ARCUATE ER ALPHA-MEDIATED ESTROGEN SIGNALING PATHWAYS AND REGULATION OF KNDY-ASSOCIATED GENE EXPRESSION IN FEMALE MICE

by

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ABSTRACT OF THE DISSERTATION

Arcuate ERα-mediated estrogen signaling pathways and regulation of KNDy-associated gene expression in female mice

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Central signaling of 17β-estradiol (E2) in the arcuate nucleus (ARC) is important in many homeostatic functions including reproduction, energy balance, and thermoregulation, among others. KNDy neurons that coexpress kisspeptin, neurokinin B, and dynorphin function in control of multiple homeostatic functions and are integrators of reproduction and energy balance. In this dissertation, I characterize ERα-mediated and ERα-independent signaling in the ARC and identify the impact of energy balance and E2 on KNDy neurons. First, I characterize ERα-mediated E2 signaling in the ARC, where E2 binds to estrogen receptor-alpha (ERα), translocates to the nucleus, and regulates gene transcription by binding to the Estrogen Response Element (ERE) on DNA through ERE-independent signaling. However, E2 also functions through ERE-independent signaling, such as interacting with nuclear transcription factors and binding to E2-responsive G-protein coupled receptors. I use three genotypes of mice: 1) wild-type, 2) knock in/knock out (KIKO), which lack ERE-dependent signaling, and 3) ERα total knock out (ERKO) to determine differential E2 signaling in the ARC. I found that multiple genes involved in reproduction and energy balance are controlled by E2 through ERE-

dependent and ERE-independent mechanisms and identify novel signaling pathways that are modulated by E2. In the last section, I examined the interactions of E2 and energy balance on KNDy-associated gene expression in the ARC. I determined that in overnutrition and undernutrition, ARC expression of KNDy neuropeptides and receptors is disrupted, leading to downstream changes in gonadotropin (LH and FSH) production. Our previous studies found that in NPY/AgRP neurons, E2 interacted with fasting and diet-induced obesity to regulate genes involved in ghrelin signaling. Overnutrition and undernutrition did not regulate these ghrelin signaling genes in *Tac2* neurons. However, E2 augments the expression of growth hormone secretagogue receptor *(*Ghsr), ghrelin's receptor, in *Tac2* neurons by six- to eight-fold, suggesting that E2 modulates ghrelin signaling in KNDy neurons. In summary, our results suggest differential E2 signaling mechanisms in the ARC are important to maintain energy homeostasis and reproduction and that expression of KNDy genes are regulated by both positive and negative energy balance, leading to downstream changes in reproduction.

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TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

LIST OF FIGURES (CONTINUED)

ASSOCIATED PUBLICATIONS

Chapter 2

Yang JA, Mamounis KJ, Yasrebi A, Roepke TA. 2015. Regulation of gene expression by 17β-estradiol in the arcuate nucleus of the mouse through ERE-dependent and ERE-independent mechanisms. *Steroids*. 107:128-138.

Chapter 4

Yang JA, Yasrebi A, Snyder M, Roepke TA. 2016. The interaction of fasting, caloric restriction, and-induced obesity with 17β-estradiol on the expression of KNDy neuropeptides and their receptors in the female mouse. *Molecular and Cellular Endocrinology. In press.*

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- Mamounis KJ, **Yang JA**, Yasrebi A, Roepke TA. 2014. Estrogen response element-independent signaling partially restores post-ovariectomy body weight gain but is not sufficient for 17beta-estradiol's control of energy homeostasis. *Steroids* 81:88-98.
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- Roepke TA, **Yang JA**, Yasrebi A, Mamounis KJ, Oruc E, Zama A, Uzumcu M. 2016. Regulation of Arcuate Genes by Developmental Exposures to Endocrine-Disrupting Compounds in Female Rats. *Reproductive Toxicology.* 62: 18-26.

CHAPTER 1: LITERATURE REVIEW

1. Literature review

1.1 Importance of the problem

The advancement of research on the topics of obesity, infertility, cancer, alcoholism, and others depends on the elucidation of numerous molecular mechanisms and environmental factors. One major difficulty in understanding the etiology of these diseases is the differences between the two sexes, with little advancement in many fields towards elucidating these differences. Research in the sciences often focuses on male rodent models, as estrogens interact with and regulate many key functions within the body, complicating female systems and diseases (Mamounis et al., 2013,Roepke et al., 2007,Roepke et al., 2008,Yang et al., 2016). Estrogens are the class of steroid hormones most commonly associated with female reproduction and are comprised of three isoforms: estrone (E1), estradiol (E2), and estriol (E3). E2 is the most biologically potent estrogen and is produced primarily in the ovary. The cyclical production of E2, along with other reproductive hormones, is critical to the maintenance of ovarian cyclicity in females, though E2 also functions in male reproduction.

In addition to reproduction, E2 functions in various roles outside of reproduction and its feedback mechanisms are involved in additional metabolic processes including, but not limited to, core body temperature, feeding, and weight gain. E2 is ubiquitous and is involved in the central nervous system, endocrine system, immune system, cardiovascular system, renal system, and musculoskeletal system, among others. Furthermore, the sex-specific differences in the prevalence of various diseases and metabolic processes are influenced by E2 as well. The expression of E2's receptors in non-reproductive tissues also suggests E2 has roles outside of reproduction (Kuiper et al., 1997,Merchenthaler et al., 2004,Shughrue et al., 1997,Sinchak and Wagner, 2012,Wilson et al., 2002). In females, E2 levels differ throughout the ovarian cycle,

complicating the use of female rodent models. It is therefore imperative to consider cyclical levels of E2 and feedback mechanisms that impact the hypothalamic-pituitary-gonadal (HPG) axis, which in females, E2 regulates through both negative and positive feedback. Lastly, E2 regulation is dependent on additional efferent pathways that intersect the HPG axis. Recent identification of kisspeptin as an important regulator of reproduction and energy balance suggests that input of kisspeptin on E2 signaling is critical. The present dissertation work therefore focuses on kisspeptin as a primary target of E2 as a regulator of reproduction and energy balance.

1.2 Estrogen signals through multiple pathways

1.2.1 ERE-dependent signaling

In the classical genomic receptor mechanism, E2 binds to its receptor, ERα (*Esr1)* or ERβ (*Esr2)* in the cytosol. Once bound, the complex will homodimerize (ERα-ERα or ERβ-ERβ) or heterodimerize (ERα-ERβ), based on tissue type and receptor concentration, before it undergoes a conformational change. Following this, the ligand-receptor complex translocates to the nucleus, acting as a transcription factor and binding to the Estrogen Response Element (ERE) on DNA to regulate RNA synthesis, protein synthesis, and ultimately, cell function. However, in addition to direct regulation of gene transcription, ERE-dependent signaling can also include interaction of the ligand-receptor complex to additional proteins and transcription factors (Kelly and Ronnekleiv, 2012). This genomic response of E2 signaling typically occurs within hours of receptor activation. In addition to the term ERE-dependent signaling, Hammes and Levin, categorize these signaling mechanisms as nuclear-initiated steroid signaling (NISS) (Hammes and Levin, 2007). Lastly, in the hypothalamus of the brain, ERα is expression is higher than ERβ (Shughrue et al., 1997). Thus, this dissertation, along with many

other studies in the brain, focus largely on ERα signaling as the dominant ERE-dependent signaling.

1.2.2 ERE-independent signaling

In addition to classical ERE-dependent signaling, ERE-independent mechanisms also function, which have been categorized by Hammes and Levine as “membrane-initiated steroid signaling” (MISS) for rapid, nongenomic pathways (Hammes and Levin, 2007). These nongenomic pathways include a multitude of rapid signaling of E2 through protein-protein interactions and activation of second messenger signaling pathways. Previous studies indicate that E2 binding to ERα and ERβ can activate second messenger systems including phosphoinositide 3-kinase (PI3K)/protein kinase B (Akt), mitogen-activated protein kinases (MAPK)/extracellular signal-regulated kinases (ERK), protein kinase A (PKA), and protein kinase C (PKC) pathways (Dos Santos et al., 2002,Filardo et al., 2002,Lee et al., 2005,Qiu et al., 2003). For example, the MAPK/ERK1/2 pathway, important in multiple cellular processes including cell proliferation and survival, is stimulated by E2 in multiple cell types including breast cancer and adipocytes (Dos Santos et al., 2002,Migliaccio et al., 1996). Similar to ERE-dependent signaling, these second messenger pathways act through either ERα or ERβ, depending on tissue-specific receptor expression. For example, in breast cancer cells, E2 activates PI3K/Akt signaling through ERα, but not ERβ (Lee et al., 2005). In addition, these second messenger systems function through multiple downstream effectors and actions. The PKA pathway leads to phosphorylation of cAMP response element binding (CREB) protein, which in turn binds to the cAMP response element (CRE) to regulate transcription. Other pathways, such as PI3K/Akt are important in regulation of downstream proteins including additional kinases such as mechanistic target of rapamycin (mTOR) (Tao et al., 2015)

In addition to the classical ER receptors, previous studies have shown that E2 functions through additional G-protein activated pathways (Carmeci et al., 1997,Filardo et al., 2002,Qiu et al., 2003,Qiu et al., 2006,Raz et al., 2008,Revankar et al., 2005). Recent candidates include two G-protein coupled receptors (GPCRs), GPR30 and G-protein coupled membrane estrogen receptor (Gq-mER) (Filardo et al., 2000,Filardo et al., 2002,Roepke et al., 2009,Roepke et al., 2010). GPR30 is a member of the 7-transmembrane receptor family that was first identified to be E2 responsive in breast cancer cells, independent of classical ER signaling (Filardo et al., 2000). In that study, addition of the ER antagonist, ICI 182, 780 did not prevent E2 signaling through activation of MAPK/ERK1/2 via GPR30 (Filardo et al., 2000). Multiple groups have cloned GPR30 in the late 1990s (Carmeci et al., 1997,Owman et al., 1996,Takada et al., 1997); within the past decade, numerous studies support the hypothesis that GPR30 is an estrogen receptor, and thus, it is also currently defined as G-protein coupled estrogen receptor 1, or GPER (Filardo et al., 2002).

Multiple studies have supported the hypothesis that there is an additional GPCR, Gq-mER that is responsive to E2 (Nag and Mokha, 2014,Roepke et al., 2009,Roepke et al., 2010,Smith et al., 2013). As reviewed in Roepke et al., Gq-mER signaling occurs through a phospholipase C (PLC) second messenger pathway, leading to activation of PKA, phosphorylation of CREB to act in CRE-dependent gene transcription, and release of calcium from the endoplasmic reticulum (Roepke et al., 2009). While this is the proposed mechanism, the putative Gq-mER may function through additional second messenger pathways. Furthermore, in electrophysiological recordings in guinea pig and mouse hypothalamic slices (POMC neurons), E2 attenuated μ-opioid and γ-aminobutyric acid ($GABA_B$) agonists in the activation of G-protein-coupled, inwardly rectifying K^+ (GIRK) channels, which function downstream of the proposed Gq-mER signaling pathway (Qiu et al., 2003) and activates K_{ATP} channels in GnRH neurons (Zhang et al.,

2010). While the Gq-mER has yet to be cloned, STX, a nonsteroidal compound that selectively targets the Gq-coupled signaling in POMC neurons has been characterized (Qiu et al., 2006). The Gq-mER signaling pathway is involved in regulation of post-ovariectomy body weight gain as well as food intake, core body temperature and bone remodeling (Qiu et al., 2006,Roepke et al., 2009,Roepke, 2009,Roepke et al., 2010). Similar to estradiol benzoate (E2B), STX attenuates the increase in body weight following ovariectomy (Qiu et al., 2006). This STX mediation of body weight is due, at least in part, to central regulation of energy balance by STX. In the hypothalamus, STX decreases *Npy* expression (Roepke et al., 2008) while increasing GABAergic signaling in NPY neurons (Smith et al., 2013) and decreases daily food intake in ovariectomized females compared to vehicle controls (Roepke et al., 2010). Furthermore, STX is involved in attenuating the increase in core body temperature in ovariectomized females (Roepke et al., 2010). Recent studies also suggest that the Gq-mER may be important in sex differences in the regulation of pain (Nag and Mokha, 2014). Furthermore, STX may be involved, at least in part, in E2's neuroprotective effects after ischemia (Lebesgue et al., 2010).

In 2002, Toran-Allerand's group suggested that another ER, which they named ER-X, is important in 17α-estradiol signaling, as opposed to E2 (17β-estradiol) (Toran-Allerand et al., 2002). ER-X is expressed in neocortical and uterine plasma membranes of postnatal mice (Toran-Allerand et al., 2002). Currently, neither the structure nor function of ER-X is known. In 2012, Kampa, et al. suggested evidence of a novel membrane receptor element (ERx, not to be confused with ER-X) that is important in a number of cell functions including apoptosis and growth (Kampa et al., 2012). As with ER-X, there are few studies that elucidate the mechanism and additional functions of ERx. With regards to these additional E2 pathways, in many studies, these additional, ERE-independent signaling cascades and putative ERs do not act independently, but

often integrate with classical ERs. Thus, studies elucidating function are often unclear and need to account for compensatory mechanisms. For example, knock down of GPR30 does not impact reproduction, unlike knock down of ERα in animals, which leads to infertility (Otto et al., 2009). In this case and in others, ERE-dependent and ERE-independent signaling function together. Distinguishing which E2 signaling mechanism regulates different pathways is imperative to understanding the vast network E2 functions through.

1.2.3 Transgenic mouse models to study ERE—dependent and –independent signaling

To study ERE-dependent and -independent signaling in a rodent model, the present work uses two transgenic mouse models. An ERα total knock out (ERKO) mouse that lacks ERα, which is the primary classical E2 receptor found in the hypothalamus following puberty, is used as a model to determine changes associated with ERα-independent signaling (Shughrue et al., 1997). In addition, an ERα knock in/knock out (KIKO or NERKI) mouse model with a defective ERE-binding domain is used to study ERE-independent signaling. However, the KIKO mouse, while unable to regulate gene transcription through ERE, is able to through other hormone response elements (HRE). Thus, future studies should include a mouse model that lacks all HRE signaling to distinguish if HRE-dependent transcription is involved in E2-dependent signaling pathways. The development of an ERE/HRE-independent ERa KIKO mouse model (EAAE-ERα-KIKO) that lacks all HRE-dependent signaling should be used to identify these differences in HRE-dependent and HRE-independent signaling (Hewitt et al., 2014).

Phenotypically, KIKO animals show features that are intermediate between WT and ERKO animals. Luteinizing hormone (LH) levels of KIKO animals are intermediate between WT and ERKO animals, while serum E2 levels are similar to WT animals

(Glidewell-Kenney et al., 2007). KIKO animals have a body weight profile similar to WT animals, unlike ERKO animals, which are heavier (Park et al., 2011). Interestingly, our lab has shown that E2 administration does not attenuate the body weight gain associated with ovariectomy in KIKO animals, unlike WT animals (Mamounis et al., 2013). Collectively, these data suggest that while ERE-independent signaling (in KIKO animals) regulates normal body weight, it is not sufficient to attenuate body weight gain in a hypoestrogenic state. Furthermore, previous studies have used WT, KIKO, and ERKO mouse models to distinguish between signaling pathways in the uterus (Hewitt and Korach, 2011). These studies show that in the uterus, ERE-dependent and ERE-independent signaling are both necessary to maintain reproductive function, thus suggesting that multiple E2 signaling pathways may be important in other E2-dependent systems.

1.3 Functions of estrogens

1.3.1 Estrogens and reproduction

The classical role of estrogens in female reproduction includes central regulation of negative and positive feedback on the HPG axis to control reproduction and ovarian cyclicity. In short, in the HPG axis, hypothalamic gonadotropin-releasing hormone (GnRH) neurons, located in the median eminence, release GnRH. GnRH binds to its receptor, GnRH receptor (GnRHR), in the anterior pituitary, which leads to release of LH and follicle stimulating hormone (FSH). LH and FSH bind to their respective receptors, located on the gonads (ovaries and testes), ultimately leading to the production of reproductive steroid hormones: in females, estrogen and progesterone, and in males, testosterone. Feedback is imperative in the HPG axis. In males, negative feedback of testosterone (and estradiol) regulates the production of the upstream hormones: GnRH, LH, and FSH.

Both negative and positive feedback are needed to maintain ovarian cyclicity in females. In females, estrogens act via negative feedback to regulate GnRH, LH, and FSH, until a threshold level is reached, leading to positive feedback and production of the LH surge to induce ovulation. In addition, the HPG axis includes additional hormones that regulate its activity. Kisspeptin is one neuropeptide hormone that regulates positive and negative feedback of E2 onto the HPG axis. As shown in Figure 1, kisspeptin is expressed in the anteroventral periventricular nucleus (AVPV), or the "surge center," where is mediates positive feedback of E2 onto the hypothalamus and the anterior pituitary. By contrast, kisspeptin expressed in the arcuate nucleus (ARC), or the "tonic center," mediates negative feedback of E2 onto the hypothalamus and the anterior pituitary. AVPV kisspeptin is sexually dimorphic in females, as males do not exhibit positive feedback of E2, and is expressed as a ratio of 10:1 (female: male). By contrast, ARC kisspeptin is not sexually dimorphic, as both sexes need to maintain negative feedback of E2 onto GnRH, LH, and FSH. Figure 1 explains the role of kisspeptin in mediating feedback of the HPG axis. Lastly, negative feedback by progesterone is also important in the regulation of the HPG axis to control GnRH, LH, and FSH levels. This elegant system regulating negative and positive feedback in the HPG axis is critical in female reproduction and allows estrogens to function as the master regulators to control reproduction.

To elucidate the role of E2 in reproduction, knock out animal models are used to determine differences in phenotype and reproductive function. These knock out models include the ERα knock out (ERKO), the animal model we use, and the ERβ knock out. Additional studies suggest that GPR30 knock out models do not show reproductive deficits (Otto et al., 2009). Future studies should include knock down of ER-X, ERx, and Gq-mER to consider additional ERE-independent reproductive pathways. Nonetheless, the phenotypes of ERα and ERβ knock out models provide a base to understand E2 in

reproduction. In both sexes, ERα KO animals are infertile, whereas in ERβ KO animals, males exhibit normal fertility and females have decreased fertility (Hewitt and Korach, 2003). LH levels (mRNA and protein) are increased in the pituitary of both ERα KO and ERβ KO animals, due to lack of negative feedback effects of E2, though ERβ KO animals have normal levels of serum LH and FSH (Couse and Korach, 1999,Couse and Korach, 1999). As expected, the infertile ERα KO animals lack corpus lutea, confirming the reproductive infertile phenotype (Schomberg et al., 1999). By contrast, ERβ KO animals appear to have normal ovaries (Krege et al., 1998). Taken together, these studies indicate a distinct role of ERa in regulation of the HPG axis.

In addition to E2 functions in female reproduction, evidence suggests involvement in male reproduction. As previously mentioned, ERα KO males are infertile, similar to their female counterparts. Furthermore, multiple studies suggest that E2 is critical in the development of the male reproductive tract as ERα KO male mice have a decreased testes size and increased testosterone levels (Hess, 2003). Thus, while the focus of E2 in the present dissertation is in females, E2 is imperative for male reproductive function as well.

