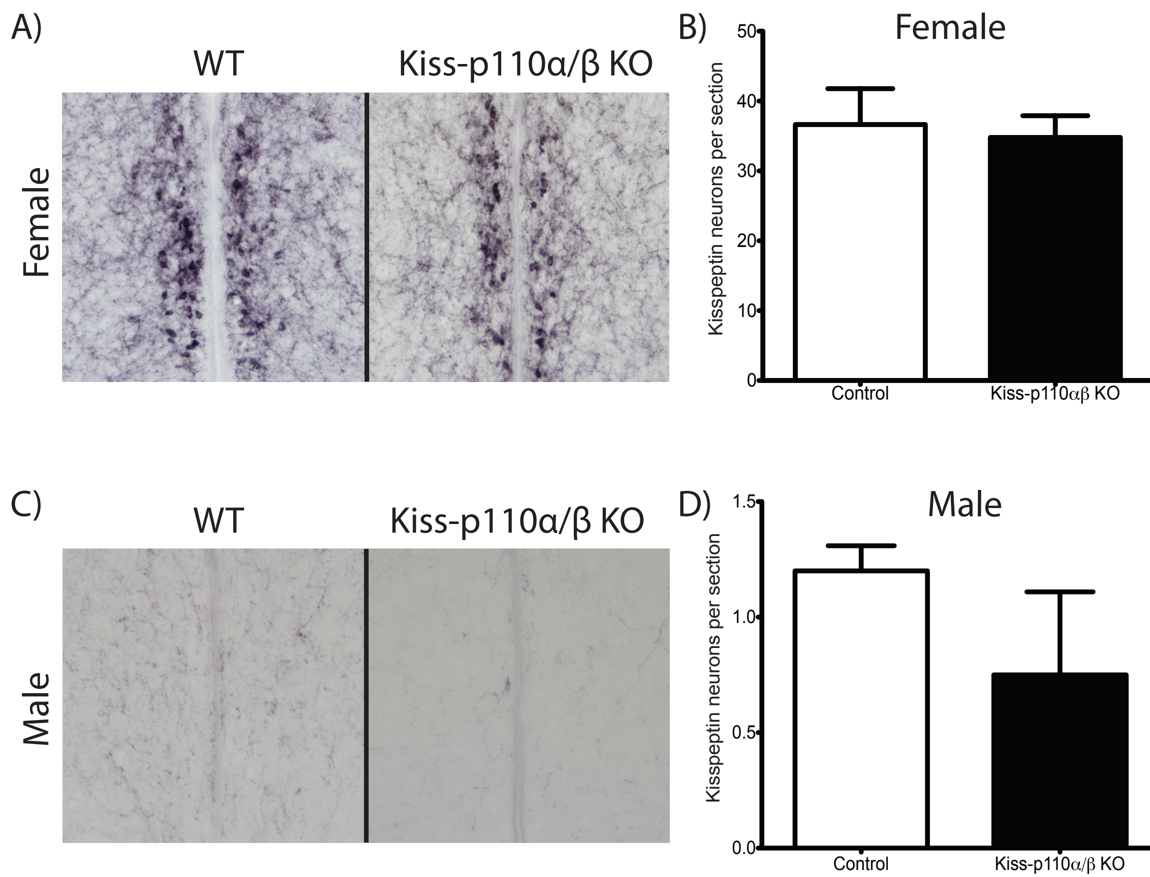


Fig. 9. *Kisspeptin Protein Expression in the AVPV.* Representative micrographs of the AVPV in WT and Kiss-p110α/β KO females (A) and males (C). Number of kisspeptin neurons per section in AVPV of females (B) and males (D).

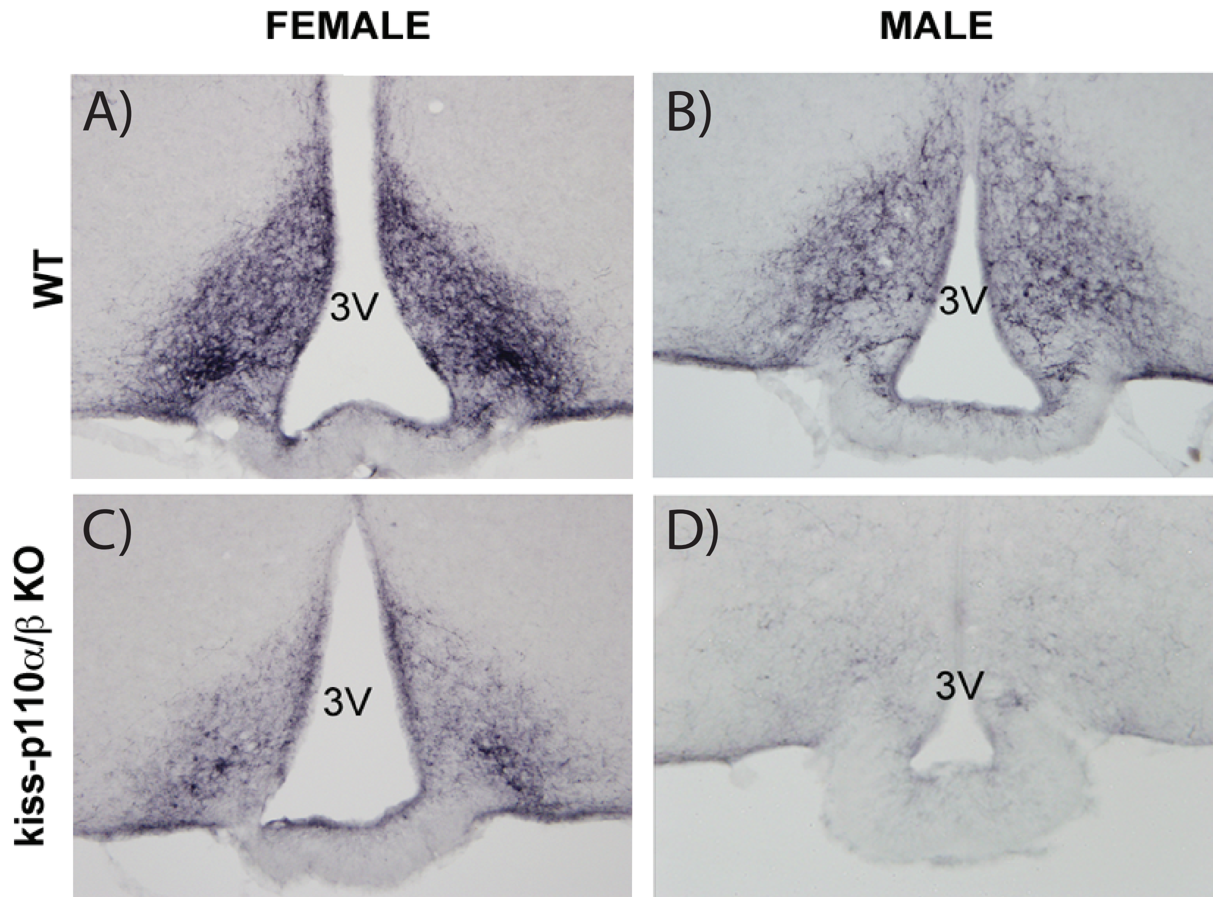


Fig. 10. *Kisspeptin Protein Expression in the ARC.* Representative photomicrographs of ARC Kiss-ir in female WT (A), female Kiss-p110α/β KO (C), male WT (B), and male Kiss-p110α/β KO (D).

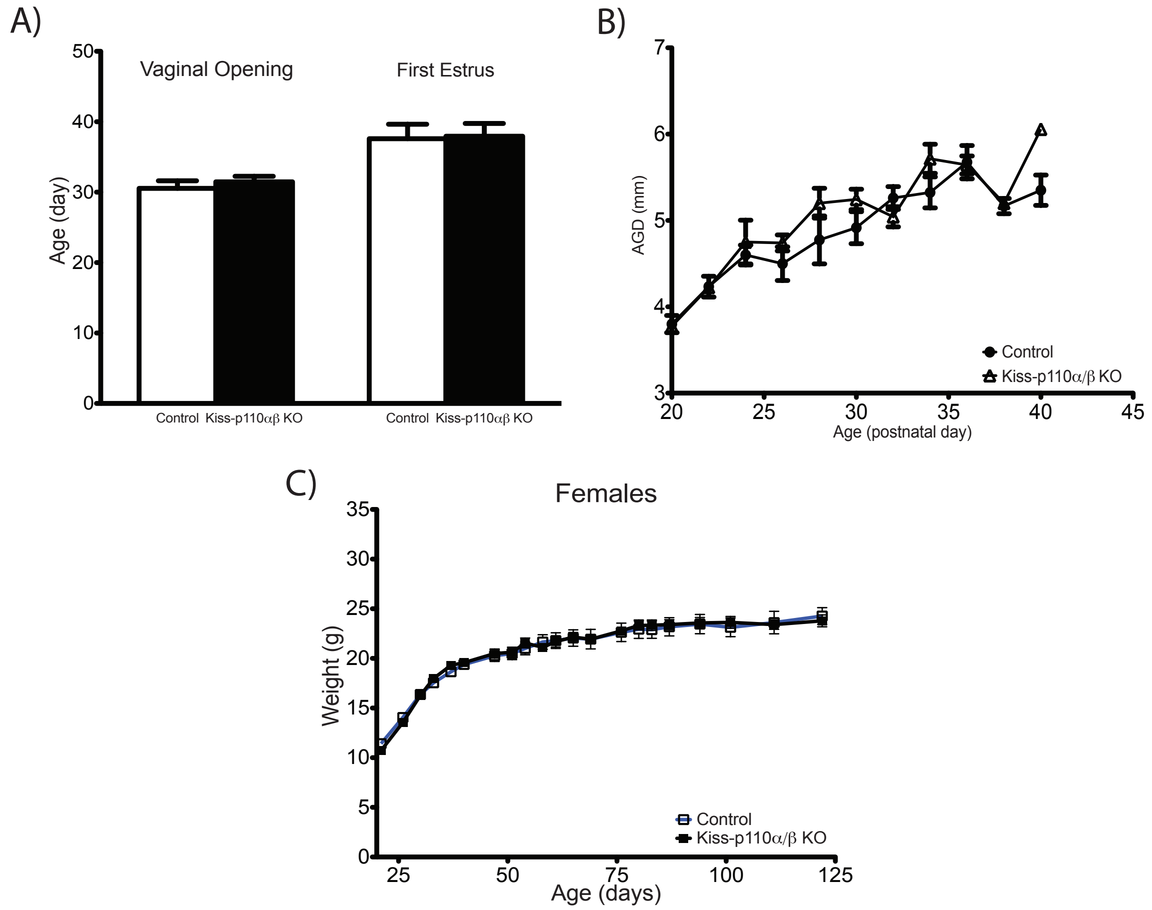
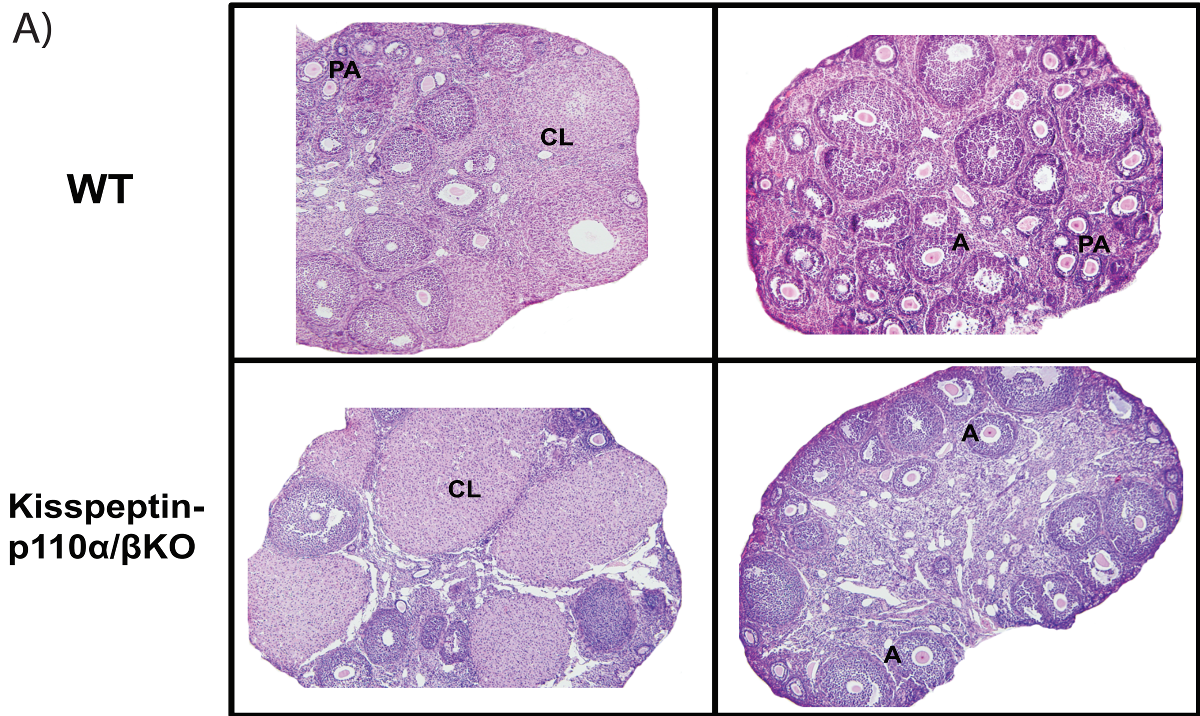