1.3.2 Estrogens and energy balance

In addition to reproduction, E2 functions in metabolism and energy homeostasis. One obvious example is present in women experiencing symptoms associated with menopause, when E2 levels decline. In these women, low E2 levels lead to increases in core body temperature associated with hot flushes and weight gain. It is suggested that hot flushes in women are regulated by E2 signaling in kisspeptin neurons, outlined in later sections (Mittelman-Smith et al., 2012). Symptoms involving changes in energy homeostasis, such as weight gain, are associated with neuroendocrine effects of E2 through the control of feeding by proopiomelanocortin (POMC)/cocaine-and-

amphetamine regulated transcript (CART) and neuropeptide Y (NPY)/agouti-related peptide (AgRP) neurons as well as changes in energy expenditure.

Dysfunctions in estrogen signaling can lead to problems including obesity, insulin resistance, and type 2 diabetes mellitus (T2DM) (Prossnitz and Barton, 2011). This is further supported by apparent differences present in insulin resistance, body composition, and energy balance between males and females. Metabolic syndrome, which is characterized by a distinct phenotype including obesity, hyperphagia, and developing insulin resistance, is accelerated during menopause, when there is a deficiency of E2 (Carr, 2003). Many of these symptoms integrate in the central regulation (and de-regulation) of E2 in energy balance and food intake. In rodent models, ovariectomy leads to an increase in body weight through ERE-dependent signaling and E2 supplementation abrogates this increase in body weight gain (Mamounis et al., 2013,Wade et al., 1985). Furthermore, the post-ovariectomy increase in body weight is due to various reasons, including hyperphagia (McElroy and Wade, 1987). E2 signaling is therefore critical in regulation of body weight, further supported by the phenotype present in ERα total knock out (ERKO) animals. ERKO mice of both sexes are obese and show an increase in body fat and decrease in lean mass compared to WT controls. In addition, ERKO animals have higher fasting blood glucose (Bryzgalova et al., 2006). ERβ knock out and KIKO animals do not show this phenotype.

The phenotype of ERKO animals suggests that E2 has important roles in energy balance, primarily through the interaction with POMC/CART and NPY/AgRP neurons in the arcuate nucleus (ARC) of the hypothalamus. In the ARC, POMC neurons are anorectic and NPY neurons are orexigenic and work antagonistically to control feeding behavior. POMC neurons express ERα and are involved in a number of metabolic processes including energy balance, reproduction, and energy expenditure (Hirosawa et al., 2008,Malyala et al., 2005,Xu et al., 2011). Furthermore, E2 increases *Pomc*

expression in the ARC as both are anorectic (Pelletier et al., 2007). This change in *Pomc* expression is supported by electrophysiological evidence of E2's increase in excitatory inputs to POMC neurons (Gao et al., 2007). Interestingly, these inputs occur through a Gq-mER pathway to activate a PLC-PKC-PKA signaling pathway (Qiu et al., 2003,Qiu et al., 2006). Furthermore, POMC neurons are regulated by changes in the menstrual cycle. POMC mRNA levels decline before the LH surge and remain high during the surge, when E2 is high (Petersen et al., 1993). ERKO animals have decreased *Pomc,* regulated by partially through leptin and insulin (Hirosawa et al., 2008).

In addition to POMC neurons, E2 also controls NPY neurons. As previously mentioned, E2 and STX both attenuate $GABA_B$ coupling to GIRK channels in POMC neurons (Qiu et al., 2003). Recent studies suggest that in NPY neurons of female mice, E2 (and STX) activate Gq-mER by enhancing the GABAergic response (Smith et al., 2013). By contrast, activation by E2 (and not STX) through ERα has an opposite effect, attenuating the GABAergic response, similar to POMC neurons (Smith et al., 2013). These results suggest that E2 signals through different mechanisms to regulate overall NPY excitability. Interestingly, less than 4% of NPY neurons colocalize with ERα, suggesting that Gq-mER signaling is critical in E2 regulation of NPY neurons (Roepke et al., 2011). Thus, in both POMC and NPY neurons, the E2 regulation of GIRK channels is important in the regulation of the feeding circuitry.

E2 regulation of NPY neurons is suggested to occur, at least partially, through the M-current, a non-inactivating outward potassium current comprised of KCNQ2, 3, and 5 subunits (Roepke et al., 2011). Thus, the M-current is an inhibitory current and its activation decreases neuronal excitability. E2 increases expression of *Kcnq5* subunit in the guinea pig ARC (Roepke et al., 2007,Roepke et al., 2008). Furthermore, E2 increases the M-current in NPY neurons, suggesting a decrease in NPY neuronal excitability (Roepke et al., 2011). Additional studies outlining the M-current in the context

of ghrelin are outlined in Chapter 4. In conclusion, while the changes associated with *Pomc* and *Npy* expression levels are not due solely to E2 levels but also due to feedback from other hormonal regulators including progesterone, E2 is an important contributor to the feeding circuitry.

1.3.3 Additional functions of estrogens

The classical estrogen receptors are expressed throughout the body in addition to the reproductive system. Furthermore, previous studies suggest that E2 is involved in the regulation of core body temperature, fluid balance, motivational behaviors, and stress physiology through central hypothalamic neural pathways. E2 is involved in the decrease in core body temperature (T_C) and lack of circulating E2 in postmenopausal women show symptoms of increased T_C including hot flushes. Studies on E2 regulation of T_C show that this occurs through a membrane estrogen receptor (Roepke et al., 2010). In addition, E2 is important in fluid balance by regulating arginine vasopressin (Forsling and Peysner, 1988). In many of these cases, E2 regulation is through control of calcium and potassium channels. For example, previous studies show that E2 regulates voltage-dependent T-type calcium channels that are important in burst firing and neurotransmitter release (Bosch et al., 2009,Qiu et al., 2006,Zhang et al., 2009). These examples are only a small fraction of the number of studies elucidating the importance of E2 in neurobiology and physiology. Due to its ubiquity in the body, dysfunction of E2 signaling can lead to a number of diseases including, but not limited to, problems in energy balance (anorexia nervosa, obesity) and cancer.

1.4 Problems in reproduction associated with energy balance

In the United States, the obesity epidemic continues to increase in severity. According to data from the CDC, as of 2014, no state in the United States has a prevalence of obesity less than 20% (CDC, 2015). Furthermore, three states: Arkansas,

Mississippi, and West Virginia had obesity rates of greater than 35% in 2014 (CDC, 2015). In addition to well defined problems associated with obesity such as decreased life expectancy, coronary heart disease, and metabolic syndrome, reproductive dysfunction is also common (Fontaine et al., 2003). In women, obesity is a risk factor for reproductive disorders such as irregular menses, infertility, and miscarriages (Moran et al., 2011,Norman and Clark, 1998). In men, obesity decreases sperm parameters (quality and quantity) and fertility (Bray, 1997,Norman and Clark, 1998). Furthermore, obesity also decreases testosterone levels in men and increases anovulation in females (Bray, 1997). In fact, projected estimates of infertility in the United States suggests that, by 2025, infertility will affect approximately 6.5 million women, roughly 12% of females (Stephen and Chandra, 1998). Infertility is not only a massive psychological problem among couples being second only to cancer for associated stress, but creates an economic burden as well. The average cost of successful assisted reproductive technologies (ART) is currently over $60,000, up to $800,000 (Katz et al., 2011). With evidence suggesting that overweight and obese females are more prone to infertility problems, it is important to consider the molecular and cellular mechanisms governing energy balance and reproduction.

In addition to positive energy balance, negative energy balance can also affect reproduction. For example, amenorrhea is prominent in athletes, largely due to the low body mass index (BMI) of this population (Helge, 2001,Jacobs, 1982). Reproduction is also compromised in other forms of under nutrition, including individuals suffering from anorexia nervosa, who often display hypothalamic amenorrhea (Bulik et al., 1999). Take together, it is well defined that reproduction is disrupted in both overweight and underweight individuals. While the mechanisms regulating and integrating reproduction and energy balance are unknown, they intersect in the hypothalamus. Furthermore,

regulation of reproduction and energy balance involves a number of hormones including E2, kisspeptin, leptin, insulin, and ghrelin.

1.5 Functions of kisspeptin

1.5.1 Kisspeptin and reproduction

Kisspeptin is a peptide hormone that was discovered in Hershey, PA, initially acting as a metastasis suppressor in cancer. Currently, kisspeptin is primarily researched in reproduction following the identification that mutation in its receptor, *Kiss1r*, or GPR54, leads to hypogonadotrophic hypogonadism (de Roux et al., 2003). Furthermore, in *Kiss1* knock out mice, similar abnormal sexual maturation is observed (Lapatto et al., 2007). Taken together, kisspeptin was identified as an important regulator in reproduction and in the onset of puberty. This also led to the phrase that puberty "starts with a kiss."

As previously stated, reproduction is controlled through the HPG axis and kisspeptin signaling integrates into the HPG axis to regulate positive and positive feedback of E2. Kisspeptin is expressed in two main regions of the brain: the anteroventral periventricular nucleus (AVPV) and the ARC. In short, AVPV kisspeptin is primarily important in positive feedback of E2 and ARC kisspeptin is primarily important in negative feedback of E2 (Brock and Bakker, 2013). The AVPV kisspeptin-expressing region is well established in rodents, though it is has rarely been found in humans and in monkeys (Rometo et al., 2007). In addition, the AVPV kisspeptin population is sexually dimorphic, with females showing a 10-fold increase in the number of cells expressing kisspeptin (Clarkson and Herbison, 2006,Kauffman et al., 2007). In rodents, AVPV kisspeptin is important in inducing the LH surge once an E2 threshold is reached, resulting in positive feedback of E2 (Brock and Bakker, 2013). Thus, the sexual dimorphism present in the AVPV kisspeptin population is important, as males do not

reach the E2 threshold needed for positive feedback. Kisspeptin cell bodies have been found in abundance in both the AVPV and preoptic nuclear (POA), where GnRH neurons are found (Clarkson et al., 2009). Furthermore, GnRH neurons express *Kiss1r* (Higo et al., 2016). Thus, as reviewed in Pinilla et al (2012), the mechanism of action in AVPV kisspeptin control of reproduction is through direct afferent projections to GnRH neurons (Pinilla et al., 2012).

By contrast, ARC kisspeptin is important in negative feedback of E2 (Navarro and Tena-Sempere, 2011,Oakley et al., 2009). ARC *Kiss1* is expressed in primates and in rodents, with no observed sexual dimorphism (Clarkson and Herbison, 2006). In addition, ARC kisspeptin is commonly co-expressed in KNDy neurons, termed for colocalization of **K**isspeptin, **N**eurokinin B (NKB), and **Dy**norphin (DYN). Coexpression of the KNDy neuropeptides in ARC kisspeptin neurons depends on species. In the sheep, nearly all DYN cells colocalize NKB or kisspeptin (Cheng et al., 2010). In goats, 99.5% of ARC kisspeptin neurons express NKB and 78% express DYN (Wakabayashi et al., 2010). Nonetheless, the KNDy population is well established in a number of species. The presented dissertation work will identify colocalization of the KNDy neuropeptides in the female mouse in the presence and absence of E2 (see Chapter 4).

The main output in KNDy neurons that functions in the regulation of GnRH is kisspeptin. In the ARC, ovariectomy increases *Kiss1 and* E2 administration abrogates this increase (Brock and Bakker, 2013). By contrast, in the AVPV, E2 regulates *Kiss1* expression by a positive feedback loop (Brock and Bakker, 2013). The two additional neuropeptides in ARC KNDy neurons, NKB and DYN, act as autoregulators to control kisspeptin output. NKB acts as a positive autoregulator to increase kisspeptin output whereas DYN acts as a negative autoregulator to decrease kisspeptin output. Taken together, the NKB and DYN interaction is important in the control of KNDy neuronal excitability (Uenoyama et al., 2014). NKB is a peptide in the tachykinin family of proteins.

In addition to in the ARC, *Tac2* is expressed in other brain regions in rodents including, but not limited to, the hippocampus, ventromedial nucleus, and preoptic area (Navarro, 2013). In the control of kisspeptin output, NKB acts via its receptor, neurokinin 3 receptor (NK3R/*Tac3r*). *Tac3r* is expressed on KNDy neurons and responds to feedback of sex steroids, including E2 (Navarro et al., 2011). Similarly, *Tac2* also responds to feedback by the sex steroids, including E2. Thus, both *Tac2* mRNA levels as well as NKB protein levels have been shown to decrease in the presence of E2 (Navarro et al., 2009,Navarro et al., 2011,Navarro et al., 2011,Oakley et al., 2009). Physiologically, E2 regulation of NKB/*Tac2* and *Tac3r* supports the negative feedback induced suppression of GnRH pulsatility by E2. As NKB increases kisspeptin output, a decrease in NKB suggests a decreased function of KNDy neuronal activity, thus decreasing it's actions as a pulse generator to control GnRH neurons.

By contrast, DYN is part of the opioid peptide family and binds to the receptor kappa opioid receptor, KOR. The gene that encodes DYN is *Dyn,* though for the present work, the propeptide, predynorphin, *Pdyn* is used for gene expression studies. While the roles of DYN and KOR are not as well defined, it has been suggested that the two function in the upstream regulation of KNDy neurons to block kisspeptin release, ultimately regulating its downstream targets: GnRH and LH (Wakabayashi et al., 2010). While *Kor* has been detected on KNDy neurons in rodents, this subset of neurons expressing *Kor* is low (Navarro et al., 2009,Navarro et al., 2011). Interestingly, the recent identification of KOR coloailzation with ~98% of ARC kisspeptin neurons in the ewe suggests that the negative regulatory effects of KOR on KNDy neurons may occur through direct pathways rather through an interneuronal pathway in seasonally reproductive animals (Weems et al., 2016). These species-specific differences are important to consider when determining the molecular mechanisms involved with KNDy

control of reproduction. Nonetheless, DYN functions as an inhibitory input to kisspeptin release through an unknown interneuronal network, either directly or indirectly.

In addition to the regulation of E2 negative feedback, ARC kisspeptin is also important in additional reproductive functions. Following puberty, defined by vaginal opening in female rodents, ARC *Kiss1* expression is increased (Semaan and Kauffman, 2015). Furthermore, a 32% knockdown of ARC kisspeptin results in abnormal estrous cyclicity and LH pulsatility (Hu et al., 2015). These data suggest that ARC kisspeptin is critical in female reproduction and reproductive development and that kisspeptin may be critical to regulation of the changing E2 circuitry during puberty (Cui et al., 2015). In addition, AVPV kisspeptin is also important in the onset of puberty and estrous cyclicity, similar to ARC kisspeptin (Hu et al., 2015).

In the control of E2 feedback on the HPG axis, it is supported that kisspeptin neurons stimulate GnRH neurons through direct contact. There are a number of experiments suggesting that kisspeptin-immunoreactive fibers extend to GnRH neurons (Clarkson and Herbison, 2006,Smith et al., 2013,Smith et al., 2008). Kisspeptin binds to its receptor Kiss1r, expressed on GnRH neurons, to regulate GnRH neuronal excitability (Goodman et al., 2013). This regulation includes kisspeptin depolarizing and exciting GnRH neurons via a PLC second messenger signaling pathway that activates transient receptor potential (TRPC) channels (Zhang et al., 2008). In addition to GnRH neuronal excitability, kisspeptin regulates GnRH pulsatility and firing rate. KNDy neurons are involved in the pulse generation of GnRH neurons, controlling episodic pulse patterns (Goodman et al., 2013). These episodic pulse patterns in GnRH neurons are necessary for LH secretion and reproduction (Belchetz et al., 1978). Thus, KNDy neurons act as upstream, master regulators of reproduction. Lastly, while kisspeptin is largely expressed in the ARC and AVPV, it is expressed in additional brain regions. The recent identification of an additional population of kisspeptin-expression neurons in the

amygdala suggests kisspeptin integrates social behavior and reproduction (Pineda et al., 2016). The role of amydala kisspeptin in reproduction is unclear and thus, pathways of kisspeptin regulation of reproduction may not be completely elucidated.

1.5.2 Kisspeptin and energy balance

KNDy neurons integrate feeding signals through input from hormones such as ghrelin, leptin, and insulin (De Bond and Smith, 2013,Fernandez-Fernandez et al., 2006). These mechanisms remain unclear; however, kisspeptin's involvement in energy balance is well established. In *ob/ob* mice that are leptin deficient, morbidly obese, and infertile, *Kiss1* mRNA levels in the ARC are low, and *Kiss1* levels are restored by leptin (Smith et al., 2006). These data therefore suggest that ARC kisspeptin neurons are involved in the feeding circuit, mediated as least partly (in positive energy balance) by leptin, though the mechanisms have yet to be elucidated.

Thus, kisspeptin expression is regulated by changes in energy balance, and in turn regulates reproduction. Interestingly, *Kiss1* expression is based on current energy level status and both acute and chronic changes in kisspeptin by energy balance can be rescued (Castellano et al., 2005,Dudek et al., 2016). As previously stated, regulation by both negative and positive energy balance is important to understand, as the mechanisms and pathways that are involved in each may differ. Previous studies on negative energy balance show that in all cases, *Kiss1* mRNA levels are decreased, lending to problems in downstream reproductive parameters (Castellano et al., 2005,Iwasa et al., 2015,Kalamatianos et al., 2008,Matsuzaki et al., 2011). In fasts ranging in from 24- to 72-hrs, hypothalamic *Kiss1* is decreased in adult and pubertal rodent models of both sexes (Kalamatianos et al., 2008,Luque et al., 2007). In addition to acute fasts, chronic energy deficiency is important to consider. As previously mentioned, individuals suffering from anorexia nervosa, one example of a chronic negative energy balance state, experience problems in reproduction including

amenorrhea (Bulik et al., 1999). Furthermore, one recent study suggests that mice on a chronic caloric restriction (modeling anorexia nervosa) show a reduction in *Kiss1* mRNA and unlike in models of obesity, leptin administration fails to rescue this disruption in *Kiss1* expression (True et al., 2011).

While there are have been a number of studies that identify negative energy balance effects on kisspeptin, there are a few important points. For one, many of these previous studies examine *Kiss1* gene expression differences at the level of the hypothalamus. With the identification of new populations of kisspeptin neurons outside of the AVPV and ARC, as well as the identification of distinct roles in AVPV and ARC kisspeptin, it is important to consider specific nuclei and populations. More recent studies are beginning to examine distinct nuclei, with additional studies suggesting that the KNDy neuronal population is regulated by negative energy balance, as decreased *Kiss1* extends to decreased *Tac2* and *Pdyn* (True et al., 2011). In addition, similar studies indicate that negative energy balance through models of anorexia nervosa and metabolic stress (by LPS) lead to similar changes in hypothalamic *Kiss1* expression (Castellano et al., 2010). Interestingly, only a few of these studies differentiate between changes in the sexes, or during different stages of the estrous cycles in females. Since it is known that sex steroids regulate *Kiss1*, it is imperative to consider feedback mechanisms, including regulation by E2 as well as progesterone (P4). Chapter 4 will examine changes in KNDy neuronal gene expression in the context of these feedback mechanisms.

In addition to negative energy balance, positive energy balance is also important in the integration of kisspeptin with reproduction. Recent studies suggests that in obese human males, the hypothalamic KISS1 system is disrupted (George et al., 2010). This evidence in humans is extended to and supported by rodent studies. In one study, DIO was shown to reduce hypothalamic *Kiss1* expression in mice (Quennell et al., 2011).

Furthermore, as with negative energy balance, feedback of sex steroids in positive energy balance is important. For example, in male rats fed a HFD, obese animals show a decrease in hypothalamic *Kiss1* that is not responsive to negative feedback expected by low T levels (Sanchez-Garrido et al., 2014). Interestingly, dysfunction of the kisspeptin system may therefore lead to dysfunction of the HPG axis and feedback systems needed for reproduction.

Kisspeptin signaling in the other organs, including the liver, is also important in energy balance. Recent work has suggested that kisspeptin signaling in the liver is important in regulation of glucagon and insulin sensitivity and therefore, dysfunction can lead to problems including T2DM. During hyperglucagonemia, which occurs at the onset of T2DM, elevated glucagon levels lead to a continuous breakdown of glucose in response to insulin insensitivity. Studies show that during this time, kisspeptin production is increased in the liver, which suppresses glucose stimulated insulin secretion (GSIS) (Song et al., 2014). In addition, DIO mice and diabetic mice (leptin defective) have increased kisspeptin in the liver and knockdown of liver kisspeptin attenuates the suppression of (GSIS) (Song et al., 2014). The pathways that regulate kisspeptin signaling in the liver to regulate energy balance are not well understood. However, evidence in DIO animals suggests that kisspeptin elevation is important in regulation of glucagon and insulin. Additional studies need to examine the kisspeptin in other examples of energy balance deregulation, including fasting and caloric restriction.

The following section will describe additional signaling pathways that intersect with kisspeptin signaling in energy balance. These pathways are important in the regulation of energy homeostasis and may act as direct or indirect pathways integrating reproduction and energy balance. Lastly, while disruptions to energy balance are important to consider, these pathways may differ to normal function of kisspeptin in the context of energy homeostasis. For example, KNDy neurons are involved in regulation of

body weight. Recent studies show that ablation of KNDy neurons in rats abrogates the weight gain associated with ovariectomy (Mittelman-Smith et al., 2012).

1.6 Additional signaling pathways intersect with kisspeptin signaling

While the integration of energy balance and reproduction is mediated, in part, by kisspeptin, the mechanism of this regulation is unknown. Thus, it is possible that both direct and indirect mechanisms are involved. In addition to considering sex steroids as candidates that regulate kisspeptin function, nutritional cues may also regulate kisspeptin. While studies have identified signaling of insulin, leptin, and ghrelin to kisspeptin, the pathways regulating these nutritional cues with kisspeptin have not been fully elucidated. Below is a summary of the nutritional cues that have been suggested to integrate with kisspeptin signaling.

1.6.1 Insulin

Insulin is a peptide hormone produced by the pancreas important in glucose absorption. Thus, insulin levels are directly proportional to glucose levels in the blood and insulin acts as an important indicator of energy status. Insulin levels are also proportional to adipose tissue in most mammals, though problems in insulin resistance can lead to diseases including metabolic syndrome and type 2 diabetes mellitus (Hill et al., 2008). Furthermore, insulin resistance is linked to disruptions of the reproductive system. Following ART treatment, insulin resistance increases the risk factor for spontaneous abortions (Tian et al., 2007). This insulin regulation of reproduction occurs through central signaling mechanisms, as neuron-specific insulin receptor knockout (NIRKO) mice show both an increase in obesity and impaired fertility (Bruning et al., 2000). To date, no *in vivo* model has shown that GnRH neurons express the insulin receptor (IR). Thus, it is hypothesized that the insulin-mediated effects on reproduction may be through an interneuronal network.

In the hypothalamus, IRs are expressed in the ARC (Hill et al., 2008). In the ARC, approximately 22% of *Kiss1* neurons colocalize with IR (Qiu et al., 2013). IR deletion from *Kiss1* neurons has no effect on reproduction, but delays puberty. (Qiu et al., 2013). This suggests that direct insulin signaling in kisspeptin neurons is not necessary for normal reproduction. Nonetheless, indirect signaling of insulin through NPY/AgRP and POMC/CART neurons may be important to consider. In fact, central intracerebroventricular (icv) injections of insulin decrease *Npy* in fasted animals (Schwartz et al., 1991), while intraperitoneal injections of insulin normalize *Pomc* expression in streptozotocin-induced diabetic rats (Kim et al., 1999). These data show that insulin signaling is important in NPY/AgRP and POMC/CART neurons. As such, additional research needs to be conducted on insulin signaling between the neurons involved in feeding (NPY/AgRP and POMC/CART) and ARC kisspeptin neurons.

1.6.2 Leptin

As previously mentioned, leptin signaling is important in energy balance and the development of the *ob/ob* transgenic mouse that lacks leptin has led to a clear advancement in the field. These *ob/ob* mice are morbidly obese and sterile and reproduction is rescued with leptin administration (Mounzih et al., 1997). Thus, sufficient levels or leptin are required for reproduction to occur. Furthermore, in patients suffering from amenorrhea due to chronic negative energy balance, leptin rescues LH and E2 levels (Hill et al., 2008). In rodent models, the development of a “skinny” mouse with leptin levels compared to obese mice led to accelerated puberty and intact fertility (Yura et al., 2000). Taken together, these data show that leptin is a critical signal in reproduction.

Leptin also mediates the functions of kisspeptin, impacting reproduction. Previous studies suggest that the leptin receptor, LepR, is not expressed on GnRH neurons, but is expressed in ~40% of ARC *Kiss1* neurons (Hill et al., 2008).

Furthermore, in *ob/ob* mice, there is a decrease in ARC *Kiss1,* which is rescued with leptin infusion (Hill et al., 2008). In *in vitro* studies, knock out of leptin in GABAergic neurons leads to problems in reproductive maturity and ovariectomized animals display significantly reduced expression of *Kiss1* (Martin et al., 2014). These data suggest that leptin regulates GABAergic neurons through a kisspeptin-mediated mechanism. Furthermore, in diabetic rodent models, insulin infusion normalizes *Kiss1* expression and normalizes downstream hormone levels, including LH and T (Castellano et al., 2006). Taken together, these data indicate that leptin signaling may be important in kisspeptin regulation. However, similar to insulin, it is important to consider leptin signaling in NPY/AgRP and POMC/CART neurons, both of which express LepR (Hill et al., 2008). As previously stated, negative and positive energy pathways may differ. In one previous study, calorically restricted animals displayed a decrease in LH and *Kiss1* levels, which were not rescued by leptin (True et al., 2011). Thus, while previous studies suggest that leptin rescues reproduction, kisspeptin regulation in different energy balance models is important to consider.

1.6.3 Ghrelin

Ghrelin is a brain-gut peptide that is involved in the regulation of feeding behavior and weight gain. Central signaling of ghrelin is important in the regulation of energy homeostasis, primarily through research conducted in POMC/CART and NPY/AgRP neurons. In the ARC, ghrelin has been shown to stimulate NPY neurons and suppress POMC neurons through inhibitory γ-aminobutyric acid (GABA)-ergic inputs (Andrews, 2011). Recent evidence also suggests that ghrelin may be involved in signaling to ARC kisspeptin neurons. Furthermore, in our lab, we show that in ovariectomized oil-treated females, fasting increases *Ghsr* expression in the ARC, but not in NPY neurons (Yasrebi et al., 2016). Lastly, in *Ghsr* KO mice, ovariectomy does not increase food intake, as is present in WT animals (Clegg et al., 2007). These data suggest that the fasting

regulation of ghrelin signaling is not through NPY neurons, and may be involved in regulation in other neurons of the ARC. We therefore hypothesize that fasting regulated increase in ARC *Ghsr* expression is primarily present in ARC kisspeptin neurons.

However, ghrelin signaling in ARC kisspeptin neurons has not been elucidated. Studies have shown that ARC *Kiss1* mRNA is not be regulated by ghrelin administration in a fed or fasted state (Forbes et al., 2009). Nonetheless, as previously stated, E2 admin increases *Ghsr* expression in the ARC, suggesting that feedback mechanisms of kisspeptin are involved (Yasrebi et al., 2016). Furthermore, recent studies examining electrophysiological changes in KNDy neurons suggest that pathways of regulation might be important to consider. In this study, ghrelin was shown to depolarize KNDy neurons, indicating a direct relationship between ghrelin and KNDy neurons (Frazao et al., 2014). In reproduction, ghrelin functions in the suppression of LH pulse frequency (Forbes et al., 2009). While both ghrelin and *Ghsr* knock out mice fail to show any reproductive defects, previous studies nonetheless show that ghrelin is important in the HPG axis (Barreiro and Tena-Sempere, 2004).

The ghrelin signaling cascade involves an AMP-activated protein kinase pathway. Ghrelin is first acylated by ghrelin O-acyltransferase (GOAT) before it binds to its receptor, growth hormone secretogogue receptor (GHSR) type 1a (Andrews, 2011). Once the ligand-receptor complex is formed, the CaMKK-AMPK-UCP2 pathway is active (Andrews, 2011). This pathway upregulates carnitine palmitoyl transferase 1 (CPT1), increasing fatty acid acyl CoA transportation to the mitochondria for oxidation (Andrews, 2011). In addition, uncoupling protein 2 (UCP2), which is involved in decreasing reactive oxygen species (ROS) that are produced during oxidation, is increased. UCP2, along with a host of additional transcription factors including forkhead box protein O1 (FOXO1), are important in transcription of genes involved in upregulating feeding (Andrews, 2011). Figure 2 depicts the ghrelin signaling cascade.

1.6.3.1 Ghrelin signaling: The M-current in POMC/CART and NPY/AgRP neurons

Future studies in our lab will examine the M-current as a mechanism that regulates ghrelin signaling in KNDy neurons. Previous studies show that the M-current is involved in ghrelin's activation of NPY neurons (Shi et al., 2013). The M-current is a non-inactivating, outward potassium current that is comprised of KCNQ2, KCNQ3, and KCNQ5 subunits. Activation of neuronal excitability is achieved through attenuation of the M-current by muscarinic receptors as well as several neurotransmitters including serotonin and dopamine (Marrion, 1997,Pfaffinger et al., 1988). In addition, many of the neurotransmitters involved in the attenuation of the M-current have been implicated to function in the hypothalamic control of energy balance. Past studies have shown that the M-current is involved in the control of POMC and NPY neuronal activity and therefore feeding behavior (Roepke et al., 2011,Roepke et al., 2012). In NPY neurons, fasting attenuates the M-current to increase neuronal excitability (Roepke et al., 2011).

To date, no studies have examined the role of the M-current in the control of KNDy neuronal activity. Recently, evidence suggests that binding of ghrelin to GHSR attenuates the M-current through a Gq-PCR pathway involving the PLC-PKC pathway (Cooper and Jan, 2003,Shen et al., 2005). In this pathway, PLC is responsible for the hydrolysis of phosphatidylinositol 4,5-bisphosphate (PIP_2). PIP_2 in the membrane causes an increase in KCNQ and therefore the M-current to be in the open state (Horowitz et al., 2005). Therefore, we hypothesize that increasing GHSR activation by ghrelin increases PIP_2 hydrolysis to attenuate the M-current and activate KNDy neurons (Figure 3).

1.6.3.2 Ghrelin signaling in Tac2 neurons

As previously mentioned, one weakness in previous studies is the focus on whole hypothalamus and not discrete nuclei involved in the control of interneuronal circuits. The presented dissertation work therefore focuses on 1) ARC gene expression

changes, as opposed to whole hypothalamic studies and 2) individual *Tac2* neurons to determine neuron-specific mechanisms. To study individual *Tac2* neurons, we use a *Tac2*-EGFP model and harvest single-neurons using fluorescent microscope visualization and a micromanipulator with an electrode. Harvested neurons are collected individually (single neurons) and in pools of five cells. Singly harvested neurons are used for qualitative single-cell PCR reactions and visualized on an agarose gel. Due to the low RNA concentration present in a single-cell, we are unable to perform quantitative real-time polymerase chain reaction (qPCR) experiments and use these neurons simply to identify colocalization patterns in single cells. Pools of five neurons are used to identify qualitative gene expression through qPCR.

One of the difficulties with discrete nuclei and single cell studies is the size and relative nucleic acid and protein concentration associated. Due to this, it is impossible to conduct protein expression studies such as Western blot unless multiple animals are grouped together to account for one sample. In lieu of this, future studies in the lab will use electrophysiological experiments to study functional changes within neurons.

1.7 Summary and objectives of the thesis

This dissertation investigates mechanisms of E2 signaling in the ARC and the regulation of *Tac2* neurons by E2, diet, and fasting. The objectives of the dissertation are summarized below:

Objective 1: To determine the mechanisms of E2 regulation of homeostasis genes in the ARC. To study E2 signaling in the ARC, three genotypes of mice were used: WT, KIKO, and ERKO females. Females were ovariectomized and treated with E2 or oil to determine differences in ARC gene expression. (Chapter 2)

Objective 2: To determine the ARC estrogenome using next-generation sequencing of the RNA transcriptome in WT, KIKO, and ERKO females. Females of three genotypes

(WT, KIKO, and ERKO) were used to determine differential regulation of E2-responsive ARC genes. (Chapter 3)

Objective 3: To determine the effects of E2, fasting, and diet on KNDy-associated gene expression in the ARC and *Tac2* neurons. Females were treated to a number of energy balance disruptions: 1) 24 h fast; 2) 30% caloric restriction; and 3) DIO through a high fat diet. We identified gene expression changes in the ARC and in individual *Tac2* neurons to determine if disruptions in energy balance lead to changes in discrete neurons. (Chapter 4)

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Figures

Figure 1. Negative and positive feedback of E2 and P4 in the female mouse. E2 and P4 from the ovaries negatively feedback onto the hypothalamus by inhibitiing AVPV kisspeptin neurons and ARC KNDy neurons. During the proestrous stage, E2 positively feedbacks onto AVPA kisspeptin neurons to excite GnRH neurons and increase LH production. In the pituitary, E2 also has a negative effect on LH production from the gonadotropes and inhibin has a negative effect on FSH production.

Figure 2. Activation of NPY/AgRP neurons through the ghrelin/*Ghsr* signaling cascade. The ghrelin signaling cascade regulates expression of energy homeostasis genes in NPY neurons.

Figure 3. Direct modulation of KCNQ channels (M-current) via Gq-GPCR-mediated pathways. We propose that activation of GHSR on KNDy neurons increases PIP_2 hydrolysis through a PLC-PKC pathway to attenuate the M-current and increase KNDy neuron function.

Figure 1.

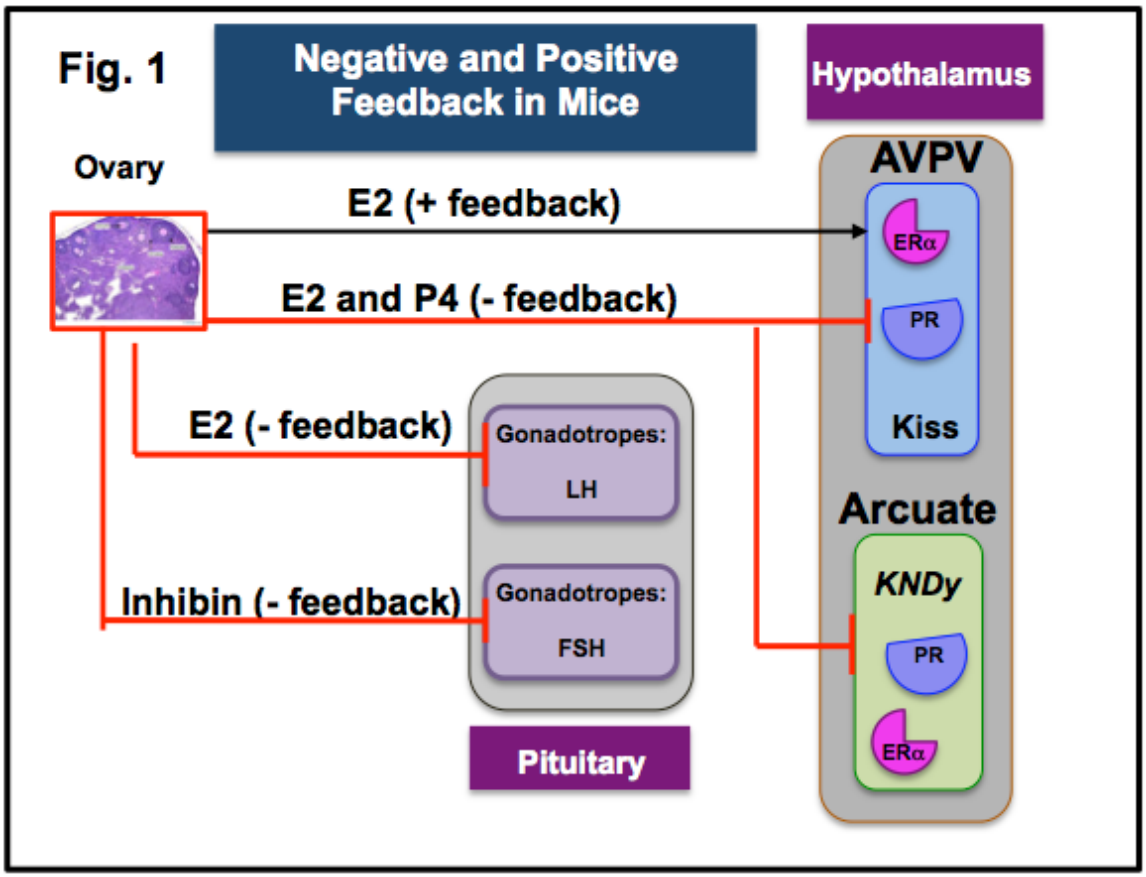


Fig. 1
Negative and Positive Feedback in Mice
Hypothalamus
Ovary
E2 (+ feedback)
AVPV
ERα
PR
Kiss
E2 and P4 (- feedback)
E2 (- feedback)
Gonadotropes: LH
Arcuate
KNDy
PR
ERα
Inhibin (- feedback)
Gonadotropes: FSH
Pituitary