Fig. 11. *Female Pubertal Development.* Age at pubertal onset in WT and Kiss-p110α/β KO female mice as measured by day of vaginal opening (VO) and day of first estrus (A). Anogenital distance (B) in WT and Kiss-p110α/β KO female mice during the peri-pubertal period. Post-weaning weight (C) in WT and Kiss-p110α/β KO female mice.



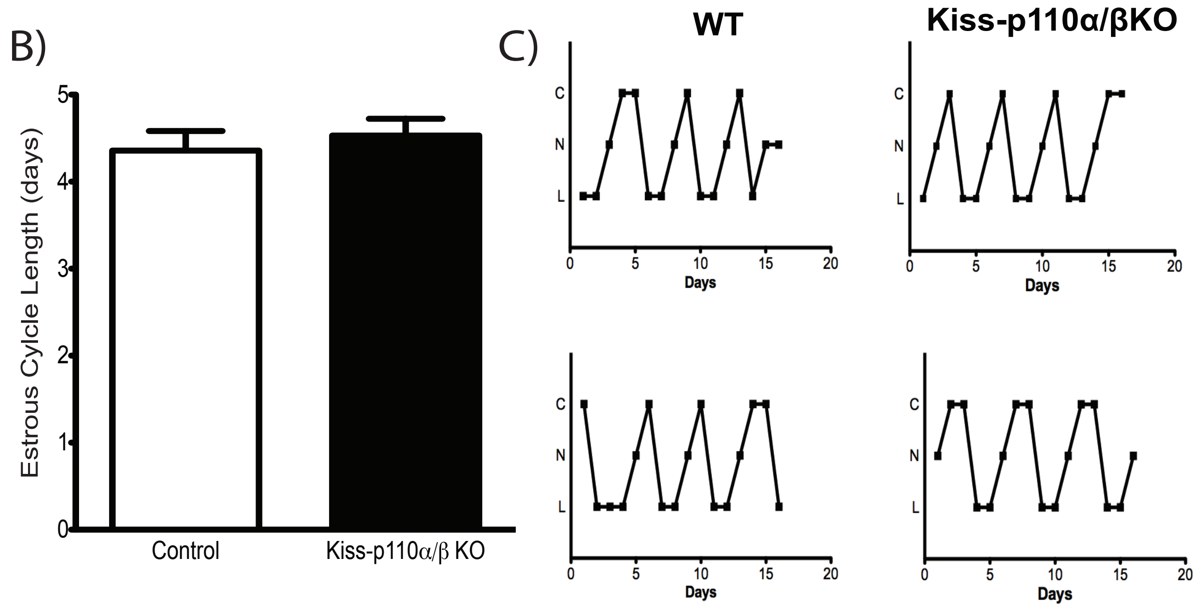


Fig. 12. *Adult Female WT and Kiss-p110α/β KO Ovarian Cyclicity.* Representative photomicrographs (A) of H&E stained ovaries from WT and Kiss-p110α/β KO females. Average cycle length in days (B) of WT and Kiss-p110α/β KO females. Representative graphs (C) showing ovarian cyclicity determined by vaginal cytology through daily vaginal smears.

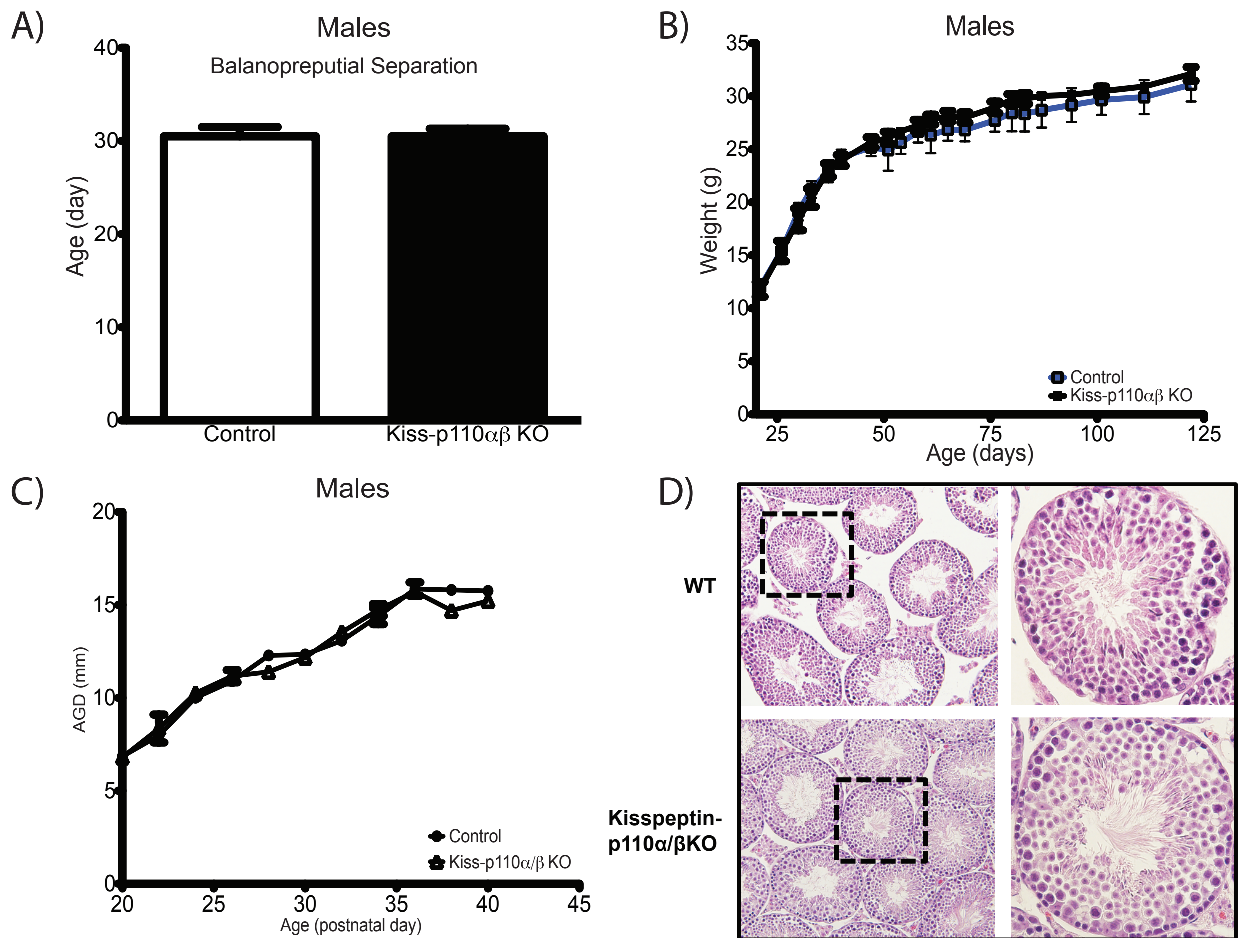


Fig. 13. *Male Pubertal Development and Adult Testicular Morphology.* Age at puberty onset (A) as determined by balanopreputial separation in WT and Kiss-p110α/β KO males. Post weaning weight gain (B) and anogenital distance (C) of WT and Kiss-p110α/β KO males. Representative photomicrographs (D) of WT and Kiss-p110α/β KO male H&E stained testes.

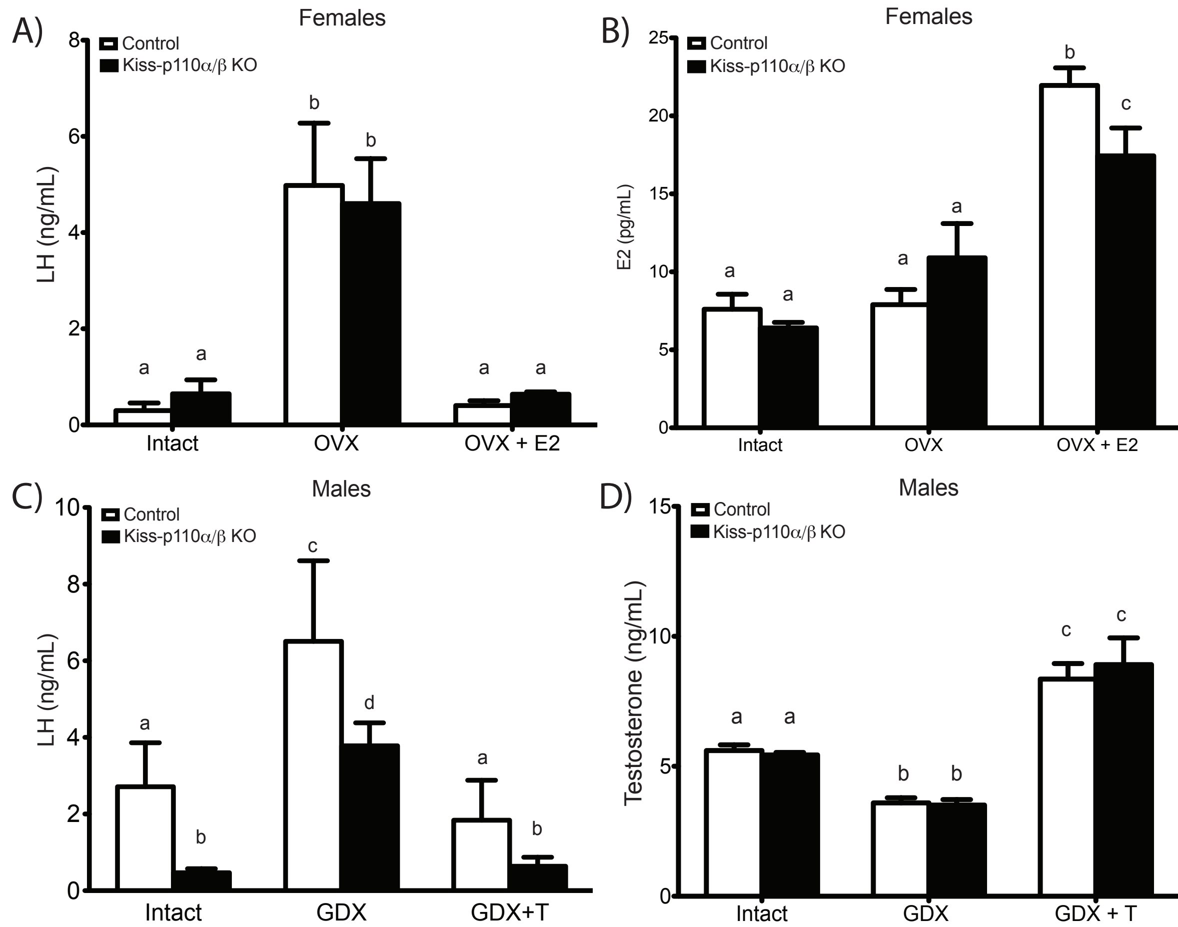


Fig. 14. *Steroid Hormone Negative Feedback on LH Levels.* LH (A) and E_2 (B) levels in intact, OVX, and OVX + E_2 WT and Kiss-p110α/β KO female mice. LH (C) and T (D) levels in intact, GDX, and GDX + T WT and Kiss-p110α/β KO male mice.