Figure 2

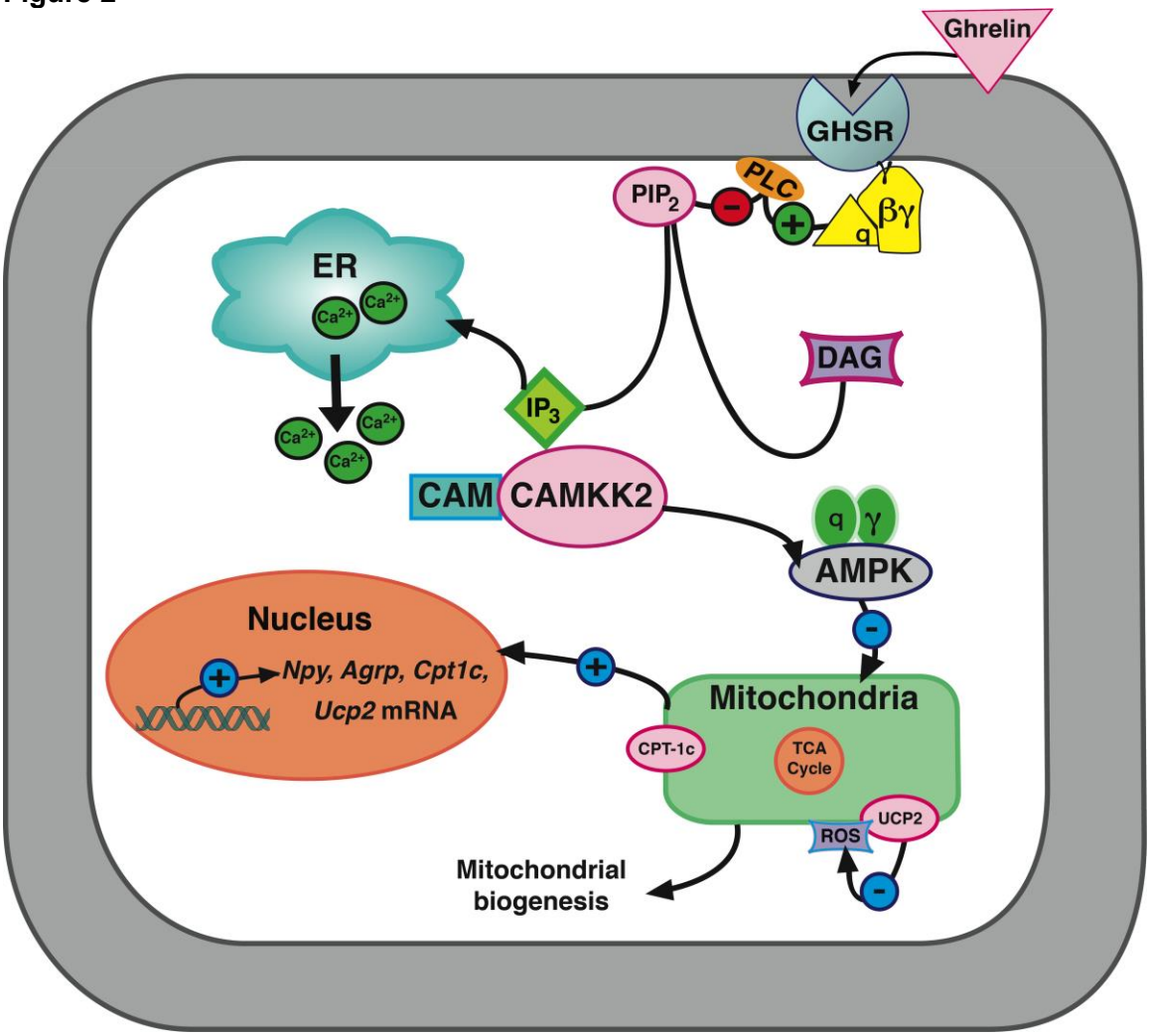
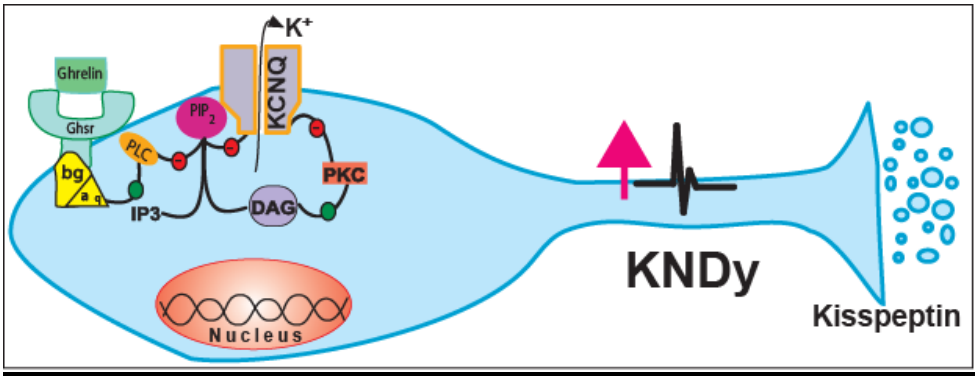

Ghrelin
GHSR
PIP2
PLC
βγ
q
ER
Ca2+
Ca2+
Ca2+
Ca2+
Ca2+
IP3
DAG
CAM
CAMKK2
q
γ
AMPK
Nucleus
Npy, Agrp, Cpt1c,
Ucp2 mRNA
Mitochondria
CPT-1c
TCA
Cycle
UCP2
ROS
Mitochondrial
biogenesis

Figure 3


K+
KCNQ
Ghrelin
Ghsr
PIP2
PLC
bg
aq
IP3
DAG
PKC
Nucleus
KNDy
Kisspeptin

CHAPTER 2: REGULATION OF GENE EXPRESSION BY 17β-ESTRADIOL IN THE ARCUATE NUCLEUS OF THE MOUSE THROUGH ERE-DEPENDENT AND ERE-INDEPENDENT MECHANISMS

2. Regulation of gene expression by 17β-estradiol in the arcuate nucleus of the mouse through ERE-dependent and ERE-independent mechanisms

2.1 Abstract

17β-estradiol (E2) controls homeostatic functions by modulating gene expression in the hypothalamic arcuate nucleus (ARC). In the ARC, estrogen receptor (ER) α is highly expressed and is an important contributor to E2's actions, controlling gene expression through estrogen response element (ERE)-dependent and -independent mechanisms. The objective of this study was to determine if known E2-regulated genes are regulated through these mechanisms. The selected genes have been shown to regulate homeostasis and have been separated into three functional categories: channels, receptors, and neuropeptides. To determine if ERE-dependent or ERE-independent mechanisms regulate gene expression, two transgenic mouse models, an ERα knock-out (ERKO) and an ERα knock-in/knock-out (KIKO), which lacks a functional ERE binding domain, were used in addition to their wild-type littermates. Females of all genotypes were ovariectomized and injected with oil or estradiol benzoate (E2B). Our results suggest that E2B regulates multiple genes through ERE-dependent and ERE-independent mechanisms. Of note, *Cacna1g* and *Kcnmb1* channel expression was increased by E2B in WT females only, suggesting an ERE-dependent regulation. Furthermore, the NKB receptor, *Tac3r*, was suppressed by E2B in WT and KIKO females but not ERKO females, suggesting that ERα-dependent, ERE-independent signaling is necessary for *Tac3r* regulation. The adrenergic receptor *Adra1b* was suppressed by E2B in all genotypes indicating that ERα is not the primary receptor for E2B's actions. The neuropeptide *Tac2* was suppressed by E2B through ERE-dependent mechanisms. These results indicate that E2B activates both ERα-dependent and

independent signaling in the ARC through ERE-dependent and ERE-independent mechanisms to control gene expression.

2.2 Introduction

The steroid hormone 17β-estradiol (E2) is known to regulate gene expression throughout the brain. E2 primarily uses two classical nuclear receptors, estrogen receptor α (ERα*, Esr1,*) and ERβ (*Esr2*) to regulate gene expression (Roepke et al., 2008). In the classical ER-mediated mechanism, ligand binding to the receptor initiates receptor recognition of the estrogen response element (ERE) to regulate gene transcription. In addition to classical regulation of gene expression, E2 also functions through ERE-independent mechanisms. As reviewed in McDevitt et al. (2008), these mechanisms include ligand-independent ER signaling, rapid effects through a membrane-associated ER, and ERE-independent signaling through protein-protein interactions (AP-1, etc.) (McDevitt et al., 2008).

In the hypothalamus, E2 regulates numerous homeostatic functions including reproduction, energy homeostasis, core body temperature, fluid balance, motivational behaviors, and stress physiology by regulating central neural pathways. Many of these pathways originate in or pass through the arcuate nucleus (ARC) of the hypothalamus (Roepke et al., 2008,Roepke et al., 2010,Santollo et al., 2011,Shughrue et al., 1997). In the ARC, ERα is highly expressed and is the primary receptor used by E2 to control many homeostatic functions (Mitra et al., 2003,Shughrue et al., 1997). Few studies have examined the physiological effects of ERα-mediated, ERE-dependent and ERE-independent signaling on hypothalamic (ARC) gene expression.

Recently, the development of an ERα knock-in/knock-out (KIKO) mouse model lacking a functional DNA-binding domain (no ERE binding) gives insight to nonclassical, ERα-mediated, ERE-independent signaling while retaining ERβ-mediated signaling and

other extra-nuclear initiated pathways (GPER, Gq-mER) (Qiu et al., 2003,Qiu et al., 2006). While KIKO females are infertile due to an absence of the LH surge (Hewitt et al., 2009,Jakacka et al., 2002), they exhibit similar body weights, feeding, activity, oxygen consumption, glucose homeostasis, and hypothalamic leptin sensitivity compared to wild-type (WT) females, unlike their total ERKO counterparts (Park et al., 2011). However, recent data from our lab suggest that ERα-mediated, ERE-independent signaling partially restores the post-ovariectomy (ovx) weight gain but is not sufficient to mediate E2's attenuation of this weight gain (Mamounis et al., 2013).

E2 control of homeostatic functions occurs, in part, through regulation of important genes in the ARC. However, only a few studies have examined which signaling mechanisms E2 utilizes to control the expression of these genes. The KIKO mouse model provides an appropriate tool to increase our understanding of how homeostatic genes are regulated by E2-induced, ERα-mediated mechanisms in the ARC. In the ARC, E2 is known to regulate the expression of a variety of cation channels including calcium channels and potassium channels (Bosch et al., 2009,Qiu et al., 2006,Roepke et al., 2007,Zhang et al., 2009). The expression and activity of these cation channels are involved in regulating ARC proopiomelanocortin (POMC) and neuropeptide Y (NPY) neurons and their neuronal excitability (Bosch et al., 2002,Bosch et al., 2009,Bosch et al., 2013,Ibrahim et al., 2003,Mamounis et al., 2013,Qiu et al., 2006,Roepke et al., 2007,Roepke et al., 2008,Roepke et al., 2011). Furthermore, E2 is known to regulate the mRNA expression of signaling molecules such as calmodulin and phosphatidylinositol-4,5-biphosphate 3-kinase (PI3K) (Malyala et al., 2004,Malyala et al., 2005,Malyala et al., 2008,Roepke et al., 2007) and neurotransmitter enzymes like tyrosine hydroxylase (TH) and glutamate decarboxylase (Curran-Rauhut and Petersen, 2002,Roepke et al., 2008). Many of these E2-regulated genes are involved in

reproduction, energy homeostasis, and hormone receptor signaling (Bosch et al., 2012,Jamali et al., 2003,Roepke et al., 2008,Roepke, 2009,Roepke et al., 2010).

E2 is also known to suppress or augment the expression of a variety of neuropeptides and receptors in the hypothalamus, depending upon the experimental paradigm. In rodents, E2 increases *Pomc* and suppresses *Npy* expression in the ARC (Roepke et al., 2008) and increases the expression of growth hormone (Addison and Rissman, 2012). It is also well known that E2 differentially regulates steroid receptors in the hypothalamus such as ERα, ERβ, and progesterone receptor (*Pgr*) (Roepke et al., 2007,Roepke et al., 2008,Zuloaga et al., 2012). Other hypothalamic hormone and neurotransmitter receptors that are regulated by E2 in the hypothalamus include growth hormone secretagogue receptor (*Ghsr*) (Frazao et al., 2014) and serotonin receptor 2C receptor (5HT2c) (Mirkes and Bethea, 2001). The mechanism underlying the regulation of all of these genes is largely unknown. Our current study focused on the regulation of ARC gene expression by E2.

E2 also regulates ARC KNDy (Kisspeptin-Neurokinin B-Dynorphin) neuronal gene expression (Brock and Bakker, 2013). Kisspeptin is involved in mediating negative and positive feedback of E2 on the hypothalamic-pituitary-gonadal (HPG) axis and potentially has a role in energy homeostasis and core body temperature (Mittelman-Smith et al., 2012). Previous studies indicate that the *Kiss1* gene is regulated by E2 through ERE-independent mechanisms in the mediobasal hypothalamus while dynorphin expression is ERE-dependent (Gottsch et al., 2009). Thus, we used these genes as positive and negative controls for ERE-dependent and ERE-independent gene expression. Nothing is known about the mechanisms regulating Neurokinin B (NKB, *Tac2*) or the KNDy receptors (*Kiss1r, Tac3r*) expression by E2 in the ARC. Therefore, the objective of this study was to determine if ER-responsive, homeostatic genes involved in reproduction and energy homeostasis are regulated by E2 in the ARC

through ERE-dependent or ERE-independent mechanisms using ovx WT, KIKO, and ERKO females.

2.3 Methods

2.3.1 Animal care

All animal procedures were in compliance with institutional guidelines based on National Institutes of Health standards and were performed with Institutional Animal Care and Use Committee approval at Rutgers University (Protocol #: 11-003). Adult C57BL/6 mice were housed under constant photoperiod conditions (12/12 h light/dark cycle) and maintained at a controlled temperature (23°C). Animals were given low phytoestrogen chow diet (<75 isoflavone ppm, Lab Diet Advanced Protocol 5V75, St. Louis, MO, USA) and water *ad libitum*. Animals were weaned on postnatal day 21 (PD21). Genotype was determined by using PCR products of extracted DNA from ear clippings, using previously published protocols (Hewitt et al., 2009). We used three genotypes of mice: WT, KIKO, and ERKO (provided by Dr. Ken Korach, NIEHS) (Hewitt et al., 2009). Crossing heterozygous WT/KI males expressing the nonclassical ERα knock-in with WT/KO heterozygous females generated WT and KIKO females. Crossing heterozygous WT/KO males and females generated WT and ERKO females. WT females used in the experiments were littermates generated from both colonies.

2.3.2 Drugs

17β-estradiol benzoate (E2B) was purchased from Steraloids (Newport, RI, USA) and dissolved in ethanol (1mg/ml) prior to mixing in sesame oil (Sigma-Aldrich). Ketamine was purchased from Henry Schein Animal Health (Melville, NY, USA) and used for sedation prior to sacrifice.

2.3.3 Ovariectomy

Adult females (7-22 weeks and > 14 g body weight) were bilaterally ovx under isoflurane anesthesia 7 days prior to sacrifice using sterile no-touch techniques according to the NIH "Guidelines for Survival Rodent Surgery." Animals were given a dose of analgesic [4 mg/kg carprofen (Rimadyl®)] one day following surgery for pain management. Animals typically lost 1-2 g of weight one day after surgery. Females were monitored daily and allowed to recover for 5 days prior to the first injection of E2B or oil. The active metabolite of E2B is 17β-estradiol. Females were injected in the morning at 1000 h on post-ovx days 5 and 6 and sacrificed on post-ovx day 7 in the morning at 1000 h.

2.3.4 Experimental design

Females of each genotype (WT, KIKO, ERKO) were ovx and separated into a control sesame oil-treated group (n=6-9 per genotype) and an E2B-treated group (n=6-9 per genotype). An E2B injection protocol was used that has been shown to alter gene expression in the hypothalamus (Bosch et al., 2013). Animals were injected subcutaneously at 1000 h on post-ovx day 5 with either 0.25 µg of E2B or sesame oil. On post-ovx day 6, a 1.5 µg dose of E2B or sesame oil was injected at 1000 h. We did not include intact females in our experimental design as neither ERKO nor KIKO females exhibit a normal estrous cycle, which makes it difficult to compare among intact WT, KIKO, and ERKO females (Park et al., 2011). Animals were sacrificed on post-ovx day 7 at 1000 h. Animals were sedated with ketamine (100 µl of 100 mg/ml stock, i.p.) and decapitated. Brains were removed and rinsed in ice-cold Sorensen's Phosphate Buffer (0.2 M sodium phosphate, dibasic; and 0.2 M sodium phosphate, monobasic) for 30-60 sec. The basal hypothalamus (BH) was cut using a brain slice matrix (Ted Pella, Inc., Redding, CA, USA) into 1-mm thick coronal rostral and caudal blocks corresponding to Plates 42 to 47 and Plates 48 to 53, respectively, from *The Mouse Brain in Stereotaxic Coordinates* (Franklin and Paxinos, 2008). The slices were transferred to a 50/50

Pyrogard water/RNA*later*® (Life Technologies, Grand Island, NE, USA) solution and fixed overnight at 4°C. The ARC tissue, found in two slices, was microdissected using a dissecting microscope, following our previous publications (Bosch et al., 2009,Franklin and Paxinos, 2008,Mamounis et al., 2013,Roepke et al., 2008). The microdissected sections represent the entirety of the ARC tissue. Dissected tissue was stored at -80 °C until RNA extraction. Trunk blood was collected at sacrifice to measure plasma E2 levels. Uteri were removed and wet weight was recorded. Wet uterine weight (mg) is an indicator of circulating E2 in WT mice.

2.3.5 Tissue extraction

RNA was extracted from ARC using Ambion RNAqueous® Micro Kits (Life Technologies, Inc., Carlsbad, CA, USA) according to the manufacturer's protocol, followed by DNase-I treatment to remove contamination by genomic DNA. RNA samples were run on a NanoDrop™ ND-2000 spectrophotometer (ThermoFisher, Inc., Waltham, MA, USA) to assess quantity, followed by an Agilent 2100 Bioanalyzer run using the RNA 6000 Nano Kit (Agilent Technologies, Inc., Santa Clara, CA, USA) to assess quality. Samples with a RNA integrity number (RIN) > 8 were used for quantitative real-time PCR (qPCR).

2.3.6 Blood preparation

Whole trunk blood was centrifuged (1300 rpm at 4 °C for 30 min). The supernatant was subjected to an additional 15 minutes of centrifugation (4 °C at 1300 rpm), then the plasma supernatant was removed and stored at -20 °C until E2 analysis. E2 concentration of plasma was analyzed using Mouse Calbiotech ELISA at the Ligand Assay and Analysis Core of the University of Virginia's Center for Research in Reproduction (Haisenleder et al., 2011). The Calbiotech ELISA is specific to E2 detection.

2.3.7 Quantitative real-time PCR

cDNA was synthesized from 200 ng of total RNA using Superscript III reverse transcriptase (Life Technologies, Inc.), 4 µl 5x buffer, 25 mM $MgCl_2$, 10 mM dNTP (Clontech Laboratories, Inc., Mountain View, CA, USA), 100 ng random hexamer primers (Promega Corporation, Madison, WI, USA), 40 U/µl Rnasin (Promega), and 100 mM DTT in DEPC-treated water (GeneMate, Bioexpress, Inc., Kaysville, UT, USA) in a total volume of 20 µl. Reverse transcription was conducted using the following protocol: 5 min at 25 °C, 60 min at 50 °C, 15 min at 70 °C. A 1:20 dilution of the cDNA was produced using nuclease-free water (GeneMate) for a final cDNA concentration of 0.5 ng/µl and stored at -20 °C. BH tissue RNA, which contains the ARC, from a male mouse was used as a positive control. A negative tissue control (BH with no reverse transcriptase) was also used.

A Taqman® Low Density Array (TLDA) (Life Technologies, Inc.) was used to analyze ARC gene expression in WT females. This array consisted of Taqman® expression assays of genes known to be E2-regulated and/or involved in reproduction and energy homeostasis (see Table 1 for a listing of genes analyzed). Data presented graphically only include genes significantly regulated by E2B, with the exception of *Gpr30*. The TLDA was designed so each expression assay was run in triplicate, including the reference gene, *Actb,* and the internal control, *18S.* On each TLDA plate, one WT experimental female sample was run in duplicate and a calibrator sample (male BH) was run on the remaining wells. The same calibrator sample was run on each plate. Taqman® primers were ordered for all genes, and KIKO and ERKO samples were analyzed. Positive and negative tissue control samples and master mix (nuclease-free water) controls were added to each run. In KIKO and ERKO qPCR plates, an additional sample (termed “pool”) was analyzed that included all the control oil samples from each respective genotype, to account for inter-plate variation (data from C_q values analyzed across plates). qPCR was performed on a StepOnePlus™ Real-Time PCR System (Life

Technologies, Inc.) using Taqman® Gene Expression Master Mix. For qPCR, we used 4 µg of cDNA (equivalent to 2 ng of total RNA). The amplification protocol for all genes was as follows: a holding stage consisted of 2 min at 50 °C and 95 °C for 10 min, followed by a cycling stage of 95 °C for 15 sec (denaturing) and at 60 °C (annealing) for 1 min for 40 cycles.

In addition to the genes analyzed in the TLDA, we also analyzed the mRNA expression of four additional genes: *Adra1b, Cart, Ghsr,* and *Chrm1*, which were found to be E2-regulated in our preliminary investigations or from the literature (Nedungadi and Briski, 2007,Silva et al., 2010). Primers for these genes were designed to span exon-exon junctions and were synthesized by Life Technologies, Inc., using Clone Manager 5 software (Sci Ed Software, Cary, NC, USA). See Table 2 for a listing of synthesized primers used for qPCR. For qPCR of these four genes, we used 4 µg of cDNA (equivalent to 2 ng of total RNA) amplified with either PowerSYBR® Green Master Mix (*Adra1b, Chrm1;* Life Technologies, Inc.) or SsoAdvanced™ SYBR Green (*Cart, Ghsr;* BioRad, Inc., Hercules, CA, USA) on CFX-Connect Real-time PCR Instrument (BioRad, Inc.). A standard curve was generated for each primer pair using serial dilutions of BH cDNA in triplicate. Efficiencies were calculated as a percent efficiency, listed in Table 2. Amplification protocol for Table 2 genes was as follows: initial denaturing 95 °C for 10 min (PowerSYBR®) or 3 min (SsoAdvanced™) followed by 40 cycles of amplification at 94 °C for 10 sec (denaturing), 60 °C for 45 sec (annealing), and completed with a dissociation step for melting point analysis with 60 cycles of 95 °C for 10 sec, 65 °C to 95 °C (in increments of 0.5 °C) for 5 sec and 95 °C for 5 sec. The reference genes used were *β-actin* (PowerSYBR®) and *Gapdh* (SsoAdvanced™). Positive, negative, and water blank controls were included in the qPCR plate design.

Analysis of qPCR was done using the comparative C_q method using a 1:20 diluted BH cDNA (equivalent to 2 ng of RNA) sample from a male as the calibrator (Livak

and Schmittgen, 2001,Pfaffl, 2001). All values were normalized to oil controls and are expressed as relative mRNA expression. In all plates, we maintained a consistent threshold level, set at the lowest but steepest slope of the exponential curve. We calculated the linear quantity of target genes using the formula $2^{-\Delta\Delta Cq}$. Data are expressed as *n*-fold difference from the calibrator, normalized to oil controls. The *n*-fold difference was used for statistical analysis.

2.3.8 Statistical analyses

qPCR data from the TLDA in WT females were initially analyzed using Data Assist® software (Life Technologies, Inc.) to determine significant differences between oil- and E2B-treated samples using a *t*-test with a false discovery rate (Benjamini-Hochberg method) set to a $p < 0.05$. All further statistical analyses were performed using GraphPad® Prism software (GraphPad Software, Inc., La Jolla, CA, USA). Data were expressed as mean ± SEM and analyzed by a two-way ANOVA (genotype x treatment), followed by a *post-hoc* Bonferroni's multiple comparison test between oil- and E2B-treated groups within each genotype (WT, KIKO, ERKO). Uterine weights were analyzed using a two-way ANOVA (genotype x treatment) followed by Bonferroni's multiple comparison test between oil- and E2B-treated groups, within each genotype (WT, KIKO, ERKO). Plasma E2 levels of all three genotypes were pooled together for oil vs. E2B treatment analysis, as there was no genotype effect (data not shown), and analyzed using a Student's *t*-test. In all experiments, a $p < 0.05$ was considered to be significant. To determine relative gene expression among genotypes, we compared oil-treated WT, KIKO, and ERKO females. These data were analyzed using a one-way ANOVA followed by a *post-hoc* Bonferroni's multiple comparison test.

2.4 Results

2.4.1 Uterine weights and plasma E2 levels

Following sacrifice, we dissected and weights the uterus of each female to confirm the hypertrophic actions of E2, an ERα-mediated process (Kurita et al., 2001). Past studies in our lab have suggested that E2B (250 ng/dose) replacement every other day for 4 weeks significantly increased the uterine weight in WT females (Mamounis et al., 2013). E2B significantly increased the uterine weight in WT females (Table 3; ANOVA: $F(2,24) = 34.75$, $p < 0.0001$), but did not increase uterine weight in KIKO and ERKO females. Body weight at sacrifice was not affected by for E2B, in any of the genotypes. The age of sacrifice of females was as follows: WT: 12-23 weeks (average: 18.2 ± 1.0 weeks); KIKO: 7-23 weeks (average: 17.2 ± 1.6 weeks); ERKO: 7-23 weeks (average: 13.9 ± 1.8 weeks). There were no age-specific effects when we analyzed the $2^{\Delta\Delta Cq}$-values for all regulated genes across genotypes (data not shown).

We pooled plasma E2 data across genotypes (WT, KIKO, ERKO) because there was no genotype effect observed (data not shown). Plasma E2 concentrations were as follows: oil-treated: 5.2 ± 0.4 pg/ml; E2B-treated: 28.82 ± 7.0 pg/ml. There was a significant increase in plasma E2 levels 24 h post-injection between oil- and E2B-treated groups ($p < 0.001$).

2.4.2 E2B dose regulates ARC gene expression

E2B-treatment in ovx WT females regulated the mRNA expression of 14 genes in the ARC (Table 4). These genes include those that function as cation channels, receptors for hormones and neurotransmitters, and neuropeptides. E2 replacement suppressed the mRNA expression of *Adra1b, Cart, Chrm1, Esr1, Kiss1, Pdyn, NKB (Tac2),* and *Tac3r* and augmented expression of *Cacna1g, Esr2*, *Ghsr, Kcnmb1, Kiss1r,* and *Pgr*. The remaining genes in Table 4 were not regulated by E2B in WT females.

After studying WT ARC mRNA expression, we then analyzed all the genes in ARC tissue from KIKO and ERKO females injected with either sesame oil or E2B using individual Taqman® assays or custom primers. In comparing the genotypes, we found

that mRNA expression of two cation channels, which are involved in neuronal excitability, were upregulated by E2B in WT females only: Cav3.1 (*Cacna1g*), a subunit of the T-type Ca^{2+} channels, (Figure 1A; ANOVA: $F(2,30) = 4.004$, $p < 0.05$); and *Kcnmb1*, the β1 regulatory subunit for Ca^{2+}-activated potassium channel (Figure 1B; ANOVA: $F(1,27) = 6.221$, $p < 0.05$). The mRNA expression of both genes was increased more than two-fold in WT females injected with E2B.

In addition, multiple receptors involved in reproduction and energy balance showed a change in gene expression with E2B treatment. The mRNA expression of the cholinergic muscarinic receptor 1 (*Chrm1)* was suppressed threefold (Figure 2A; ANOVA: $F(2,30) = 25.64$; $p < 0.0001$), and ERα (*Esr1)* by ~35% (Figure 2B; ANOVA: $F(2,29) = 4.143$; $p < 0.05$) in WT females. E2B increased expression of growth hormone secretagogue receptor (*Ghsr*) (Figure 2C; ANOVA: $F(2, 30) = 28.45$; $p < 0.0001$) and progesterone receptor (*Pgr*) (Figure 2D; ANOVA: $F(2,29) = 13.43$; $p < 0.0001$) in WT females. Expression of *Tac3r,* the NKB receptor, was suppressed twofold in both WT and KIKO females by E2B (Figure 2E; ANOVA: $F(1,29) = 40.58$, $p < 0.0001$). Expression of adrenergic receptor, *Adra1b,* was suppressed by E2B across all genotypes (Figure 2F; ANOVA: $F(1,34) = 17.64$, $p < 0.001$). Expression of the kisspeptin receptor, *Kiss1r,* was increased by E2B across all genotypes (Figure 2G; ANOVA: $F(1,29) = 35.49$, $p < 0.0001$). Finally, ERβ (*Esr2*) expression was augmented by E2B in both WT and KIKO females (Figure 2H; ANOVA: $F(2,28) = 4.452$, $p < 0.05$).

E2B also regulated several important neuropeptide genes in the ARC. In WT females only, prodynorphin (*Pdyn*) and neurokinin B (*Tac2*) expression were suppressed by E2B twofold (Figure 3A: ANOVA: $F(1, 29) = 9.322$, $p < 0.01$) and fivefold (Figure 3B; ANOVA: $F(2, 29) = 5.183$, $p < 0.05$), respectively. Kisspeptin (*Kiss1*) gene expression was suppressed fivefold in WT females and twofold in KIKO females (Figure 3C;

ANOVA: $F(1,28) = 27.63$, $p < 0.0001$). Interestingly, *Cart* expression was suppressed by E2B in WT and KIKO females, but was increased by E2B in ERKO females (Figure 3D; ANOVA: $F(2,42) = 7.719$, $p < 0.01$). Surprisingly, we did not find a significant change in other E2-regulated arcuate genes including POMC and TH, which may be due to differences in treatment paradigms or rodent models (Blum et al., 1987,Priest and Roberts, 2000,Roepke et al., 2008).

2.4.3 ARC genes are differentially expressed in KIKO and ERKO ovx oil-treated females

Differences in gene expression across these genotypes may provide insight into signaling and feedback mechanisms involved in ERE-dependent, ERE-independent and ERα-independent signaling in the ARC. To determine if mRNA expression of the selected genes was different among the genotypes, we calculated the relative mRNA expression for each gene by normalizing the KIKO and ERKO oil groups to the WT oil group. Data were analyzed using a one-way ANOVA followed by *post-hoc* Bonferroni's multiple comparison tests between WT vs. KIKO, WT vs. ERKO, and KIKO vs. ERKO females. See Table 5 for results.

The mRNA levels of multiple genes were lower in KIKO and ERKO oil-treated females as compared to WT females. Interestingly, *Cart* expression was lower in KIKO females but not ERKO females compared to WT females. *Cart* expression in KIKO females was lower than in ERKO females (ANOVA: $F(2, 14) = 19.43$, $p < 0.0001$). For eight genes, mRNA expression in KIKO and ERKO females was lower compared to WT females, with no difference observed between KIKO and ERKO females. These genes include: *Abcc8* (the regulatory subunit for K_{ATP} channels; ANOVA: $F(2,14) = 15.63$, $p < 0.001$), *Chrm1* (ANOVA: $F(2,14) = 5.657$, $p < 0.05$), *Gad2* (glutamate decarboxylase 2; ANOVA: $F(2,14) = 181.8$, $p < 0.0001$), *Htr2c* (the 5HT2c serotonin receptor, ANOVA: $F(2,14) = 30.72$, $p < 0.0001$), *Kiss1* (ANOVA: $F(2,14) = 10.43$, $p < 0.01$), *Pomc* (ANOVA:

F(2,14) = 12.48, p < 0.001), *Tac2* (ANOVA: F(2,14) = 26.99, p < 0.0001), and *Th* (ANOVA: F(2,14) = 47.44, p < 0.0001). For another two genes, mRNA expression was lower in KIKO and ERKO females compared to WT females, with mRNA expression also lower in ERKO females than in KIKO females. These genes were *Bcl2* (the anti-apoptotic gene B cell leukemia/lymphoma 2; ANOVA: F(2, 14) = 124.8, p < 0.0001), and *Sirt1* (Sirtuin 1 or NAD-dependent deactylase; ANOVA: F(2, 14) = 16.58, p < 0.001).

The mRNA expression of three other genes was lower in ERKO females, but not KIKO females, compared to WT females. These genes include *Gpr30* (ANOVA: F(2, 14) = 10.05, p < 0.01), *Kcnmb4* (Ca^{2+}-activated potassium channel subunit β4; ANOVA: (2, 14) = 10.04, p < 0.01), and *Mtor* (mammalian target of rapamycin; ANOVA: F(2, 14) = 10.19, p < 0.01). Additionally, six genes were expressed at lower levels in ERKO females compared to both WT and KIKO females. These genes were *Cacna1h* (a subunit of the T-type Ca^{2+} channels; ANOVA: F(2, 14) = 10.92, p < 0.001), *Esr1* (ANOVA: F(2,14) = 22.96, p < 0.0001), *Gad1* (glutamate decarboxylase 1; ANOVA: F(2,14) = 15.48, p < 0.001), *Kcnj11* (Kir6.2, the channel subunit for K_{ATP} channels; ANOVA: F(2,14) = 20.18, p < 0.0001), *Ncoa* (a coactivator of transcription; ANOVA: F(2,14) = 15.17; p < 0.001), and *Pik3r3* (PI3K p55γ subunit; ANOVA: F(2,14) = 6.116, p < 0.05). Interestingly, expression of calmodulin (*Calm1*) was lower in ERKO females compared to KIKO females, but not WT females (ANOVA: F(2,14) = 5.163, p < 0.05).

We observed other gene-expression differences between genotypes. *Adra1b* expression was higher in KIKO females compared to WT females (ANOVA: F(2,14) = 10.82, p < 0.01). Additionally, expression of *Cacna1g* (ANOVA: F(2,14) = 14.64, p < 0.001) and *Pgr* (ANOVA: F(2,14) = 14.04, p < 0.001) was higher in both KIKO and ERKO females compared to WT females. Expression of *Kiss1r* (ANOVA: F(2,12) = 16.29, p < 0.001) and *Kcnmb1* (ANOVA: F(2,14) = 18.41, p < 0.001) was higher in ERKO females compared to both WT and KIKO females. Interestingly, expression of

Pdyn was higher in KIKO females compared to WT females and lower in ERKO compared to KIKO females (ANOVA: $F(2,14) = 16.41$, $p < 0.001$). Finally, there was no change observed in *Esr2*, *Ghsr, Npy, Tac3r,* and *Kcnq5* (a subunit of the potassium channel that produces the M-current) in the ARC across oil-treated females in WT, KIKO, and ERKO genotypes.

2.5 Discussion

Previous studies have demonstrated that E2 controls homeostatic functions through ERα-mediated mechanisms by regulating ARC gene expression. In the present study, to distinguish between ERE-dependent and ERE-independent transcriptional mechanisms, we compared gene expression in WT, ERα KO and an ERα KIKO mouse models. Previously, this KIKO model, which lacks ERα-mediated ERE-dependent signaling, has been used to delineate such ERα-mediated signaling in the uterus (Hewitt and Korach, 2011) and the HPG axis (Glidewell-Kenney et al., 2007,Gottsch et al., 2009). Studies on osteoblasts suggested that E2 regulation of gene transcription occurs through both ERE-dependent and ERE-independent mechanisms (including non-genomic mechanisms) (Roforth et al., 2014). The classical ERα signaling pathway is genomic ERE-dependent gene transcription in which E2 binds to ERα in the nucleus and then ERα binds to ERE to regulate expression of multiple genes. In addition to the classical ERα regulation of gene expression, there are ERE-independent mechanisms that include ERα/β-mediated, non-genomic second messenger pathways, and ERα/β-independent signaling through membrane estrogen receptors (Qiu et al., 2003,Qiu et al., 2006). ERE-independent signaling includes PI3K and mitogen-activated protein kinase second messenger signaling cascades from membrane-associated ERα/β, protein-

protein interactions with other transcription factors, and ligand-independent mechanisms (McDevitt et al., 2008).

Recent evidence suggests that E2 signals through multiple membrane ERs including GPER (GPR30) (Roepke et al., 2008,Roepke et al., 2010). However, there are few studies that determine how E2 signals through ERE-dependent and ERE-independent mechanisms to regulate gene expression in the hypothalamus. The present study compared gene expression in WT, KIKO, and ERKO mouse models to characterize mechanisms of E2 regulation of ARC gene expression. We determined that regulation of ARC gene expression by E2B, with E2 being the active hormone, occurs through both ERE-dependent and ERE-independent mechanisms. The genes regulated by ERα-mediated, ERE-dependent and ERE-independent mechanisms in this study include cation channels, receptors, and neuropeptides associated with reproduction, energy balance, stress, and other homeostatic functions. However, it is necessary to note that our ERE-independent KIKO mouse model is nonselective to other hormone response element (HRE) motifs and may bind to other HREs to regulate transcription (Hewitt et al., 2014). The development of an “EAAE” ERα mouse that lacks ERE- and HRE-dependent signaling would be useful in future studies to distinguish between those two types of signaling (Hewitt et al., 2014).

In the current study, E2 (or treatment with E2B) increased channel expression of *Cacna1g* and *Kcnmb1* in WT females only, a finding supported by previous studies, but not in KIKO or ERKO (Bosch et al., 2013,Roepke et al., 2007,Roepke et al., 2011). Voltage-dependent T-type calcium channel subunit alpha-1G (*Cacna1g*, also known as *Cav3.1*) is one subunit of low voltage-activated (T-type) calcium channels that is important in burst firing and neurotransmitter release. The elevated expression of *Cacna1g* expression in KIKO and ERKO females suggests that ERα-mediated, ERE-

dependent signaling independent of ligand differentially regulates *Cacna1g* expression. Past studies have suggested that E2 regulates expression of the *Cav3* subunits (Qiu et al., 2006,Zhang et al., 2009). For example, in GnRH neurons of the preoptic area (POA), a high E2B dose increased *Cacna1g* expression transiently (Zhang et al., 2009). Increased expression of *Cacna1g* in the ARC led to increased neuronal excitability and burst firing to regulate hypothalamic neurons involved in reproduction and energy homeostasis (Qiu et al., 2006,Zhang et al., 2009).

E2 has been found to regulate mRNA expression of a range of potassium channels in the guinea pig (Roepke et al., 2007). In the current study, we found that expression of *Kcnmb1*, a calcium-activated potassium channel (a MaxiK channel), was increased by E2B treatment in WT females only. Collectively, these studies emphasize the role of E2 regulation of channel expression in the ARC, which may translate to regulation of electrophysiological properties of ARC neurons (Roepke et al., 2011). Future studies in our lab will investigate the neurophysiological effects of the E2-induced gene expression. Furthermore, the results of our study indicate that in the mouse ARC, *Kcnmb1* MaxiK channel expression is regulated through ERE-dependent mechanisms. Elevated expression of *Kcnmb1* in oil-treated ERKO females also indicates that the loss of ERα positively affects ARC *Kcnmb1* gene expression.