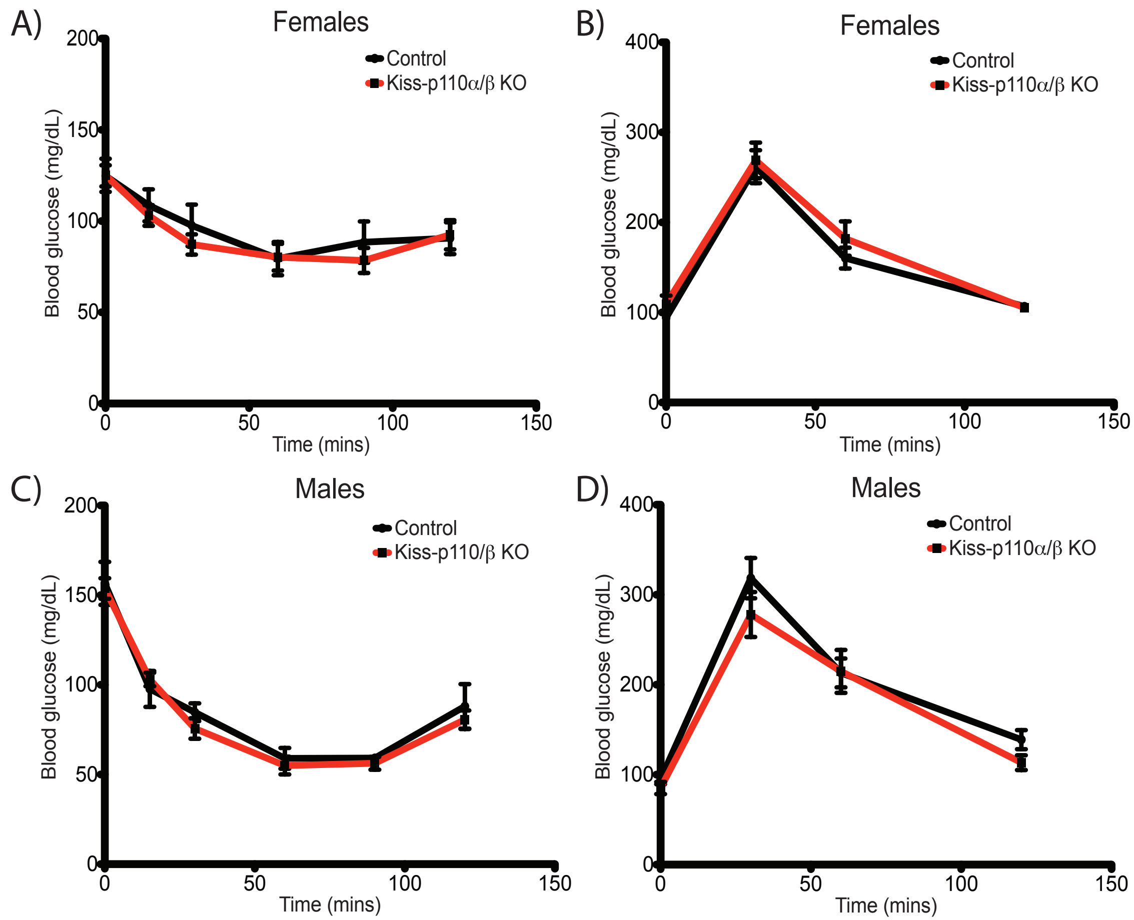


Fig. 15. *Glucose and Insulin Sensitivity in WT and Kiss-p110α/β KO mice.* Ability to clear exogenously administered glucose in WT and Kiss-p110α/β KO female (A) and male (C) mice. Blood glucose response to exogenously administered insulin in WT and Kiss-p110α/β KO female (B) and male (D) mice.

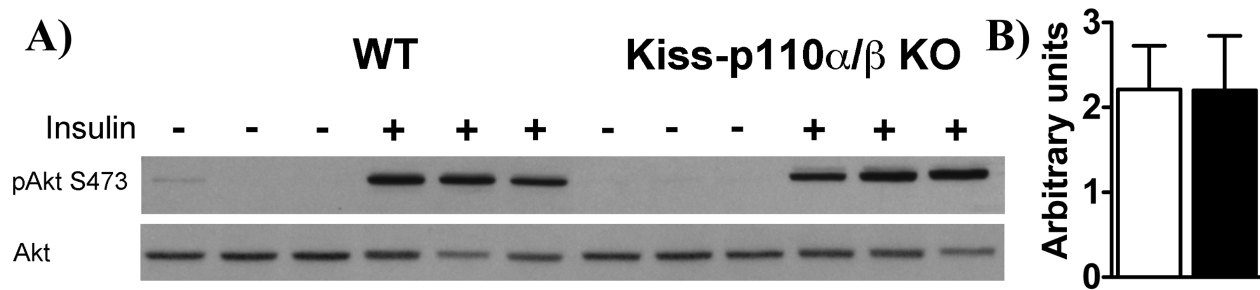


Fig. 16. *Peripheral Insulin Sensitivity in WT and Kiss-p110α/β KO male livers.* Western Blot analysis of pAkt in WT and Kiss-p110α/β KO male mice after insulin treatment (A). Densitometry analysis of western blot with arbitrary units (B). Open bar = WT; Black bar = Kiss-p110α/β KO.

CHAPTER 3

PHOSPHOINOSITIDE 3-KINASE SINGALING IN GALANIN-LIKE PEPTIDE EXPRESSING NEURONS

INTRODUCTION:

As with kisspeptin, GALP seems to be another interesting candidate for investigating how metabolic signals, such as insulin and leptin, regulate the HPG axis. To this extent we sought to determine whether PI3K signaling in GALP neurons is part of the downstream signaling mechanism activated by peripheral metabolic cues that influence reproduction, such as insulin. In addition, we investigated the role of GALP-neuron-specific PI3K signaling in the regulation of hypothalamic *GALP* mRNA expression and the metabolic control of LH release in male and female mice. Our data demonstrate that GALP neurons are direct targets of steroid hormones and that PI3K signaling regulates hypothalamic *GALP* mRNA expression and LH levels in a sex-specific fashion.

MATERIALS AND METHODS:

Animals:

Animals were housed at Stony Brook University, Division of Laboratory Animal Resources (DLAR) under a 12-hr light, 12-hr dark cycle and had access to water and rodent chow *ad libitum*. All procedures were approved by the Institutional Animal Care and Use Committee at Stony Brook University Medical Center in accordance with the NIH Guide for the Care and Use of Laboratory Animals.

Generation of GALP- p110α/β cKO:

All animals used were of a 129Sv-C57BL/6 mixed genetic background. To generate mice in which the genes for the PI3K catalytic subunits p110α (PIK3CA) and p110β (PIK3CB), are specifically deleted in GALP neurons, double floxed mice, p110$\alpha^{flx/flx}$/p110$\beta^{flx/flx}$ were mated with mice carrying the Cre transgene under the control of the GALP promoter (GALP-Cre). Double floxed animals bear loxP sites flanking exon 1 of the PIK3CA gene and exons 3 and 4 of the PIK3CB gene (Lu 2009). Mice generated by the first breeding were then intercrossed to generate GALPCre-p110$\alpha^{flx/flx}$/p110$\beta^{flx/flx}$ and GALPCre^{+}p110$\alpha^{flx/flx}$/p110$\beta^{flx/flx}$ animals, referred to here as WT and GALP-p110α/β cKO, respectively. Animals were screened for the presence of Cre and floxed p110α and p110β by PCR of isolated genomic tail DNA as described previously (Lu 2009, Rosati 2011). In addition, using genomic DNA from various tissues, including the hypothalamus, the presence or absence of Cre-mediated recombination was detected via PCR. Bands indicating deletion of both the p110α and p110β alleles were gel extracted, subcloned and sequenced, to verify that they were indeed the expected PCR products.

Generation of FoxO1GFP-GALP mice:

To monitor the activity of the PI3K-Akt signaling pathway in GALP neurons, FoxO1GFP reporter mice were crossed with GALP-CRE+ mice. FoxO1GFP is expressed under the control of a ubiquitous promoter that is silenced by a loxP flanked transcriptional blocker (Fukuda *et al.* 2008). Thus, the resulting FoxO1GFP-GALP-CRE+ animals expressed the FoxO1GFP protein in GALP-CRE+ neurons only. To confirm that the deletion of PI3K catalytic subunits ablated PI3K activity, FoxO1GFP-GALP-CRE+ mice were crossed with GALP-p110α/β cKO animals (GALPCre^{+} p110$\alpha^{flx/flx}$/p110$\beta^{flx/flx}$). The resulting heterozygous animals were intercrossed until

Cre+ mice homozygous for all three floxed alleles (FoxOGFP, p110α, and p110β) were obtained: FoxO1GFP-GALP-p110α/β cKO. The genotype of mice was obtained by PCR as described previously (Fukuda *et al.* 2008).

Effects of icv infusion of insulin on FoxO1GFP subcellular localization in GALP neurons:

Adult FoxO1GFP-GALP mice were anesthetized with ketamine (80 mg/kg ip; Butler Schein Animal Health, Dublin, OH) and xylazine (32 mg/kg ip; LLOYD Laboratories, Shenandoah, IA), and stereotaxic surgery was used to target a guide cannula (Plastics One, Roanoke, VA) to the right lateral ventricle (coordinates: 0.34 mm caudal to Bregma, 2.5 mm ventral to the skull, 0.9 mm lateral). Six days later, correct placement of the cannula was verified by observing their response to icv infusion of 10 ng of angiotensin II. Mice were fasted overnight with *ad libitum* access to water. The following day animals were infused with human insulin (100 pmol, Humulin R; Eli Lilly Corp., Indianapolis, IN) or saline. Thirty minutes later, mice were intracardially perfused with 0.1M PBS followed by 4% paraformaldehyde. Brains were removed and postfixed in 4% paraformaldehyde overnight, followed by immersion in 30% sucrose cryoprotectant. Then, 25 μm sections were cut in series of 3 using a cryostat and placed in tissue culture wells containing cryoprotectant solution and stored at -20°C until processed for staining.