In addition to the above findings related to channel expression regulation, the results of this study indicate that, through multiple ER-mediated mechanisms, E2B regulates several hormone and neurotransmitter receptors in the ARC. These receptors are involved in a number of functions including reproduction, neuronal excitability, and energy balance. Collectively, our data suggest that E2B regulates the expression of several receptors involved in feeding, stress, and neurotransmission through multiple receptor-mediated mechanisms. *Chrm1* is a muscarinic acetylcholine receptor involved in a number of functions including memory consolidation, neuronal excitability, and

signal transduction (Nathanson, 2000). Only a few studies to date have examined *Chrm1* expression in the ARC. In the present study, E2B suppressed *Chrm1* expression in the ARC through ERE-dependent signaling. Past studies in the rat hippocampus also suggest that *Chrm1* is decreased in response to immediate E2 replacement following ovx (Cardoso et al., 2010). The current study used a different E2 replacement paradigm, yet it would be informative to examine *Chrm1* expression at different time points after E2B administration in the ARC to determine the time-dependent regulation of *Chrm1* expression. We also found suppressed *Chrm1* expression in KIKO and ERKO females compared to WT. This difference may result in a decrease in muscarinic signaling in the ARC of KIKO and ERKO females, which can be examined in future experiments using electrophysiology to assess muscarinic activity in ARC neurons. Furthermore, the role of muscarinic receptors in the control of homeostatic functions in the ARC has not been previously investigated and the effects of the suppression by E2 in these functions are unknown.

Expression of another neurotransmitter receptor, *Adra1b,* was suppressed by E2B treatment in WT, KIKO, and ERKO females. Alpha-1B adrenergic receptor (*Adra1b)* is a receptor for catecholamines (norepinephrine/epinephrine) involved in arousal, feeding behaviors, cell growth, and proliferation. Previous studies in rats report that E2 increases *Adra1b* expression in the hypothalamus and POA (Karkanias et al., 1996,Petitti et al., 1992,Quesada and Etgen, 2002). E2 was administered via subcutaneous injection 24- and 48-hours prior to sacrifice, similar to the present study. The difference between those studies and the current study indicate the differential effects of E2 on adrenergic signaling between rodent species and between discrete hypothalamic nuclei. Furthermore, previous reports suggest that E2 inhibits catecholamine secretion *in vitro* (Kim et al., 2000,Machado et al., 2002). It should be noted that *Adra1b* suppression occurs in ERKO animals, suggesting that this is an ERα-

independent mechanism. A decrease of *Adra1b* expression in the ARC would suppress, in part, the actions of noradrenergic/adrenergic signals from the hindbrain that control hypothalamic functions including arousal, feeding behavior, and energy expenditure (Bienkowski and Rinaman, 2008,Rinaman, 2003). Genotype-specific analysis indicates that *Adra1b* expression and activity is different between KIKO and WT females, which may be a mechanism behind the differences in feeding behavior between the genotypes (Mamounis et al., 2013). Future studies should examine if the difference in expression correlates with changes in noradrenergic signaling in ARC neurons, specifically NPY neurons.

The growth hormone secretagogue receptor, GHSR, is a GPCR involved in ghrelin signaling. E2B treatment has been shown to increase *Ghsr* expression in the ARC in mice (Frazao et al., 2014). We determined that the increase in ARC *Ghsr* expression by E2 is regulated by ERE-dependent mechanisms, as E2B-treatment did not affect *Ghsr* expression in KIKO or ERKO females. Ghrelin signaling in the hypothalamus illuminates the relationship of E2 on feeding behavior control by ARC neurons. *Ghsr* activation by ghrelin excites NPY neurons (Cowley et al., 2003) and initiates a signaling cascade that increases transcription of NPY and AgRP (Nogueiras et al., 2010). Activation of NPY neurons is associated with an increase in food intake, which is opposed by the actions of E2 (Hillebrand et al., 2002). However, *Ghsr* is expressed throughout the heterogeneous population of ARC neurons including POMC, TH, and KNDy neurons (Frazao et al., 2014,Guan et al., 2008,Pirnik et al., 2014), and the increase in ARC *Ghsr* expression by E2 is most likely involved in other homeostatic functions of these neurons. Cell-type specific analysis will be necessary to address this contradictory information of E2's regulation of GHSR signaling in the ARC.

Our results also indicate that steroid receptors associated with reproduction are regulated by E2 through both ERE-dependent and ERE-independent mechanisms. The

classical role of E2 in the control of reproduction is through feedback on the HPG axis and regulation of steroid receptor expression. The results of this study agree with past studies on E2 regulation of the estrogen receptor, ERα (*Esr1).* In the hypothalamus, E2 has been shown to decrease expression of *Esr1* (Pinzone et al., 2004). This is supported by our study, which suggests that the E2-mediated *Esr1* suppression occurs through ERE-dependent mechanisms. There are no previous studies that identify the mechanism for this E2-mediated *Esr1* decrease. Furthermore, E2B treatment augmented the expression of ERβ (*Esr2*) in WT and KIKO females. These data indicate that, unlike with ERα, ERβ is regulated in the ARC by ERE-independent, ERα-mediated signaling. GPER/GPR30, a membrane estrogen receptor, was not regulated by E2 in any genotype. ERα (*Esr1*) expression was lower in ERKO compared to WT and KIKO females. GPER (*Gpr30)* expression was lower in ERKO compared to WT females. Lastly, there was no difference in expression of ERβ (*Esr2*) between all genotypes.

In addition, E2B treatment increased expression of *Pgr* in WT females only. These results are consistent with multiple studies that suggest E2B increases *Pgr* in the hypothalamus, priming the brain for progesterone's reproductive and behavioral actions (Diotel et al., 2011,Moffatt et al., 1998), but also suggest that *Pgr* is regulated through ERE-dependent, ERα-mediated signaling pathway. *Pgr* expression is two- to threefold higher in both KIKO and ERKO females compared to WT females. Interestingly, the relative expression of *Pgr* in oil-treated KIKO and ERKO females is equal to the relative levels of *Pgr* in E2B-treated, WT females. Higher expression of *Pgr* in the ARC may play a role in the lack of normal estrous cycles in KIKO and ERKO females.

In the present study, E2B treatment regulated multiple neuropeptides that are coexpressed in KNDy neurons of the ARC and their receptors. Kisspeptin-expressing neurons are expressed in two main regions in the rodent hypothalamus (Brock and

Bakker, 2013). The first region is the anteroventral periventricular (AVPV) nucleus, which is referred to as the surge center for its role in the LH surge in female rodents (Brock and Bakker, 2013). The second region is the ARC, which contains kisspeptin neurons that coexpress Kisspeptin (*Kiss1)*, Neurokinin B (*Tac2)* and Dynorphin (Dyn, *Pdyn)* (Oakley et al., 2009). These KNDy neurons are known for their contribution to negative feedback of E2 on the HPG axis and are hypothesized to be the pulse generator for the secretion of GnRH into the median eminence (Lehman et al., 2010,Navarro and Tena-Sempere, 2011). Additional studies suggest that kisspeptin neurons directly contact GnRH neurons in the median eminence to control GnRH excitability and pulsatility (Lehman et al., 2010). Since then, KNDy neurons have also been shown to integrate feeding signals through input from peptides such as ghrelin, leptin, and insulin (De Bond and Smith, 2013,Fernandez-Fernandez et al., 2006). Our results corroborate previous data demonstrating that *Kiss1* regulation by E2 is nonclassically mediated (Gottsch et al., 2009). *Kiss1* expression also was lower in both KIKO and ERKO females than WT females. Because ARC *Kiss1* is involved in negative feedback, perhaps the lower expression indicates that negative feedback is augmented in these genotypes, in part, due to lower expression of *Kiss1* (Lehman et al., 2010,Navarro and Tena-Sempere, 2011)..

Amongst the other two KNDy neuropeptides, Neurokinin B (*Tac2)* expression is suppressed by E2 in WT females only. Our data suggest that *Tac2* expression is primarily controlled by ERE-dependent signaling much like *Pdyn* (Kurita et al., 2001,Zuloaga et al., 2012). As with *Kiss1*, *Tac2* expression is lower in both KIKO and ERKO females. The suppressed expression of *Tac2* in these genotypes also supports the hypothesis that lower expression of both *Kiss1* and *Tac2* plays a role in the disruption of negative feedback in these genotypes. Because Neurokinin B (NKB, *Tac2*) is involved in GnRH pulse generation, the lower expression in KIKO and ERKO

contributes to the dysregulation of the GnRH actions on the gonadotropes in these genotypes (Lehman et al., 2010,Navarro and Tena-Sempere, 2011). Finally, *Pdyn* expression, the other KNDy neuropeptide, is suppressed by E2B through ERE-dependent transcription. These data support previous studies and was used in this study as a "negative" control for ERE-independent signaling (Gottsch et al., 2009). Interestingly, *Pdyn* expression was higher in KIKO females compared to WT females unlike *Kiss1* and *Tac2* expression. Elevated *Pdyn* may further disrupt the pulse generator in this genotype as this neuropeptide is considered a negative regulator of the pulse generator (Lehman et al., 2010,Navarro and Tena-Sempere, 2011). Because *Kiss1* is the only KNDy gene that is regulated by ERα ERE-independent mechanisms, the KNDy pulse generator (NKB, Dyn) for GnRH secretion is primarily controlled by ERE-dependent mechanisms.

KNDy-associated receptors are also regulated by E2. *Tac3r,* the NKB receptor, is suppressed in WT and KIKO females by E2B treatment, suggesting that, like *Kiss1, Tac3r* suppression is through an ERα-mediated, ERE-independent pathway (Sinkevicius et al., 2008). These results suggest that both ERE-dependent and ERE-independent mechanisms are involved in the control of KNDy neuropeptides and their receptors and are necessary to maintain a functional HPG axis. In fact, current evidence suggests that mutations in *Tac3r* lead to problems in reproductive development including hypogonadotrophic hypogonadism, similar to mutations in *Kiss1r* (Gianetti et al., 2010,Topaloglu et al., 2009). The decrease in *Tac3r* and *Tac2* associated with E2B treatment may both be involved in KNDy-mediated control of negative feedback and the GnRH pulse generator.

Kiss1r, the receptor for Kiss1*,* is increased by E2B treatment in all three genotypes. This upregulation suggests that E2 regulation of *Kiss1r* expression is ERα-independent and potentially mediated by ERβ, GPER, or perhaps the putative STX-

responsive, Gq-coupled mER (Gq-mER) (Mizukami, 2010,Roepke et al., 2007,Roepke et al., 2008). Alternatively, the increase in *Kiss1r* expression may be due, in part, to a decrease in the desensitization of the receptor, as Kiss1 peptide and gene expression is highly reduced by E2 in the ARC (Bosch et al., 2012,Brock and Bakker, 2013,Eghlidi et al., 2010). Future studies should examine E2 regulation of Kiss1r peptide expression selectively in the median eminence, where KNDy neurons contact GnRH neurons expressing the kisspeptin receptor (Bosch et al., 2013,Oakley et al., 2009). *Kiss1r* expression was higher in ERKO females compared to their WT and KIKO females, which correlated with a decrease in the ligand expression in ERKO females. However, the effect of E2 across the genotypes was similar, suggesting that the E2-mediated increase is robust and not dependent on relative baseline expression levels.

Lastly, *Cart* gene expression, which produces a neuropeptide involved in reward, stress, and feeding behavior (Hill et al., 2008,Koylu et al., 2006), is differentially regulated by E2 in the three genotypes. CART activity has been shown to suppress feeding much like E2 in rodents and primates (Hill et al., 2008,Koylu et al., 2006). However, in this experiment, E2B treatment suppressed *Cart* in WT and KIKO females. These results are supported by previous studies that have reported the suppression of *Cart* by E2 in the rodent ARC (Silva et al., 2010) and suggest that the suppression of the *Cart* gene in the ARC is a compensatory homeostatic response for the increase in CART protein activity in the paraventricular nucleus to suppress feeding (Dandekar et al., 2012). Interestingly, E2B augmented *Cart* expression in ERKO females. The mechanism behind this effect of E2B is unknown, but certainly involves the other ER in the ARC. To date, there have been no studies that examine which ER-mediated mechanism regulates *Cart* expression or their contribution to the physiological effects of CART in the hypothalamus.

There are a number of additional receptors that can be involved in ERα-independent, E2 signaling and may be involved in the control of *Kiss1r*, *Adra1b*, and *Cart*. In addition to ERα, the classical receptor ERβ can also regulate E2-responsive genes through ERE-dependent and ERE-independent signaling. Although ERα is more highly expressed in the ARC compared to ERβ, studies suggest that ERβ signaling is still present (Roepke, 2009) and may function as a compensatory mechanism for gene regulation by E2 in the ERKO since *Esr2* is expressed in ARC during development (Wilson et al., 2002). In addition, non-nuclear ER-mediated E2 signaling can also regulate gene expression through G-protein coupled membrane estrogen receptors (GPER) and the putative Gq-mER (Qiu et al., 2008). Evidence suggests that GPER is activated by E2 and is important for rapid cell signaling and homeostatic functions (Davis et al., 2014,Revankar et al., 2005). Gq-mER activates PLC-PKC signal transduction pathway and functions in energy homeostasis, bone remodeling, and core body temperature including activation of hypothalamic neurons (Qiu et al., 2003) as well as control of ARC gene expression (Roepke et al., 2008).

We found that there were a number of genes that were not regulated by E2B but did show differences in expression amongst genotypes. These genes include *Abcc8, Bcl2, Cacna1h, Calm1, Gad1, Gad2, Gpr30, Htr2c, Kcnj11, Kcnmb4, Mtor, Ncoa1, Pik3r3, Pomc, Sirt1,* and *Th.* The range of expression differences amongst the genotypes differs for each gene. While there is no easily discernable pattern of expression differences, these changes may result in deleterious effects of ERα loss (knockout) on reproductive and energy homeostasis. Furthermore, it is important to note that the present study only examined differences in gene expression among oil-treated genotypes, which may not produce functional differences in ARC neurons or in the protein of these enzymes, signaling molecules, receptors, and cation channels. Future experiments will be needed to confirm these potential effects and if these differences are

due to a developmental role of ERα signaling, both ERE-dependent and ERE-independent, in ARC gene expression. Nonetheless, comparison of gene expression across genotypes is an important tool for enhancing our understanding of ERα's role in ARC gene expression and homeostatic functions.

While past studies have examined regulation of gene expression by E2 in the ARC, few studies to date have used the ERα KIKO transgenic mouse model as a tool to identify molecular mechanisms of E2 regulation. The results of our study indicate that genes involved in hypothalamic functions such as reproduction, energy homeostasis, and neuronal excitability are regulated by E2 through multiple ERα-mediated and ERα-independent pathways as has been previously suggested (Roepke et al., 2008). It is important to note that gene expression in our study is a measurement of the steady-state mRNA expression. Unfortunately, due to the small size of the ARC nucleus in mice, it is not possible to conduct immunoblotting of protein expression within a single animal. Because mRNA and protein levels do not always correlate when comparing gene expression and immunohistochemistry, such as with kisspeptin, (Bosch et al., 2012), future studies will examine the expression and activity of many of these genes, especially the cation channels and GPCRs, using electrophysiology. These experiments would further characterize the role of ERα-mediated, ERE-dependent and -independent signaling on ARC gene expression and neuronal functions. Determining these signaling pathways is key to understanding the physiological effects of estrogens during the reproductive cycle and in hormone replacement therapies.

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Tables

Table 1. List of genes analyzed in the Taqman® Low Density Array (TLDA)

Gene Name	Gene Abbreviation	Taqman Assay #
ATP-binding cassette, subfamily C	*Abcc8*	Mm00803450_m1
B cell leukemia/lymphoma 2	*Bcl2*	Mm00477631_m1
Cav3.1	*Cacna1g*	Mm00486572_m1
Cav3.2	*Cacna1h*	Mm00445382_m1
Calmodulin 1	*Calm1*	Mm01336281_g1
Estrogen receptor α	*Esr1*	Mm00433149_m1
Estrogen receptor β	*Esr2*	Mm00599821_m1
Glutamate decarboxylase 1	*Gad1*	Mm04207432_g1
Glutamate decarboxylase 2	*Gad2*	Mm00484623_m1
G protein-coupled receptor 30	*Gpr30*	Mm01194815_m1
Growth hormone	*Gh*	Mm00433590_g1
Serotonin receptor 2C	*Htr2c*	Mm00434127_m1
Calcium-activated potassium channel subunit β1	*Kcnmb1*	Mm00466621_m1
Kir6.2	*Kcnj11*	Mm00440050_s1
Calcium-activated potassium channel subunit β4	*Kcnmb4*	Mm00465684_m1
KCNQ5 (Kv7.5)	*Kcnq5*	Mm01226041_m1
Kisspeptin 1	*Kiss1*	Mm03058560_m1
Kisspeptin receptor	*Kiss1r*	Mm00475046_m1
Neuropeptide Y	*Npy*	Mm03048253_m1
Prodynorphin	*Pdyn*	Mm00457573_m1
Progesterone receptor	*Pgr*	Mm00435628_m1
PI3K p55γ	*Pik3r3*	Mm00725026_m1
Proopiomelanocortin	*Pomc*	Mm00435874_m1
Tachykinin 2	*Tac2*	Mm00436885_m1
Tachykinin 3 receptor	*Tac3r*	Mm00445346_m1
Tyrosine hydroxylase	*Th*	Mm00447557_m1
Nuclear receptor coactivator 1	*Ncoa*	Mm01318933_m1
Mammalian target of rapamycin	*Mtor*	Mm00444968_m1
Sirtuin 1	*Sirt1*	Mm00490758_m1
Beta actin	*Actb*	Mm01205647_g1
18s ribosomal RNA	*18S*	Hs99999901_s1

Genes that were analyzed using a TLDA and their respective Taqman® assay #s are listed. For KIKO and ERKO analysis, identical Taqman® assays were used.

Table 2. List of genes using designed primers

Gene Name	Product length	% Eff	Primer sequence	Base pair #	Accession #
Adra1b	84	100	F: CTTCATCGCTCTCCCACTTG R: TAGCCCAGCCAGAACACT	1174-1193 1240-1257	NM_007416
β-actin	63	100	F: GCCCTGAGGCTCTTTTCCA R: TAGTTTCATGGATGCCACAGGA	849-867 911-990	NM_007393.3
Cart	169	93	F: GCTCAAGAGTAAACGCATTCC R: GTCCCTTCACAAGCACTTCAA	277-297 425-445	NM_013732
Chrm1	272	110	F: AGCAGCTCAGAGAGGTCACAGCCA R: GGGCCTCTTGACTGTATTTGGGGA	1331-1354 1580-1603	NM_001112697
Gapdh	98	93	F: TGACGTGCCGCCTGGAGAAA R: AGTGTAGCCCAAGATGCCCTTCAG	852-875 106-125	NM_008084
Ghsr	122	110	F: CAGGGACCAGAACCACAAAC R: AGCCAGGCTCGAAAGACT	1003-1022 1107-1124	NM_177330

The genes listed are those that were not included in the TLDA. Sense primer is listed first with the antisense primer below. Primer pairs were ordered from Life Technologies and designed to span exon-exon junctions using Clone 5 software. *Adra1b,* alpha-1B adrenergic receptor; *β-actin,* beta actin; *Cart*, cocaine- and amphetamine- regulated transcript; *Chrm1*, cholinergic muscarinic 1 receptor; *Gapdh,* glyceraldehyde-3-phosphate dehydrogenase; *Ghsr*, growth hormone secretagogue receptor.

Table 3. Body and uterine weights

Genotype	Treatment	Body weight (g)	Uterine weight (mg)
WT	Oil	24.0 ± 0.6	27.5 ± 0.9
	E2B	23.6 ± 0.7	98.3 ± 2.1****
KIKO	Oil	22.1 ± 1.6	38.8 ± 5.3
	E2B	22.4 ± 0.9	43.0 ± 5.1
ERKO	Oil	19.5 ± 0.3	10.0 ± 1.3
	E2B	21.0 ± 0.9	13.2 ± 2.4

Body weights (g) and uterine weights (mg) are expressed as mean ± SEM. Data were analyzed with a two-way ANOVA with Bonferroni's multiple comparison tests (****$p < 0.0001$). E2B-treated females were compared to oil-treated females, within each genotype.

Table 4. TLDA Gene Expression Analysis

Gene Name	Functional Name (Protein)	Oil	E2B	Significance
Abcc8	ATP-binding cassette, subfamily C member 8	1.02 ± 0.10	0.97 ± 0.11	-
Adra1b	Alpha-1B adrenergic receptor	1.02 ± 0.04	0.67 ± 0.03	****
Bcl2	Apoptosis regulator Bcl-2	1.00 ± 0.03	0.99 ± 0.06	-
Cacna1g	Voltage-dependent T-type calcium channel subunit alpha-1G	1.01 ± 0.08	1.61 ± 0.17	***
Cacna1h	Voltage-dependent T-type calcium channel subunit alpha-1H	1.01 ± 0.05	1.03 ± 0.06	-
Calm1	Calmodulin	1.01 ± 0.07	1.13 ± 0.05	-
Cart	Cocaine- and amphetamine-regulated transcript protein	1.08 ± 0.08	0.54 ± 0.02	*
Chrm1	Muscarinic acetylcholine receptor M1	1.08 ± 0.03	0.33 ± 0.04	****
Esr1	Estrogen receptor α	1.01 ± 0.06	0.66 ± 0.08	*
Esr2	Estrogen receptor β	1.03 ± 0.13	1.60 ± 0.12	*
Gad1	Glutamate decarboxylase 1	1.01 ± 0.06	0.94 ± 0.07	-
Gad2	Glutamate decarboxylase 2	1.01 ± 0.05	1.03 ± 0.06	-
Gh	Growth Hormone (Somatotropin)	1.03 ± 0.11	1.01 ± 0.11	-
Ghsr	Growth hormone secretagogue receptor type 1	1.16 ± 0.09	2.47 ± 0.15	****
Gpr30	G-protein coupled estrogen receptor 1	1.03 ± 0.12	0.84 ± 0.04	-
Htr2c	5-hydroxytryptamine receptor 2C	1.01 ± 0.09	1.20 ± 0.14	-
Kcnj11	ATP-sensitive inward rectifier potassium channel 11	1.01 ± 0.07	0.92 ± 0.05	-
Kcnmb1	Calcium-activated potassium channel subunit beta-1	1.16 ± 0.25	2.45 ± 0.41	**
Kcnmb4	Calcium-activated potassium channel subunit beta-4	1.03 ± 0.11	1.30 ± 0.07	-
Kcnq5	Potassium voltage-gated channel subfamily KQT member 5	1.00 ± 0.04	1.12 ± 0.11	-
Kiss1	Kisspeptin	1.02 ± 0.11	0.12 ± 0.02	***
Kiss1r	Kisspeptin receptor	1.00 ± 0.02	1.29 ± 0.05	**
Mtor	serine/threonine-protein kinase mammalian target of rapamycin	1.00 ± 0.04	1.08 ± 0.06	-
Ncoa	Nuclear receptor coactivator 1	1.00 ± 0.05	1.07 ± 0.04	-
Npy	Neuropeptide Y	1.13 ± 0.25	0.99 ± 0.19	-
Pdyn	Prodynorphin	1.04 ± 0.15	0.55 ± 0.03	**
Pgr	Progesterone receptor	1.00 ± 0.04	2.12 ± 0.20	****
Pik3r3	Phosphatidylinositol 3-kinase regulatory subunit gamma	1.00 ± 0.03	1.12 ± 0.06	-
Pomc	Pro-opiomelanocortin	1.01 ± 0.08	0..94 ± 0.10	-
Sirt1	NAD-dependent protein deacetylase sirtuin-1	1.00 ± 0.04	1.06 ± 0.03	-
Tac2	Tachykinin 2	1.04 ± 0.16	0.19 ± 0.03	****
Tac3r	Tachykinin 3 receptor	1.04 ± 0.15	0.35 ± 0.06	****
Th	Tyrosine hydroxylase	1.04 ± 0.13	1.01 ± 0.05	-

Data were analyzed by a Students *t*-test for each gene (*$p < 0.05$; **$p < 0.01$; ***$p < 0.001$; ****$p < 0.0001$).

Table 5. Comparison of gene expression between genotypes in oil-treated females

Gene List	WT	KIKO	ERKO
Abcc8	1.02 ± 0.10	0.32 ± 0.14 b	0.21 ± 0.08 c
Adra1b	1.03 ± 0.12	3.97 ± 0.64 b	2.64 ± 0.29
Bcl2	1.00 ± 0.03	0.24 ± 0.07 d	0.02 ± 0.01 d D
Cacna1g	1.01 ± 0.08	1.97 ± 0.15 b	2.36 ± 0.24 c
Cacna1h	1.01 ± 0.05	0.82 ± 0.21	0.09 ± 0.02 c B
Calm1	1.01 ± 0.07	1.07 ± 0.35	0.18 ± 0.05 A
Cart	1.02 ± 0.10	0.19 ± 0.05 d	0.77 ± 0.12 B
Chrm1	1.10 ± 0.31	0.34 ± 0.07 a	0.34 ± 0.06 a
Esr1	1.01 ± 0.06	0.97 ± 0.09	0.44 ± 0.03 c C
Esr2	1.03 ± 0.13	1.80 ± 0.45	2.00 ± 0.32
Gad1	1.01 ± 0.06	0.66 ± 0.16	0.16 ± 0.06 c A
Gad2	1.01 ± 0.05	0.15 ± 0.03 d	0.09 ± 0.02 a
Ghsr	1.01 ± 0.07	1.58 ± 0.42	1.11 ± 0.14
Gpr30	1.03 ± 0.12	0.61 ± 0.21	0.12 ± 0.03 b
Htr2c	1.01 ± 0.09	0.26 ± 0.09 d	0.18 ± 0.07 d
Kcnj11	1.01 ± 0.07	0.83 ± 0.15	0.13 ± 0.06 c C
Kcnmb1	1.16 ± 0.25	6.87 ± 1.45	14.04 ± 1.91 d A
Kcnmb4	1.03 ± 0.11	0.50 ± 0.20	0.13 ± 0.06 b
Kcnq5	1.00 ± 0.04	0.85 ± 0.33	0.20 ± 0.06
Kiss1	0.92 ± 0.27	0.02 ± 0.01 b	0.17 ± 0.05 a
Kiss1r	1.48 ± 0.53	0.45 ± 0.11	6.84 ± 1.66 b C
Mtor	1.00 ± 0.04	0.54 ± 0.18	0.23 ± 0.06 b
Ncoa	1.00 ± 0.05	0.67 ± 0.16	0.18 ± 0.04 c A
Npy	1.13 ± 0.25	0.87 ± 0.49	0.45 ± 0.23
Pdyn	1.04 ± 0.15	2.39 ± 0.23 c	1.50 ± 0.10 B
Pgr	1.00 ± 0.04	3.42 ± 0.35 c	2.59 ± 0.38 a
Pik3r3	1.00 ± 0.03	1.01 ± 0.28	0.24 ± 0.09 a A
Pomc	1.01 ± 0.08	0.44 ± 0.17 a	0.17 ± 0.07 c
Sirt1	1.00 ± 0.04	0.59 ± 0.15 a	0.18 ± 0.02 c A
Tac2	1.08 ± 0.18	0.01 ± 0.01 d	0.13 ± 0.05 c
Tac3r	1.38 ± 0.83	0.05 ± 0.02	0.12 ± 0.03
Th	1.04 ± 0.13	0.23 ± 0.05 d	0.05 ± 0.01 d

Data were normalized to WT oil and analyzed by a one-way ANOVA followed by post-hoc Bonferroni's multiple comparison test, within gene. Lowercase letters denote changes observed between WT and KIKO or ERKO (**a**=$p < 0.05$; **b**=$p < 0.01$; **c**=$p < 0.001$; **d**=$p < 0.0001$). Uppercase letters sign denotes changes observed between KIKO and ERKO females (**A**=$p < 0.05$; **B**=$p < 0.01$; **C**=$p < 0.001$; **D**=$p < 0.0001$).

Figures

Figure 1. E2B regulates channel gene expression in the ARC (A) *Cacna1g*; (B) *Kcnmb1.* Results of qPCR analyses represent gene expression of oil- (black bars) and E2B- (gray bars) treated females in WT, KIKO, and ERKO genotypes. The number of animals in each treatment group is listed within each bar. Genes are expressed as relative *n*-fold changes, normalized to oil controls, within each genotype (WT, KIKO, ERKO). A two-way ANOVA (genotype x treatment) followed by *post-hoc* Bonferroni's multiple comparison test was used to determine significant differences between treatments, within genotype. **p < 0.01; ***p < 0.001.

Figure 2. E2B regulates receptor gene expression in the ARC (A) *Chrm1*; (B*) Esr1*; (C) *Ghsr*; (D) *Pgr;* (E) *Tac3r;* (F) *Adra1b;* (G) *Kiss1r*; (H) *Esr2* and (I) *Gpr30.* Results of qPCR analyses represent gene expression of oil- (black bars) and E2B- (gray bars) treated females in WT, KIKO, and ERKO genotypes. The number of animals in each treatment group is listed within each bar. Genes are expressed as relative *n*-fold changes, normalized to oil controls, within each genotype (WT, KIKO, ERKO). A two-way ANOVA (genotype x treatment) followed by *post-hoc* Bonferroni's multiple comparison test was used to determine significant differences between treatments, within genotype. *p < 0.05; **p < 0.01; ***p < 0.001; ****p < 0.0001.

Figure 3. E2B regulates neuropeptide gene expression in the ARC (A) *Pdyn;* (B) *Tac2;* (C) *Kiss1;* (D) *Cart.* Results of qPCR analyses represent gene expression of oil- (black bars) and E2B- (gray bars) treated females in WT, KIKO, and ERKO genotypes. The number of animals in each treatment group is listed within each bar. Genes are expressed as relative *n*-fold changes, normalized to oil controls, within each genotype

(WT, KIKO, ERKO). A two-way ANOVA (genotype x treatment) followed by *post-hoc* Bonferroni's multiple comparison test was used to determine significant differences between treatments, within genotype. *$p < 0.05$; **$p < 0.01$; ***$p < 0.001$; ****$p < 0.0001$.

Figure 1

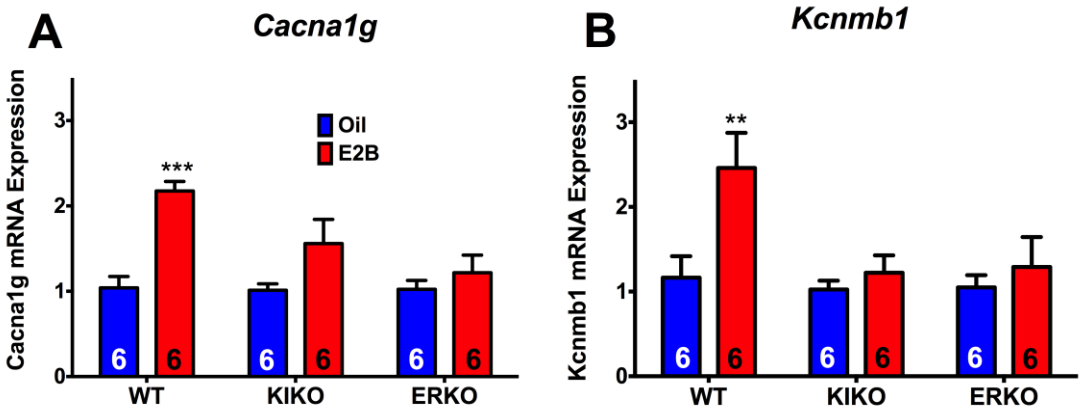

A
Cacna1g
Cacna1g mRNA Expression
Oil
E2B
***
6
WT
KIKO
ERKO
B
Kcnmb1
Kcnmb1 mRNA Expression
**

Figure 2

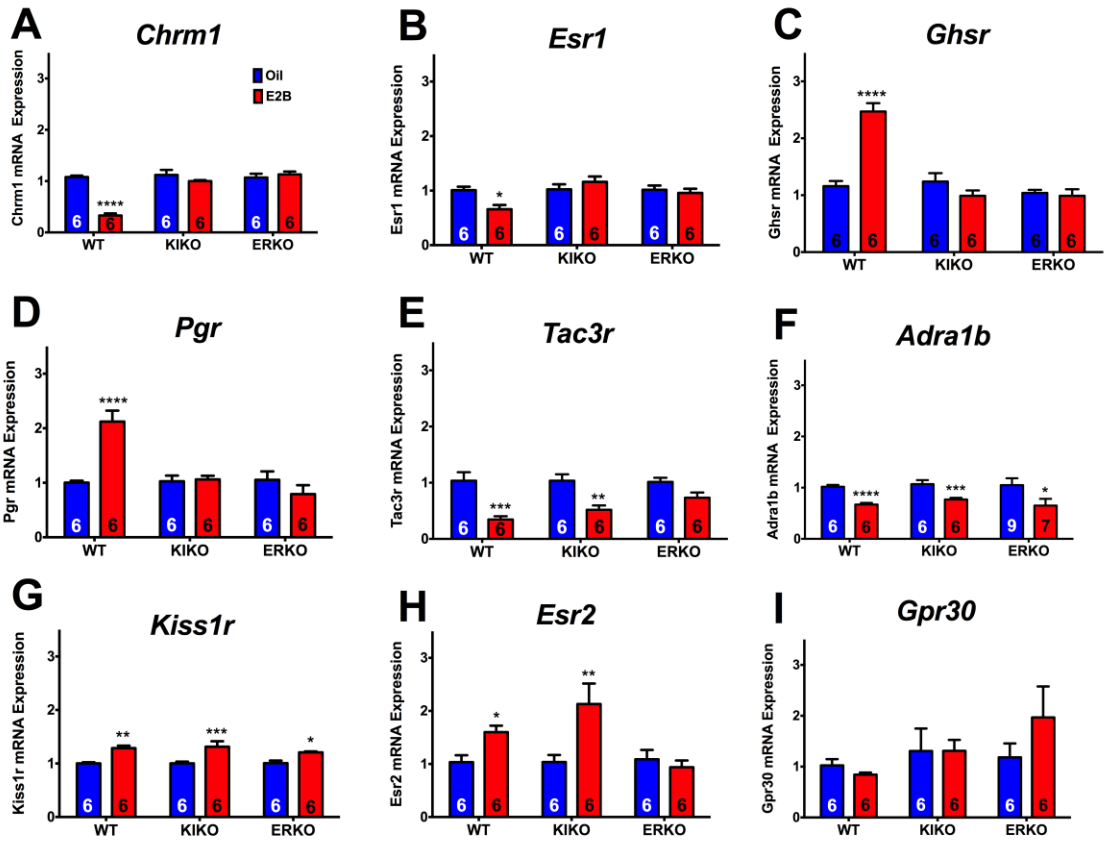
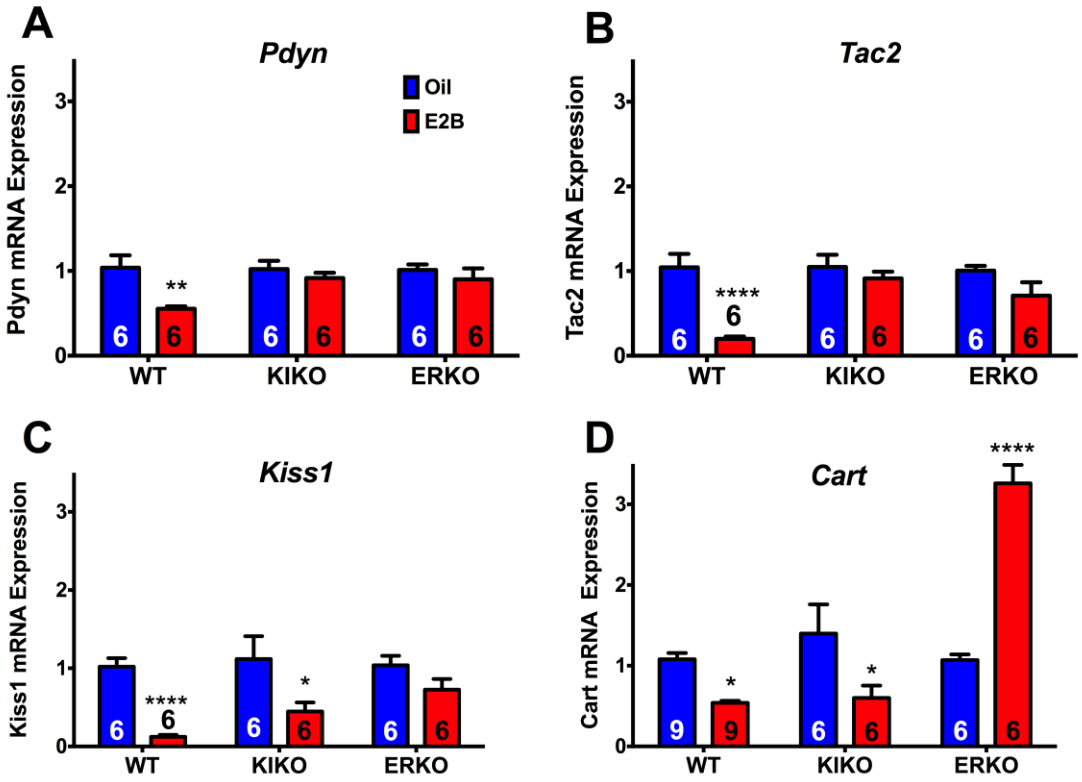

A
Chrm1
Chrm1 mRNA Expression
Oil
E2B
B
Esr1
Esr1 mRNA Expression
C
Ghsr
Ghsr mRNA Expression
D
Pgr
Pgr mRNA Expression
E
Tac3r
Tac3r mRNA Expression
F
Adra1b
Adra1b mRNA Expression
G
Kiss1r
Kiss1r mRNA Expression
H
Esr2
Esr2 mRNA Expression
I
Gpr30
Gpr30 mRNA Expression
WT
KIKO
ERKO

Figure 3


A
Pdyn
Oil
E2B
Pdyn mRNA Expression
**
WT
KIKO
ERKO
B
Tac2
Tac2 mRNA Expression
****
C
Kiss1
Kiss1 mRNA Expression
****
*
D
Cart
Cart mRNA Expression
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CHAPTER 3: THE ARCUATE ESTROGENOME: ESTROGEN RESPONSE ELEMENT-DEPENDENT AND –INDEPENDENT SIGNALING OF ERα

3. The Arcuate Estrogenome: Estrogen Response Element-dependent and -independent signaling of ERα

3.1 Abstract

17β-estradiol (E2) signaling in the arcuate nucleus occurs primarily through ERα-mediated, ERE-dependent and ERE-independent mechanisms. In the current study, we identify the differential regulation of E2 in the mouse estrogenome by using three genotypes of mice: 1) wild-type 2) ERα knock in/knock out (KIKO), which signal through ERE-independent mechanisms and 3) total ERα knock out (ERKO), which signal through ERα-independent mechanisms. Females of each genotype were ovariectomized and injected with oil or E2. We used RNA sequencing to identify genes that are regulated by E2 in WT, KIKO, and ERKO females. Our results show that E2 regulates numerous genes involved in energy homeostasis including those that function in cell signaling, cytoskeleton structure, inflammation, neurotransmission, neuropeptide production, and transcription. Furthermore, ERE-independent signaling regulates genes expressed in arcuate kisspeptin neurons and transcription factors that control the hypothalamic pituitary gonadal (HPG) axis. Interestingly, a number of genes involved in mitochondrial oxidative respiration were regulated by E2 through ERα-independent signaling. A comparison within oil- and E2-treated females across the three genotypes suggests that genes involved in cell growth and proliferation, extracellular matrices, neuropeptides, receptors, and transcription are among those differentially expressed across the genotypes. We conclude that the mouse arcuate estrogenome is regulated by multiple receptor-mediated mechanisms to modulate the central control of energy homeostasis and reproduction including novel E2-responsive pathways.