Immunofluorescence:

Free floating sections were washed several times in PBS, then incubated for 48 hours at 4°C in a rabbit anti-GFP primary antibody (1:20,000, Invitrogen, Carlsbad, CA) with 0.25% Triton X-100 in 0.1M PBS. After washing in PBS, the tyramine signal amplified fluorescence

method was used as previously described using streptavidin conjugated Alexa 488 (Invitrogen) (Hoffman *et al.* 2008). To investigate ERa and AR expression in GALP neurons, GALP-Cre mice were mated with ROSA26-YFP mice (kindly provided by S. Srinivas, University of Oxford, Oxford, United Kingdom). Animals were intracardially perfused and the brains were processed as described above. Coronal sections (14-μm) were obtained using a cryostat. Then, sections were blocked in 1X PBS, 0.025% TX-100, 5% horse serum, and then treated with rabbit anti-estrogen receptor α (ERα) (1:1,000; Millipore, MA) antiserum or with rabbit anti- AR (1:1000, Santa Cruz, Dallas, TX) overnight at 4 °C, followed by Cy3-donkey anti-rabbit IgG (1:500; Jackson Labs, Birmingham, AL) for 1 hr at room temperature. Sections were then coverslipped with Fluoromount-G (Southern Biotech).

Image analysis:

Fluorescence images were acquired using an ApoTome imaging system (Imager Z1; Zeiss, Thornwood, NY) with a 20X objective. Quantification of nuclear and cytoplasmic fluorescence was done as previously described (Fukuda *et al.* 2008). Briefly, fluorescent intensity (pixel intensity) was measured within the cytoplasm and the nuclear region, as well as outside the cell (background) using the AxioVision 4.1 software. After correction for background, the mean pixel intensity was used to determine the nuclear:cytoplasmic (N:C) ratio of fluorescence intensity. Neurons with an N:C ration of <1:2 were classified as having cytoplasmic FoxO1GFP staining, an N:C ratio of 2:1 or more signified nuclear, whereas an N:C ratio between 1:2 and 2:1 meant that the neuron had both nuclear and cytoplasmic FoxO1GFP staining. The mean N:C ratio was determined from 100 neurons in the ARC for each treatment. After analysis of neurons from both sides of the ARC (bilateral), the data were plotted as the

percentage of neurons with cytoplasmic FoxO1GFP (# of neurons with a cytoplasmic N:C ratio / total # of neurons).

The effects of 48 hr fasting on serum LH levels:

WT and GALP-p110α/β cKO adult (5 months) males were randomly assigned to either a 48 hr fast or *ad libitum* fed (control group). At the end of the experiment animals were weighed and their glucose levels recorded. Animals were killed in the morning between 9:00 and 10:30 am and their blood was collected via cardiac puncture. Plasma was obtained and stored at -20° C for LH, insulin, and leptin measurements. Brains were removed, and the MBH was dissected and immediately frozen in dry ice.

The effects of 24 hr fasting on serum LH levels:

In this experiment each animal served as its own control. Initial body weight and glucose levels were recorded from adult (3 months) WT and GALP-p110α/β cKO females. Between 9:00 and 10:30 am 100-150 μl of blood was collected from the facial vein using the Lancet method(Golde *et al.* 2005). Seven days later, animals were fasted for 24 hours beginning at 9:00 am. After the fast, blood and tissue was collected. Serum was stored at -80°C until assays were performed. A separate cohort of age-matched females was used to obtain MBH for GALP RT-PCR studies.

Gonadectomies and steroid hormone replacement:

Adult female mice from each genotype were randomly assigned to one of two treatment groups: ovariectomy (OVX) + vehicle, or OVX + E_2, (n=6-7 / genotype / treatment). Surgery

was performed under isoflurane inhalation anesthesia. Immediately after OVX, animals received subcutaneous SILASTIC capsules (1.5 cm in length plugged with silicone adhesive on each end to leave 1 cm to fill with treatment, inner diameter 1.47 mm; outer diameter 1.95 mm) containing sesame oil (OVX + V) or SILASTIC capsules containing 1mg/ml E_2 in sesame oil (OVX + E_2)(Gottsch *et al.* 2009). Seven days after surgeries, animals were killed between 9:00 – 10:30 am and tissue and brains obtained. Serum was assayed for E_2 and LH levels.

Male mice of both genotypes were castrated under isoflurane anesthesia. Immediately after surgery, animals received subcutaneous SILASTIC capsules (inner diameter 1.02; outer diameter 2.16 mm) pack with 5 mm of testosterone (T) (Sigma-Aldrich, St. Louis, MO), or left empty. Animals were killed 7 days after surgery between 9:00 – 10:30 am and tissue and brains obtained. Serum was assayed for LH and T.

GALP real-time RT-PCR:

To measure GALP mRNA, we dissected the MBH with the aid of a precision brain slicer for adult mouse brain (Braintree Scientific, Braintree, MA). We obtained coronal sections of brain tissue (2 mm) in which the posterior edge of the optic chiasm and the beginning of the mammillary bodies served as rostral and caudal boundaries, respectively. Then, under a dissection microscope two bilateral parasagittal cuts, each 1.5 mm lateral to the third ventricle, and one horizontal cut 2.0 mm dorsal to the ventral surface were made. These margins were chosen to ensure that each tissue sample contained the entire GALP neuronal population from the ARC of each animal.

Total RNA was extracted using Trizol (Invitrogen) according to the manufacturer instructions. RNA (2.5 μg) was reverse-transcribed to cDNA using the SuperScript VILO

cDNA synthesis kit (Applied Biosystems, Foster City, CA). Real-time PCR was performed at the Genomics core facility, Stony Brook University, with an ABI 7300 Real-Time PCR system (Applied Biosystems) using TaqMan probe-based gene expression analysis and primer sequences specific for the *Galp* gene (Id: Mm00626135-m1, Applied Biosystems). A 100 ng total RNA equivalent cDNA was used for TaqMan PCR (Applied Biosystems, TaqMan Universal Master Mix). Samples were run in duplicates to obtain an average threshold value (Ct). Quantities of GALP mRNA were normalized to 18s ribosomal RNA gene (Id: Mm03928990_g1). To determine the PCR reaction efficiencies for the *Galp* and the 18S gene, we ran a standard curve for each gene, using a 1:5 dilution of RNA. The difference between average target gene (*Galp*) Ct and average control gene (18S) Ct (ΔCt) for each dilution was plotted against the log input cDNA amounts. Because the PCR efficiencies of the target gene (*Galp*) and control gene (18S) reactions were different (slope of the curve more than 0.1), we used the standard curve method for relative quantification of the transcript level. Samples prepared without reverse transcriptase were used as negative controls.

Hormone assays:

Serum measurements of LH from male animals used in the 48 hr fast experiment and mice used in the E_2 and T studies were conducted at the University of Virginia Core Facility. Plasma LH levels were determined by RIA using reagents obtained from the National Institute of Diabetes and Digestive and Kidney Diseases (National Institutes of Health, Bethesda, MD), including LH reference (RP-3) and anti-rat LH antibody (S-11). The assay had a lower limit of detection of 0.2 ng/ml. Intra-assay and inter-assay coefficients of variance were 8.28 and 9.66%, respectively. Serum T levels were measured by ELISA (R&D Systems, Minneapolis, MN),

sensitivity of 0.041 ng/mL. Serum E_2 was measured by ELISA (Calbiotech, Spring Valley, CA) with sensitivity of <3 pg/ml. Serum leptin levels were measured by a solid phase sandwich ELISA (R&D Systems), with a sensitivity range of 62.5 - 4,000 pg/mL. Serum insulin levels were measured by ELISA (Millipore, Billerica, MA), with a sensitivity range of 0.2 -12.8 ng/mL. For experiments using fasted females, serum LH levels were measured using a Milliplex MAP immunoassay (mouse panel; Millipore) in the Luminex 200.

Evaluation of glucose homeostasis:

Glucose measurements were obtained from tail blood using a glucometer (One-Touch Ultra Mini, Lifescan, Milpitas, CA). For glucose tolerance tests, mice were fasted overnight and blood glucose levels were measured immediately before (t=0) and 30, 60, and 120 min after injection with D-glucose (2 g/kg BW). For insulin tolerance tests 4 hr-fasted male mice were injected ip. with 1mU/g human insulin and female mice received 0.5 mU/g. Blood glucose was measured before and 15, 30, 60, 90, and 120 min after insulin injection.

Energy homeostasis phenotype: metabolic chambers:

Food intake, ambulatory activity, O_2 consumption, CO_2 production, and heat production, were simultaneously recorded using a combined indirect calorimetry system (Comprehensive Lab Animal Monitoring System, Oxymax Columbus Instruments, Columbus, OH). Animal motion (ambulatory movements) was detected using a triple action (infrared) IR photocell technology, in which interruption of an IR beam will accrue one "count". The respiratory exchange ratio (amount of VCO2 consumed/VO2 produced) was also provided. Food and water were provided *ad libitum*. Mice were housed individually at room temperature under a 12 hr

light, 12 hr dark cycle. All parameters were recorded for at least 96 hours, and data obtained in the last 24 hours were used in the final analysis.

Statistical analyses:

Data are expressed as mean ± SEM unless stated otherwise. Statistical analysis was done using Sigma Plot 10 (San Jose, CA). Differences between WT and GALP-p110α/β cKO were analyzed using a two-way ANOVA, followed by Holm-Sidak, multiple comparison method. Two-way repeated measures ANOVA was used to analyze data obtained using mice as their own control. GraphPad Prism 4 (Graph-Pad, San Diego, CA) was used for graphic illustrations. For all statistical analyses, $P < 0.05$ was considered significant.

RESULTS:

Deletion of insulin-induced PI3K-Akt signaling in GALP neurons:

To monitor the PI3K-Akt signaling pathway in GALP neurons we assessed the localization of FoxO1GFP in reporter mice (FoxO1GFP-GALP mice). Single label immunohistochemistry for FoxO1GFP on sections through the MBH showed that FoxO1GFP was selectively expressed in the ARC-ME in accordance with previous reports for hypothalamic GALP expression in mice (Juréus *et al.* 2001). Thirty minutes after insulin infusion, FoxO1GFP was translocated from the nucleus to the cytoplasm in >55% of GALP neurons (Fig. 17B, 17C). This is in contrast to nuclear to cytoplasmic translocation of signal in <12% of GALP neurons from saline-treated FoxO1GFP-GALP animals. To confirm that deletion of PI3K catalytic subunits in GALP neurons abolished PI3K activity, we crossed FoxO1GFP-GALP reporter mice with GALP-p110α/β cKO animals, in which both catalytic subunits were deleted in GALP

neurons. ICV infusion of insulin failed to exclude FoxO1GFP from the nucleus of GALP neurons from GALP-p110α/β cKO animals (Fig. 17B, 17C).

The selective deletion of p110α and p110β in GALP neurons was also validated through PCR of genomic DNA from various tissues of Cre+ animals. The p110α and p110β alleles were deleted from the genome in the MBH, but not from GALP-negative tissue such as the tail, liver, ovary, and uterus (Fig. 17A).

GALP-p110α/β cKO does not affect pubertal onset or fertility:

We recorded puberty onset in GALP-p110α/β cKO and WT control mice using external markers such as balanopreputial separation in males and day of vaginal opening and first day of estrus in females. The time of balanopreputial separation was not significantly different between GALP-p110α/β cKO and WT males (27.7 ± 0.7 days vs. 26.2 ± 0.4 GALP-p110α/β cKO and WT, respectively, n = 13, $P > 0.05$). Similarly, the day of vaginal opening was not significantly different between GALP-p110α/β cKO and WT females (27.2 ± 0.6 v. 27.4 ± 0.6 GALP-p110α/β cKO and WT, respectively, (n = 14). In addition, genotype did not affect the first day of estrus (41.9 ± 1.2 vs. 41.4 ± 1.2 GALP-p110α/β cKO and WT, respectively, n = 14)

Assessment of cyclicity through vaginal cytology indicated that adult GALP-p110α/β cKO females have estrous cycles similar to those of their WT littermates (data not shown). Furthermore, GALP-p110α/β cKO females when paired with a WT male were able to become pregnant, deliver healthy litters, and raise them to weaning age. Likewise, GALP-p110α/β cKO males successfully impregnated WT females.

Hypothalamic GALP mRNA levels are reduced in fasted and non-fasted GALP-p110α/β cKO males:

We hypothesized that ablation of PI3K signaling in GALP neurons will impair their ability to respond to changes in peripheral metabolic signals such as insulin. Hence, we reasoned that compared to WT, the gonadotropic axis of GALP-p110α/β cKO males will be more susceptible to the negative effects of fasting. To test this hypothesis we randomly assigned WT and GALP-p110α/β cKO males to either a fed group or a group fasted for 48 hours. There were no significant differences in the body weight of GALP-p110α/β cKO and WT mice that were fed *ad libitum* (Fig 18A). Males subjected to a 48 hr fast weighed significantly less than fed males, and there was no effect of genotype on weight loss (Fig 18A). Similarly, compared to non-fasted controls, a 48 hr fast significantly decreased blood glucose and serum leptin levels in animals of both genotypes (Fig. 18B and Fig. 18C). There was no significant effect of genotype on serum glucose or leptin levels (ANOVA: genotype, $P = 0.42$ and 0.45 for glucose and leptin levels, respectively). However, there was a significant interaction between genotype and treatment on serum insulin levels (ANOVA: $P = 0.03$). Whereas a 48-hour fast decreased serum insulin levels in WT animals, serum insulin levels were not different between fed and fasted GALP-p110α/β cKO mice (Fig. 19A). No effect of food manipulation or genotype was observed on serum LH concentrations in males (2-way ANOVA; $P > 0.05$; Fig. 19B). Serum T levels were not different between non-fasted WT and non-fasted GALP-p110α/β cKO animals (4.8 ± 2.2 vs. 4.9 ± 1.3 ng/ml, GALP-p110α/β cKO vs. WT, respectively).

Deletion of p110α and p110β in GALP neurons resulted in a 5.2-fold decrease in MBH *GALP* mRNA levels (2-way ANOVA, genotype, $P < 0.001$; Fig. 19C). However, no significant interaction between genotype and treatment on *GALP* mRNA expression in the MBH was

observed ($P > 0.05$). Fasting decreased MBH *GALP* mRNA expression in WT animals ($P < 0.05$), whereas fasting did not further reduce *GALP* mRNA levels in the MBH of GALP-p110α/β cKO.

LH levels are significantly higher in GALP-p110α/β KO females before and after fasting:

Previous studies have suggested that females are more susceptible to nutritional stressors than males (Acosta-Martinez 2011). Therefore, we chose a 24-hr fasting model to investigate whether GALP-p110α/β cKO females have increased susceptibility to the suppressive effect of negative energy balance on the gonadotropic axis. There was no significant difference in initial body weight between GALP-p110α/β cKO and WT female mice (Fig. 18D). The 24 hr fast caused a similar and significant weight loss in females of both genotypes (Fig. 18D, ANOVA, $P < 0.001$).

Fasting significantly decreased serum glucose (2-way ANOVA: fed vs. fasted, $P < 0.05$; Fig. 18E). There was no significant effect of genotype on serum glucose levels (ANOVA: genotype, $P = 0.86$). Initial leptin concentrations did not differ between GALP-p110α/β cKO and WT females (Fig. 18F). After 24 hr of fasting leptin concentrations were significantly lower in WT female mice (Fig. 18F). However, serum leptin levels were not significantly lower after the fast in the GALP-p110α/β cKO females. Similarly, initial insulin levels were not different between GALP-p110α/β cKO and WT mice (Fig. 19D). However, 24 hr of fasting failed to significantly decrease serum insulin levels in mice of either genotype (ANOVA: treatment, $P = 0.47$).

In WT females LH levels declined after a 24 hr fast, but the decrease was not statistically significant (0.50 ± 0.13 ng/ml before vs. 0.33 ± 0.04 ng/ml after the fast). However, there was a

significant genotype effect on serum LH concentrations (Fig. 19E, ANOVA: genotype, $P < 0.05$). Mean serum LH levels were higher in GALP-p110α/β cKO mice. However, statistical comparisons for factor (genotype) within each treatment group were not significant ($P > 0.05$). In addition, there was no significant genotype effect on *GALP* mRNA levels from the MBH of females that were fasted for 24 hours or in a separate cohort of intact non-fasted female mice (Fig. 19F). In the latter group, serum E_2 levels were within the physiological range and no genotype effect was observed (9.85 ± 1.8 vs. 10.6 ± 1.6 pg/ml, GALP-p110α/β cKO vs. WT, respectively).

Hypothalamic GALP mRNA expression in gonadectomized WT and GALP- p110α/β KO mice:

The regulation of hypothalamic *GALP* mRNA levels by steroid hormones was investigated using gonadectomy of male and female animals. Compared to vehicle-treated castrated mice, T treatment decreased MBH *GALP* mRNA levels in males of both genotypes (Fig. 20A). However, GALP mRNA levels in GALP-p110α/β cKO were not significantly different from their WT controls in each treatment group (ANOVA genotype, $P = 0.09$). As expected, compared to GDX + T treated group, serum LH levels are significantly higher in the GDX + V treated males of both genotypes (Fig. 20B). The difference was significant by treatment ($P < 0.001$) but not genotype. However, within the GDX + V group, serum LH levels are significantly different between WT and GALP-p110α/β cKO mice (Fig. 20B, $P = 0.03$, Holm-Sidak multiple comparison test). Measurements of serum T levels also confirmed the effectiveness of the treatment; mice that were castrated and implanted with T filled capsules had elevated T levels relative to castrated animals treated with empty capsules (2.1 ± 0.1 and 1.8 ±

0.1, GDX + empty capsule, in GALP-p110α/β cKO and WT mice, respectively; 6.5 ± 0.4 ng/ml and 7.0 ± 0.4, in GDX + T-treated GALP-p110α/β cKO and WT mice, respectively).

Similar to the effects of T in males, E_2 treatment significantly reduced *GALP* expression in the MBH of WT and GALP-p110α/β cKO females when compared to vehicle-treated controls (Fig. 20C). Serum levels of LH in OVX + V treated mice were elevated relative to those in OVX + E_2-treated mice of both genotypes (Fig. 20D). ANOVA showed a significant treatment effect on LH levels ($P < 0.0001$) whereas no genotype effect was observed ($P = 0.62$). Serum E_2 levels were elevated in OVX mice treated with E_2-filled capsules, further confirming the effectiveness of the E_2 treatment (12.8 ± 1.9 and 11.0 ± 1.5 in GDX + vehicle-treated GALP-p110α/β cKO and WT mice, respectively; 46.6 ± 3.5 and 40.8 ± 3.6 in GDX + E_2-treated GALP-p110α/β cKO and WT mice, respectively).

To investigate whether GALP neurons in the ARC express steroid hormone receptors we performed immunofluorescence analysis on ARC sections obtained from GALP-Cre/Rosa-YFP mice. In intact WT GALP-Cre/Rosa-YFP mice approximately 32% of GALP cell bodies colabeled with anti-ERα antiserum (32 ± 1.4%; n = 2) and approximately 15% co expressed ARs (14.7% ± 1.8%; n = 3) (Fig. 21).

GALP-p110α/β cKOs have normal metabolic regulation and tolerance to insulin and glucose:

Deletion of PI3K signaling in GALP neurons did not affect post-weaning body weight in male or females (Fig. 22A and 22B). Because GALP plays a role in the neuroendocrine regulation of feeding, body weight, and body temperature, weight-matched GALP-p110α/β cKO and WT mice were placed in metabolic chambers. No genotype effect was observed on food intake or activity levels over a 24 hr period in either sex (Fig. 22C and 22G). In addition, GALP-

p110α/β cKO and WT mice of both sexes had similar energy expenditures as measured by O_2 consumption, heat production, and respiratory exchange ratio (Fig. 22D, 22E, and 22F). Food intake, ambulatory activity, heat production, and oxygen consumption were not different between GALP-p110α/β cKO and WT mice when data were analyzed during 12 hr dark cycle and 12 hr light cycle (data not shown).

Glucose tolerance tests (GTT) and calculated areas under the curve (AUC) did not show differences between GALP-p110α/β cKO (n = 12-14) and WT (n = 12-13) animals of either sex (data not shown). There were no differences in fasting glucose levels at the start of the GTTs. In addition, the insulin tolerance test (ITT) showed that GALP-p110α/β cKO mice display insulin sensitivity comparable to WT littermates at 5 months of age (data not shown).

DISCUSSION:

The effects of GALP on the reproductive axis, such as stimulation of gonadotropin release and sexual behavior, seem to be sex-specific (Castellano *et al.* 2006; Rich *et al.* 2007). For example, adult male rats but not females respond to central administration of GALP, which increases sexual behavior and LH levels (Castellano *et al.* 2006).

It has been suggested that the effects of GALP on LH levels in females might be steroid hormone dependent unlike in males (Castellano *et al.* 2006; Rich *et al.* 2007). Hence, the physiological explanation for the sex-specific effects of GALP on the neuroendocrine axis has not been clear. T and E_2 decrease the expression of *GALP* expression in both WT and GALP-p110α/β cKO male and female mice, indicating that steroid hormone signaling acts independent of PI3K signaling to regulate *GALP* expression. This is in contrast to our findings in Kiss-p110α/β KO mice, where Kiss-p110α/β KO males have significantly decreased *Kiss1* expression

compared to WT regardless of gonadal status and Kiss-p110α/β KO females only have a significant change in *Kiss1* in agonadal conditions. This suggests that the interactions between PI3K and steroid hormone signaling pathways may not have as significant of an effect in GALP expressing neurons as it does in kisspeptin neurons.

The molecular mechanisms by which GALP and other hypothalamic neurons, such as kisspeptin, relay information about the metabolic status to the GnRH system are still not fully realized. PI3K signaling is used by neurons to detect changes in peripheral energy stores, which in turn is reflected by changes in serum leptin and insulin levels (Belgardt *et al.* 2008; Plum *et al.* 2009). The fact that in the GALP-p110α/β cKO females LH levels remained high even after the fast, suggest that the absence of PI3K signaling in GALP neurons alters their ability to receive input from peripheral metabolic cues, and in turn impairs communication to GnRH neurons. Thus, we propose that PI3K signaling in GALP neurons serves as an integrator of metabolic cues that in turn relay metabolic status to the reproductive axis. This may be in contrast to the role of Kiss-PI3K signaling that may not have a significant role in relaying low to normal metabolic cues to the GnRH system. However, both models should be assessed under conditions of over-nutrition since PI3K signaling is activated by the presence of leptin and insulin. By blocking PI3K signaling in these neurons we may change the response to the metabolic signals seen in conditions of over-nutrition such as a treatment with a high fat diet.

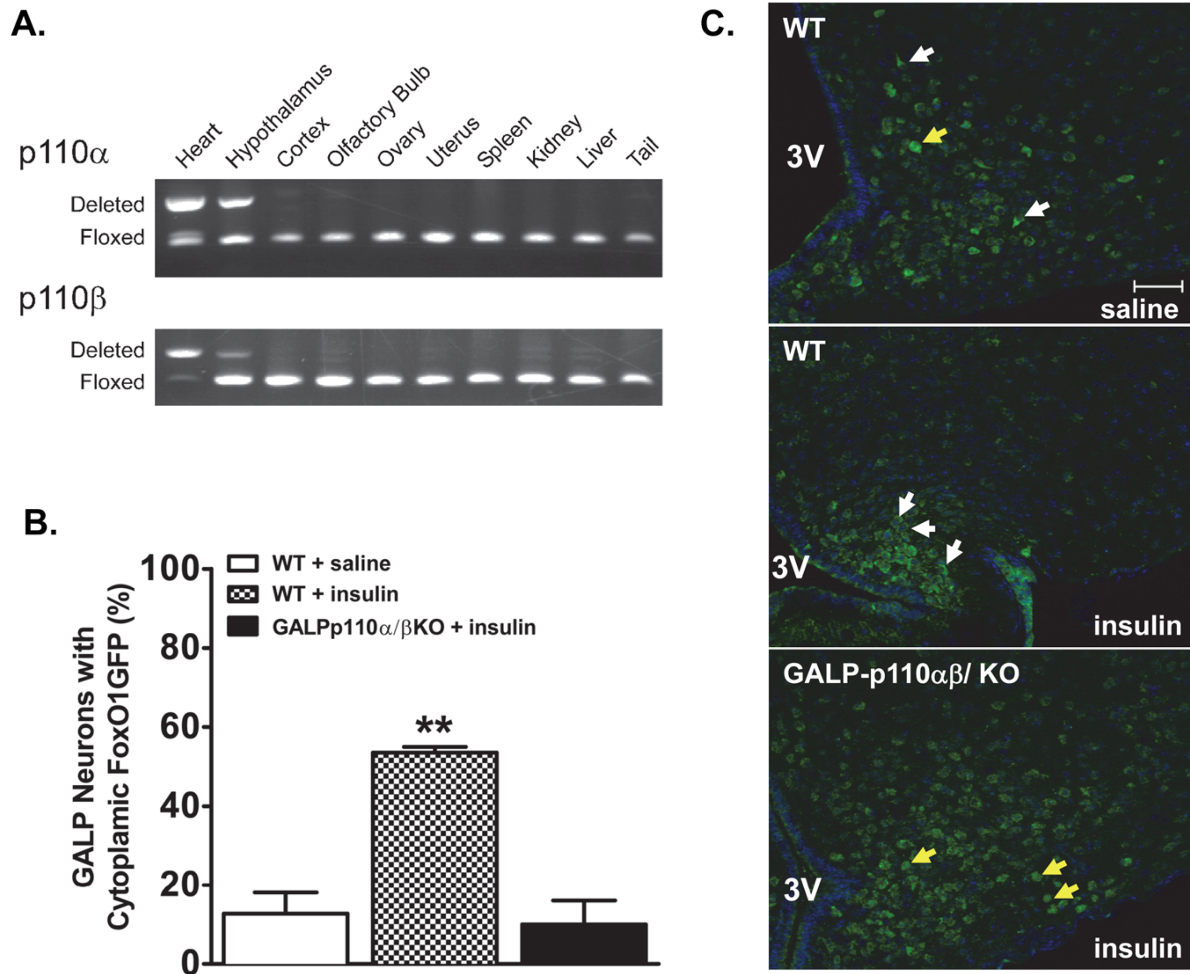


Fig. 17. *The GALP-neuron specific deletion of PI3K catalytic subunits p110α and p110β abolishes the insulin-induced nuclear export of FoxO1GFP within GALP neurons*. **(A)** PCR products showing site of Cre-mediated DNA recombination in tissues from a Cre positive mouse. Deletion of p110α and p110β was detected in the hypothalamus. DNA from the heart of heart-specific p110α/β cKO mouse served as a positive control (first lane) **(B)** Quantification of FoxO1GFP nuclear translocation in saline-treated WT, insulin-treated WT, and insulin-treated FoxO1GFP-GALP-p110α/β cKO animals (n = 3-4). Subcellular localization of FoxO1GFP is plotted as the percentage of neurons with cytoplasmic FoxO1GFP ** $P < 0.001$, One-way ANOVA, followed by Newman-Keuls multiple comparison test **(C)** Subcellular localization of FoxO1GFP in GALP neurons from saline and insulin-treated FoxO1GFP-GALP and FoxO1GFP-GALP-p110α/β cKO animals. After an overnight fast, mice were infused with saline or insulin (100 pmol, 30 min). White and yellow arrows point to GALP neurons with cytoplasmic and nuclear FoxO1GFP localization, respectively. 3V, third ventricle. Scale bar, 50 µm.

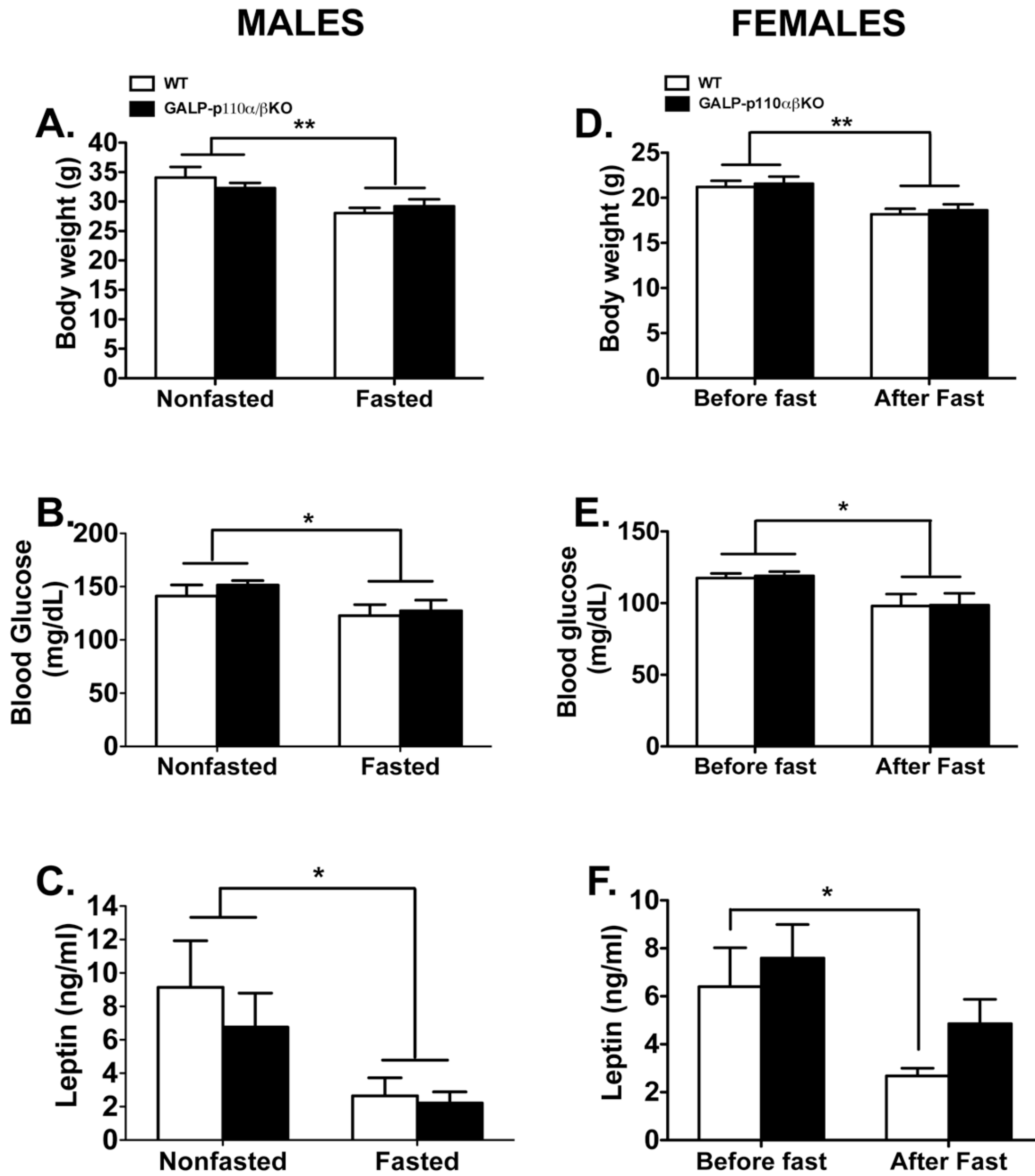


Fig. 18. *Effects of a 48-hr and of a 24-hr fast on metabolic parameters of WT and GALP-p110α/β cKO male and female mice, respectively.* Fasting reduced body weight **(A, D)** and glucose **(B, E)** levels in WT and GALP-p110α/β cKO mice of both sexes. A 48 hr fasting reduced serum leptin **(C)** levels in WT and GALP-p110α/β cKO males, whereas a 24 hr fasting reduced leptin levels in WT females **(F).** (Males: 2-Way ANOVA; Females: 2-way ANOVA with repeated measures, *$P < 0.05$, **$P < 0.001$); n = 7-8 genotype/treatment. There was no significant effect of genotype or the interaction between food manipulation and metabolic parameters.

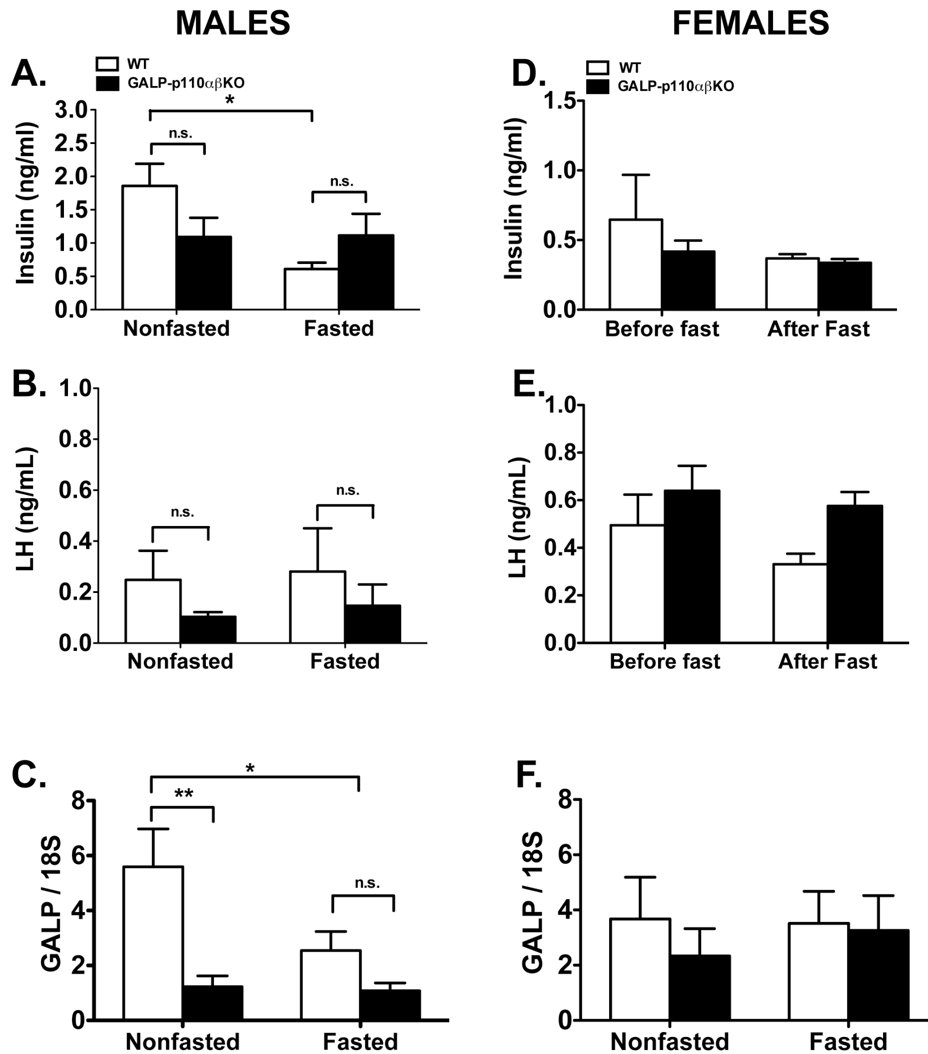


Fig. 19. *Effects of a 48-hr and of a 24-hr fast on serum insulin* (**A, D**), serum LH levels (**B, E**), and on hypothalamic GALP mRNA expression (**C, F**) in WT and GALP-p110α/β cKO male and female mice. **(A)** In males, there was a significant interaction between food manipulation and genotype on serum insulin levels (2-way ANOVA, $P < 0.05$); multiple comparison test revealed a significant effect of fasting on serum insulin levels in WT animals (* $P < 0.05$) but not in GALP-p110α/β cKO males **(D)** A 24 hr fast did not affect serum insulin levels in females of either genotype. **(B)** A 48 hr fast did not affect serum LH levels in WT or in GALP-p110α/β cKO males and no genotype effect was observed in males subjected to either food manipulation. **(E)** A 24 hr fast did not affect serum LH levels in female animals of both genotypes. However, compared to WT littermates, GALP-p110α/β cKO females have higher serum LH levels, (2-way ANOVA, repeated measures, $P < 0.05$). **(C)** There was a significant genotype effect on hypothalamic GALP mRNA levels in males (2-way ANOVA, **$P < 0.01$); compared to fed WT mice, GALP mRNA levels from the MBH of GALP-p110α/β cKO males were significantly lower regardless of the food treatment, (Holm-Sidak multiple comparison test, $P < 0.05$). **(F)** Hypothalamic GALP mRNA levels were similar in fasted vs. non-fasted females of either genotype. Data were expressed as ± SEM.

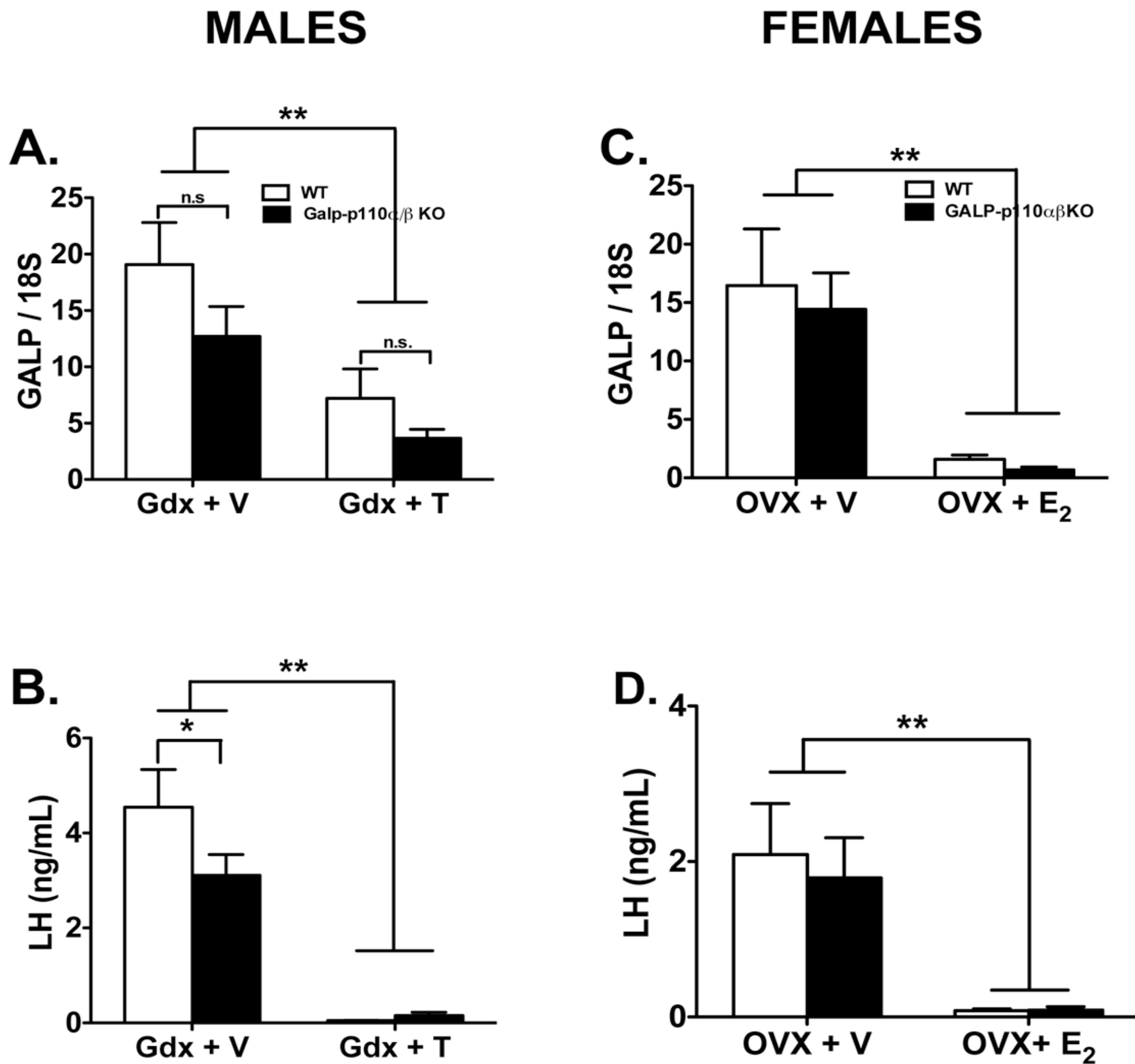


Fig. 20. *Hypothalamic GALP mRNA expression* (**A, C**), and serum LH levels (**B, D**), in WT and GALP-p110α/β cKO mice that were gonadectomized (GDX) and treated with vehicle- (GDX + V for males, OVX + V, females,), or GDX and treated with steroid hormone (GDX + T males, OVX + E_2, females) for 7 days. **(A)** *GALP* mRNA levels in the MBH of males that were castrated and treated with vehicle were significantly higher than those in the castrated and T-treated group of either genotype (2-way ANOVA, $^{**}P < 0.001$)**.** **(C)** *GALP* mRNA levels in the MBH of females that were OVX and treated with vehicle were significantly higher than those in the OVX + E_2 treated group of either genotype (2-way ANOVA, $P < 0.001$). No significant effect of genotype or a significant interaction between genotype and steroid treatment was found in males or females. Compared to GDX + vehicle treated mice, serum LH levels were increased after GDX in males (**B**) and in females (**D**) of both genotypes, (ANOVA, $^{**}P < 0.001$). In addition, within the GDX + V treated group and compared to WT, serum LH levels were significantly lower in GALP-p110α/β cKO males. Data were expressed as ± SEM.

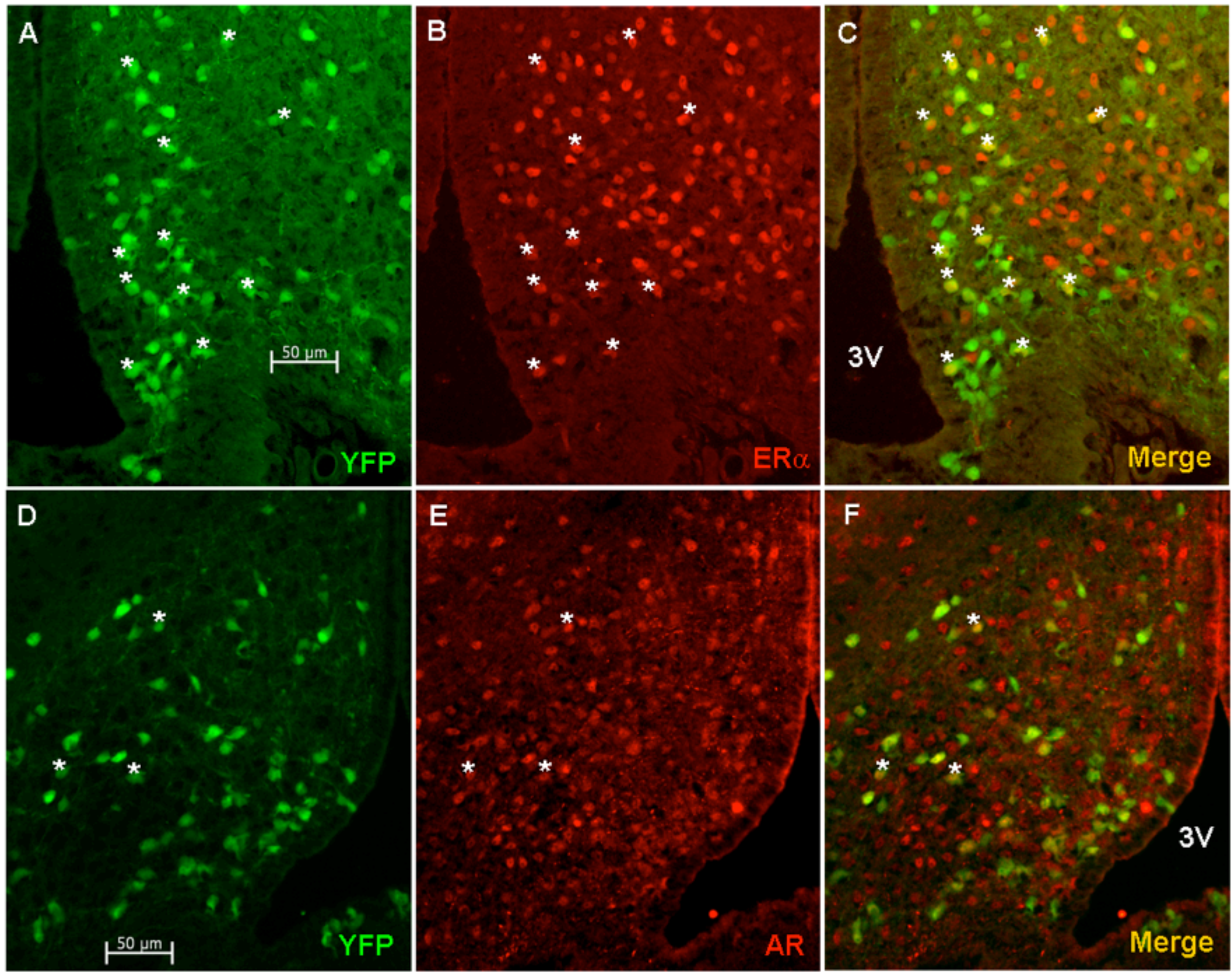


Fig. 21. *ERα and AR expression in GALP neurons.* Immunofluorescence analysis of ARC sections from GALP-CRE/R26-YFP mice using antibodies against ERα and AR show that a subset of GALP neurons (green) expresses ERα (32%, red, A-C) or AR (15%, red, D-F). Scale bar 50 µm.

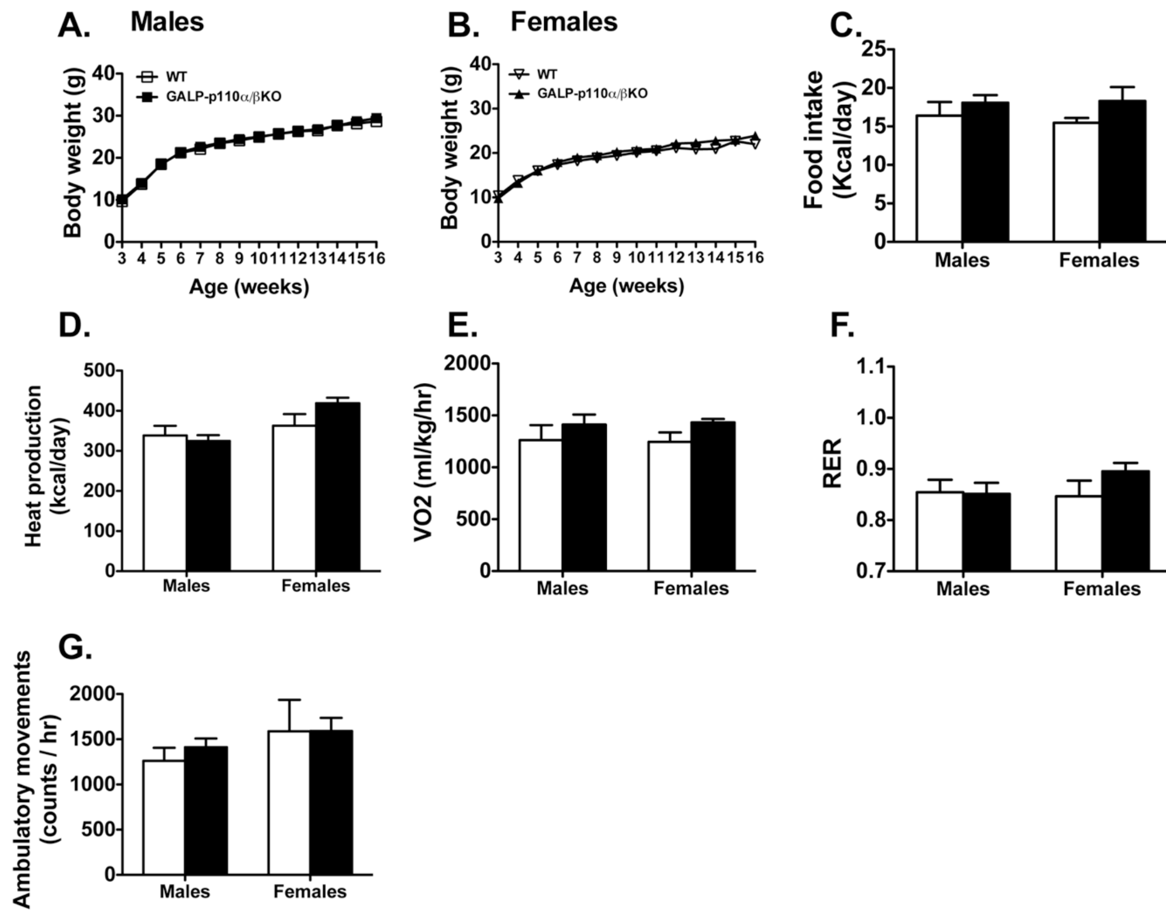


Fig. 22. *Deletion of PI3K catalytic subunits, p110α and p110β, in GALP neurons does not affect body weight gain or metabolic parameters in male and female mice.* Postweaning body weight gain in GALP-p110α/β cKO males **(A)** and females **(B)** and WT littermates on normal chow ($P > 0.05$, n =13-15). **(C)** Food intake **(D)** heat production, **(E)** volume of oxygen consumed (VO2), **(F)** respiratory exchange ratio (RER), and **(G)** ambulatory movement of 5-month old chow-fed GALP-p110α/β cKO male (n = 9) and female (n = 8) mice, and their littermate controls (6-11) were measured by a combined Oxymax indirect calorimetric system over a 24-hr time period. Values are mean ± SEM.

Chapter 4

CONCLUSIONS AND FUTURE DIRECTIONS

Conclusions:

We found that ablating PI3K signaling in kisspeptin neurons in mice decreases *Kiss1* expression in the ARC of gonadectomized males and females. However, this decrease only correlated with LH levels in males but not females. Also, LH levels in intact and T-replaced animals showed a significant decrease in Kiss-p110α/β KO males compared to WT. Since there is little evidence of which region in the rodent brain definitively controls the pulsatile release of LH/GnRH it might be likely that in males this is controlled primarily in the ARC whereas in females it might be controlled in the AVPV which is already established to be necessary for the E_2-induced LH surge. In males ARC *Kiss1* neurons show slow irregular firing whereas ARC *Kiss1* neurons in diestrus or ovariectomized females are nearly silent (de Croft *et al.* 2012). These data in conjunction with our current findings could suggest that kisspeptin-specific PI3K signaling might be involved in the sexually dimorphic function of ARC kisspeptin neurons.

In conclusion much work is still needed in characterizing the effects of disrupted PI3K signaling within kisspeptin neurons. These future findings could be seminal in beginning to understand the mechanisms that regulate *Kiss1* expression on a cellular level, however it is clear that PI3K signaling plays an important role in the regulation of *Kiss1*. Future studies using this model should focus on the molecular mechanisms of PI3K signaling within kisspeptin neurons.

Many factors play a role in regulating gene expression, such as epigenetic alterations, intracellular signaling as well as central and peripheral cues that have the ability to activate or inhibit the necessary signaling pathways.

Our findings also indicate that the role of PI3K signaling within kisspeptin or GALP neurons may different between males and females. However, the exact abundance of p110α or p110β is unknown in both types of neurons. This is crucial for determining the nature of effects we see in these models of disrupted PI3K signaling. Because we do see differences in the phenotypes of males and females, as well as regional differences between the ARC and AVPV nuclei it is likely that differences in the relative abundance of p110α and p110β may underlie these effects. Alternatively, the differences we see in the phenotypes of males versus females may also be due to differences in the specific pathways that PI3K activate in kisspeptin or GALP neurons.

Sexual Dimorphism in PI3K Signaling and Expression in Kisspeptin Neurons:

To fully deduce the effects of deleting PI3K subunits the normal expression profile within these cells needs to be established. The unfortunate limitation to this is the abundance of other neuronal populations within both the ARC (for GALP and Kisspeptin) and AVPV (Kisspeptin) that might express p110α or p110β to a certain extent. Techniques such as the use of a fluorescent reporter, for example the Kiss-EYFP mice, can be used to isolate these neurons and subsequently perform gene expression assays for a variety of intracellular signaling components.

Epigenetic regulation of kisspeptin gene expression has recently emerged as an important factor in the dynamic expression of kisspeptin in the brain. Better understanding how the

alterations in promoter methylation or histone acetylation status will piece together how peripheral factors might regulate the HPG axis through kisspeptin gene expression. Because we see significant alterations in *Kiss1* expression upon PI3K disruption it will be important to establish the precise mechanism through which this occurs. One avenue of investigation is looking at changes in methylation status of the *Kiss1* proximal promoter region as well as intronic CpG sites. Methylation of CpG islands in the promoters and introns of genes is typically thought to be a repressing factor, so it is tempting to hypothesize that disrupting PI3K greatly increased the methylation of the *Kiss1* promoter in Kiss-p110α/β KO mice compared to wild type. However, more studies need to be conducted defining typical methylation status of the *Kiss1* gene in normal conditions in order to fully understand the actions of PI3K.

Role of Kiss-PI3K in the E_2-induced LH Surge:

Preliminary findings suggested that Kiss-p110α/β KO females decreased fertility rate. Kisspeptin is expressed in peripheral tissue of males and females as well as the brain; this includes the ovaries, uterus, as well as the placenta. During pregnancy kisspeptin is expressed in the placenta, however its exactly role is still unclear. Given this possible decreased fertility rate in our Kiss-p110α/β KO females it would be interesting to investigate if placental *Kiss1* expression is altered during pregnancy in Kiss-p110α/β KO females causing loss of the fetus possibly resulting in smaller litter size or even the loss of an entire litter.

The decreased fertility rate might also be due to disruption of the E_2-induced pre-ovulatory LH surge. Even though we do not see a decrease in expression of kisspeptin within the AVPV of females, which is the proposed site for the E_2-induced pre-ovulatory LH surge

activation it is quite possible that PI3K has a role in regulating AVPV kisspeptin neuronal activity. If this is the case it could be a possible mechanism for the decrease in Kiss-p110α/β KO female fertility rate. Determining whether Kiss-p110α/β KO females have an altered pre-ovulatory LH surge in response to E_2 will help in further elucidating the effects of PI3K signaling in fertility.

Investigate the Effect of a High Fat Diet on the HPG Axis of Kiss-p110α/β KO Mice:

Even though differences in LH levels were not seen between Kiss-p110α/β KO and WT mice during 24 or 48 hour fasts, effects on the susceptibility of the HPG axis may not be realized in our Kiss-p110α/β KO model until treated with over nutrition, such as a high fat diet (HFD). Since factors such as leptin and insulin, which are potent activators of PI3K signaling, are often at increased levels during treatment with a HFD the disruption of PI3K signaling within these cells may prevent the effects typically seen upon treatment with a HFD, such as advancement the timing of vaginal opening, advanced increase in LH pulse frequency, and advanced increase in *Kiss1* expression. However care should be taken with choosing the type of diet and timing of treatment because different effects have been seen in treatments of differing percentage of calories from fat in the diets chosen as well as the timing during which the treatments were administered.

The Role of Other Signaling Pathways in Kiss-PI3K Signaling:

Given our current data we are unable to establish what specific downstream effectors of PI3K are at work in this system. PI3K signaling can activate several downstream pathways

depending on the signaling constituents within a given cell. Although the downstream Akt-FoxO1 pathway is often utilized by PI3K there are other effectors that should be investigated especially with regards to neuronal activation. Prominent signaling pathways such as PI3K often have multiple divergent effects upon activation. A single signaling pathway, such as PI3K, can control neuronal activation and firing as well as gene expression. One way to look at neuronal activation without performing electrophysiology or immunohistochemistry for c-fos would be to use new techniques such as Phospho-trap. Phospho-trap utilizes phosphorylation of the C-terminal tail of the ribosomal subunit S6, which becomes phosphorylated through the possible activation of intracellular signaling pathways. This technique would be useful in determining both if certain peripheral factors activated kisspeptin neurons and in conjunction with knock out models such as our Kiss-p110α/β KO mice. The beginnings of understanding the intracellular mechanisms of hormone action on kisspeptin neurons can be discovered. Because we see a more significant decrease in animals that have undergone gonadectomies it is likely that PI3K signaling and E_2 or T signaling have some interactions within kisspeptin neurons. It is also likely that because steroid hormones, especially E, have such strong effects on *Kiss1* expression that any effects due to alterations in PI3K signaling may be confounded. Which specific peripheral factors activate PI3K signaling within kisspeptin neurons is unknown. Other hypothalamic neuropeptide activity is regulated by factors such as insulin and leptin through PI3K activity in POMC and NPY expressing neurons.

Our data from ISH clearly shows that there is a decrease in gene expression conceptualized through the decrease in detectable kisspeptin neurons, however our results from Kiss-EYFP reporter mice show that there is no difference between WT and Kiss-p110α/β KO kisspeptin neuronal counts in the brain. Given these initially conflicting results it is likely that the

decrease in kisspeptin cells detected using ISH is not due to the neurons dying out because of the disruption of PI3K signaling but instead that they cease to fully express kisspeptin sometime during development. In order to confirm this non-radioactive in situ hybridization paired with immunofluorescence for EYFP should be performed. Comparing the amount of co-localized *Kiss1* mRNA and EFYP expressing neurons in WT versus Kiss-p110α/β KO mice will help determine whether negating PI3K signaling in kisspeptin cells has affects cell survival or merely causes a decrease of *Kiss1* gene expression.

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