3.2 Introduction

The hypothalamus is the critical brain center in the control of reproduction, energy homeostasis, stress, temperature, and other homeostatic functions. It is well established that the gonadal steroid, 17β-estradiol (E2) modulates these functions through central hypothalamic signaling. Classically, E2 binds to its nuclear receptors, ERα and ERβ, to regulate gene transcription by binding to the Estrogen Response Element (ERE) binding domain located on DNA. This "ERE-dependent" signaling is critical to many of these hypothalamic functions with the effects lasting hours to days. In addition to ERE-dependent signaling, ERE-independent signaling regulates cellular signaling and gene expression through the activation of second-messenger signaling cascades and/or protein-protein interactions. ERE-independent signaling regulates second messenger systems including phosphoinositide 3-kinase (PI3K), mitogen-activated protein kinases (MAPK)/extracellular signal-regulated kinases (ERK), protein kinase C (PKC), and protein kinase A (PKA) pathways, that control gene transcription and protein-protein interaction (Dos Santos et al., 2002,Lee et al., 2005,Raz et al., 2008,Zhang et al., 2010). Furthermore, G-protein coupled receptors such as the G-protein coupled receptor 30 (GPR30), now defined as the G-protein coupled estrogen receptor (GPER) and the putative Gq-coupled membrane estrogen receptor (Gq-mER), which regulates rapid responses to E2 in various hypothalamic neurons (Carmeci et al., 1997,Filardo et al., 2002,Prossnitz and Barton, 2011,Roepke et al., 2009,Roepke et al., 2010,Smith et al., 2013), are involved in E2 regulation of gene expression.

Previously, ERα knock in knock out (KIKO) models, which lack a functional ERE-binding domain, have been used to study ERE-independent signaling (Hewitt et al., 2009,Hewitt and Korach, 2011,Mamounis et al., 2013,Yang et al., 2016). Likewise, total ERα knock out (ERKO) animals are important in identification of ERα-independent signaling. Previous studies in our lab demonstrated that ERE-dependent and –

independent signaling are important in the regulation of selected genes (neuropeptides, receptors, cation channels, etc.) in the arcuate nucleus (ARC) (Yang et al., 2016). The ARC is a critical nucleus in the regulation of energy balance, through regulation by pro-opiomelanocortin (POMC) and agouti-related peptide (NPY) neurons, and reproduction, through KNDy (Kisspeptin-Neurokinin B-Dynorphin) neurons (Kelly and Ronnekleiv, 2012,Mittelman-Smith et al., 2012,Pelletier et al., 2007,Roepke et al., 2010,Smith et al., 2013). While our previous study used a Taqman Low-Density Array to determine regulation through these mechanisms, the objective of the current study is to examine the ARC "estrogenome" using a standard E2 replacement paradigm in KIKO and ERKO females compared to their WT littermates (Yang et al., 2016).

In addition to neuropeptides, hormone receptors, and cation channels, E2 has previously been shown to regulate ARC expression of signaling molecules and genes involved in cell communication, metabolism, cell growth, transcription, translation, and other cellular functions (Roepke et al., 2007,Roepke et al., 2008). We hypothesize that distinct canonical signaling pathways will be regulated by E2 through both ERE-dependent and –independent mechanisms. Furthermore, while ERα is the primary receptor involved in E2 signaling in the ARC, we expect to see differential gene expression in ERKO animals, suggesting additional receptor-mediated mechanisms of E2's action. Defining the estrogenome in the mouse ARC is important to understand and distinguish differential mechanisms of E2 signaling that will further elucidate ARC regulation of multiple homeostatic processes.

3.3 Methods

3.3.1 Animal care

All animal procedures were performed in accordance with the guidelines based on National Institutes of Health standards and were performed with Institutional Animal

Care and Use Committee approval at Rutgers University. Adult female mice were housed under constant photoperiod conditions (12/12 h light/dark cycle) and maintained at a controlled temperature (23°C). Animals were given low phytoestrogen chow diet (<75 isoflavone ppm, Lab Diet Advanced Protocol 5V75, St. Louis, MO, USA) and water *ad libitum*. Females were weaned on postnatal day 21 (PD21). To determine genotype, PCR products of extracted DNA from ear clippings were used, following previously published protocols (Hewitt et al., 2009). Three genotypes of mice were used: WT (C57BL/6), KIKO, and ERKO (provided by Dr. Ken Korach, NIEHS) (Hewitt et al., 2009). Crossing heterozygous WT/KI males expressing the nonclassical ERα knock-in with WT/KO heterozygous females generated WT and KIKO females. Crossing heterozygous WT/KO males and females generated WT and ERKO females. WT females used in the experiments were littermates generated from both colonies.

3.3.2 Drugs

17β-estradiol benzoate (E2B) was purchased from Steraloids (Newport, RI, USA) and dissolved in ethanol (1mg/ml) prior to mixing in sesame oil (Sigma-Aldrich). Ketamine was purchased from Henry Schein Animal Health (Melville, NY, USA) and used for sedation prior to sacrifice.

3.3.3 Ovariectomy

Adult female mice were bilaterally ovariectomized (ovx) under isoflurane anesthesia 7 days prior to sacrifice using sterile no-touch techniques according to the NIH “Guidelines for Survival Rodent Surgery.” Animals were given a dose of analgesic [4 mg/kg carprofen (Rimadyl®)] one day following surgery for pain management. Females were monitored daily and allowed to recover for 5 days prior to the first injection of E2B or oil.

3.3.4 Experimental design

Following ovx, females of each genotype (WT, KIKO, ERKO) were separated into a control sesame oil-treated group (n=4 per genotype) and an E2B-treated group (n=4 per genotype). An E2B injection protocol was used that has been shown to alter gene expression in the hypothalamus (Bosch et al., 2013,Yang et al., 2016). We did not include intact females in our experimental design as neither ERKO nor KIKO females exhibit a normal estrous cycle, which makes it difficult to compare among intact WT, KIKO, and ERKO females (Park et al., 2011). Animals were injected with 0.25 µg of E2B and 1.5 µg dose of E2B on 5 and 6 days post-ovx, respectively, at 1000 h while oil-treated animals received oil at each injection. Animals were sacrificed on post-ovx day 7 at 1000 h after a 1 h fast. Animals were sedated with ketamine (100 µl of 100 mg/ml stock, i.p.) and decapitated. Brains were removed and rinsed in ice-cold Sorensen's Phosphate Buffer (0.2 M sodium phosphate, dibasic; and 0.2 M sodium phosphate, monobasic) for 30-60 sec. The basal hypothalamus (BH) was cut using a brain slice matrix (Ted Pella, Inc., Redding, CA, USA) into 1-mm thick coronal rostral and caudal blocks corresponding to Plates 42 to 47 and Plates 48 to 53, respectively, from *The Mouse Brain in Stereotaxic Coordinates* (Franklin and Paxinos, 2008). The slices were transferred to a 50/50 Pyrogard water/RNA*later*® (Life Technologies, Grand Island, NE, USA) solution and fixed overnight at 4°C. The ARC tissue, found in two slices, was microdissected using a dissecting microscope, following our previous publications (Bosch et al., 2009,Franklin and Paxinos, 2008,Mamounis et al., 2013,Roepke et al., 2008). Dissected tissue was stored at -80 °C until RNA extraction in 50/50 Pyrogard water/ RNA*later*®.

3.3.5 Tissue extraction

RNA was extracted from ARC using Ambion RNAqueous® Micro Kits (Life Technologies, Inc., Carlsbad, CA, USA) according to the manufacturer's protocol, followed by DNase-I treatment to remove contamination by genomic DNA. RNA samples

were analyzed on a NanoDrop™ ND-2000 spectrophotometer (ThermoFisher, Inc., Waltham, MA, USA) to assess quantity, followed by an Agilent 2100 Bioanalyzer run using the RNA 6000 Nano Kit (Agilent Technologies, Inc., Santa Clara, CA, USA) to assess quality. We chose 3 samples within each treatment and genotype that had the highest RIN numbers (>8) to use for RNA sequencing analysis.

3.3.6 RNA sequencing (RNAseq)

RNA libraries for RNAseq analysis were prepared from 400ng of extracted total RNA, as assessed on NanoDrop spectrophotometer concentration readings, by the JP Sulzberger Columbia Genome Center. Poly(A) mRNA enrichment was performed using TruSeq RNA Sample Prep Kit V2 prior to sequencing to allow for target enrichment. RNA was sequenced on an Illumina HiSeq 2500 system to produce 30 million 100 bp single end reads. For all samples, there was an average of 81% unique mapped reads.

3.3.7 Bioinformatics

Data were obtained from JP Sulzberger as .fastq files and analyzed using the Tuxedo suite (Trapnell et al., 2012). Data were mapped and aligned to the mouse genome, mm_10 mouse genome, using TopHat v2.0 to produce mapped reads (.bam file) (Kim et al., 2013). Files were merged using Cuffdiff to determine treatment and genotype, and used for differential expression analysis (.diffout file). To visualize differential expression between oil and E2B-treated females within each genotype (WT, KIKO, ERKO), the R Bioconductor package cummeRbund was used (Bioconductor, Boston, MA, USA) (Goff, 2013). To determine if there were any outliers in the data, principle components analysis (PCA) and scatter matrices were created and evaluated. Genes that were differentially regulated met the following guidelines: $p < 0.05$; fragments per kilobase of transcript per million mapped reads (FPKM) values > 1; fold-change (FC) > 1.5. In order to evaluate FC > 1.5, the cutoff for downregulated genes was 0.667 and for upregulated genes, 1.5. To visualize differences in differential gene expression using

FPKM, bar graphs were created in cummeRbund. Ingenuity Pathway Analysis (IPA; Qiagen, Redwood City, CA, USA) was used to determine the top 10 canonical pathways that were differentially regulated.

Transcript analysis was conducted using Integrative Genomics Viewer (IGV; Broad Institute of Massachusetts Institute of Technology and Harvard, Cambridge, MA, USA) to determine differential transcripts within steroid and treatment (Robinson et al., 2011,Thorvaldsdottir et al., 2013). Mapped reads replicates within each group (genotype, treatment) were merged, sorted, and indexed using SAMtools (Li et al., 2009). IGV was used to view alignments and differential transcripts.

To determine differences across genotype, within steroid treatment, DESeq2 (Bioconductor) analysis was used to compare WT vs. KIKO, WT vs. ERKO, and KIKO vs. ERKO, within both oil and E2B-treated females (Love et al., 2014). Genes that had a FC > 1.5 were determined to be differentially regulated. Lastly, to visualize overlap of genes in the dataset, Venny 2.1 (Oliveros, 2007) was used to produce venn diagrams of the data.

3.3.8 Availability of datasets

Data from RNA sequencing have been deposited in NCBI's Gene Expression Omnibus (GEO) repository (Edgar et al 2002, Nucleic Acid Res) and are available at GEO Series accession number #GSExxxxxx (http://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSExxxxx). This will be submitted at the time of publication.

3.4 Results

3.4.1 Regulation of the estrogenome in WT, KIKO, and ERKO females

The goal of this study was to identify and characterize E2-dependent gene expression patterns controlled by ERα through the nuclear ERE-dependent, nuclear ERE-independent, or through ERα-independent signaling (e.g. ERβ, GPER1, Gq-mER) in the ARC of the female mouse using global RNA sequencing (RNAseq). A flow chart illustrating these different transcriptional pathways can be found in Figure 1A. To investigate these mechanisms, we utilized two transgenic ERα mouse models, total ERαKO (KO) and ERα knockin/knockout (KIKO) that express an ERα mutation that eliminates DNA binding through ERE, and their wild-type littermates (Hewitt et al., 2009). By comparing the pattern of E2-induced gene expression in these mutants, we can identify genes and canonical pathways elicited by each type of ERα signaling. Our analysis will also identify potentially novel signaling mechanisms and cellular processes that E2 can impact in the ARC to control hypothalamic homeostatic functions such as energy balance and reproduction.

We compared qPCR gene expression data (Chapter 2) with RNA sequencing data (Chapter 3) in the verification table (Table 1), to determine differences between the two different techniques. To determine the clustering of the data, PCA plots were constructed in cummeRbund to determine if oil- and E2B-treated groups were different within each genotype (Figure 1B). To determine the ratio of the number of genes differentially regulated by E2B and the total number of genes sequenced, volcano plots were constructed using cummeRbund (Figure 1C). The red dots on each plot signify genes differentially expressed, FC > 1.5, $p < 0.05$, FPKM > 1. As viewed, WT females had the greated number of regulated genes (132, Figure 1D), followed by ERKO females (35, Figure 1D), and KIKO females (22, Figure 1D). Figure 1D shows overlap of genes differentially expressed across the three genotypes. WT and KIKO females had an overlap of 10 genes: *Ptgds* (prostaglandin D2 synthase), *Bdnf* (brain-derived

neurotrophic factor), *C1q12* (complement component 1, q subcomponent-like 2), *Kiss1* (kisspeptin), *Tac2* (tachykinin 2), *Pcsk1* (proprotein convertase subtilisin/kexin type 1), *Tac3r* (tachykinin 3 receptor), *Gata2* (GATA binding protein 2), *Pou2f1* (POU domain, class 2, transcription factor 1), and *Sox3* (sex determining region Y-box 3). WT and ERKO females had an overlap of 3 genes: *Ccdc85b* (coiled-coil domain containing 85B), *Alox5ap* (arachidonate 5-lipoxygenase activating protein), and *Vip* (vasoactive intestinal peptide). There was no overlap between KIKO and ERKO, and therefore no overlap among all three genotypes.

There were 132 total genes regulated in the WT estrogenome, 60 that were decreased with E2B and 72 that were increased with E2B (Table 2). In addition to the genes listed in Table 2, additional predicted genes and genes of unknown function were regulated by E2B (data not shown). These genes were grouped into the following functions (number of genes): calcium regulation (2), cell adhesion (3), cell signaling (2), chromosome structure (3), cytoskeleton (6), enzyme (6), extracellular matrix (12), growth factor (3), immune/inflammation (11), ion channel (4), neurodevelopment (5), neuropeptide (12), neurotransmission (7), neurotrophic factors (2), protein modification (3), protein trafficking (2), receptor (7), transcription factor (19), translation (3), and WNT signaling (2).

There were 22 genes regulated by E2B in KIKO females (Table 3), grouped into functions similar as WT females (Table 2). Of these 22 genes, 10 were decreased with E2B and 12 were increased with E2B. Many of the genes regulated by E2B in KIKO females were enzymes, neuropeptides, and transcription factors involved in reproduction and energy balance. Lastly, there were 35 genes regulated by E2B in ERKO females (Table 2). Of these 35 genes, 4 genes were decreased with E2B and 31 were increased with E2B. Interestingly, a number of genes involved in mitochondrial oxidation and respiration were regulated by E2B in ERKO females only. These mitochondrial oxidation

genes include: *Cox5b* (cytochrome c oxidase subunit 5B), *Ndufa11* (NADH:ubiquinone oxidoreductase subunit A11), *Ndufb10* (NADH:ubiquinone oxireductase subunit B10), *Ndufb7* (NADH:ubiquinone oxidoreductase subunit B7), *Uqcr11* (cytochrome c reductase, complex III subunit XI), and *Uqcr1* (cytochrome c reductase core protein 1). All these genes were increased by E2B, suggesting an increase in mitochondrial function and ATP-generation in ARC neurons. In addition to mitochondrial genes, numerous ribosomal proteins that comprise both the small and large ribosomal subunits were increased with E2B. Many of the genes regulated by ERKO females did not overlap with WT females and none overlapped with KIKO females.

3.4.2 Distinct canonical pathways are regulated by E2B across WT, KIKO, and ERKO females

To determine if there were pathways that were regulated by E2B in each of the three genotypes, we used IPA to determine relationships among genes of interest. Table 5 lists the top 10 canonical pathways within each genotype. In WT females, many of the pathways are involved in diseases, including Alzheimer's disease, atherosclerosis, and endometrial cancer. In KIKO females, many pathways involved in inmmune/inflammation and energy balance were regulated, including: prostanoid biosynthesis, eicosanoid signaling, and glucocorticoid signaling. In ERKO females, many genes involved in oxidative phosphorylation and mitochondrial dysfunction are regulated by E2B.

3.4.3 Differential expression of genes across genotypes

We used DESeq to compare across genotypes, within treatment (oil and E2B). Many of the genes that were different between genotypes (WT vs. KIKO and WT vs. ERKO) in oil-treated animals were also different in E2B-treated animals. The main changes in gene expression were between WT vs. KIKO and WT vs. ERKO females, as indicated in Supplementary Table 1 & 2 and illustrated in Figure 3. As expected, there were many differences in genes involved in reproduction and energy balance across

genotypes including 31 genes that overlapped between all four treatments (1: WT vs. KIKO oil; 2: WT vs. KIKO E2B; 3: WT vs. ERKO oil; 4: WT vs. ERKO E2B; Figure 3). These genes include: *Pck1, Ptgs2, Tac3r,* and *Map3k15,* among others. In addition, there were 22 genes that were increased or decreased between WT and KIKO females in both oil and E2B comparisons, including *Tlr4* (toll-like receptor 4) and *Grik1 (*glutamate receptor, ionotropic, kainite 1). Finally, there were 74 genes that were increased or decreased between WT and ERKO females in both oil and E2B comparisons, including: *Vgf, Bdnf, Sox3, Pomc, Tac2, Pgr,* and *Ghsr*.

3.4.4 The KNDy neuropeptide genes are regulated through differential transcript variants

Previous reports have identified E2 regulation of 2 of the 3 neuropeptides co-expressed in KNDy (Kisspeptin-Neurokinin B-Dynorphin) neurons. These studies found that E2 regulated *Kiss1* (kisspeptin) through ERE-independent signaling and *Dyn* (dynorphin) through ERE-dependent signaling (Gottsch et al., 2009,Yang et al., 2016). We used cummeRbund to generate gene expression graphs of the KNDy neuropeptide genes. In our study, we saw that E2B decreased expression of *Tac2* (neurokinin B) and *Kiss1* in WT and KIKO females (Figure 2A). Interestingly, we did not see a change in *Pdyn,* or prodynorphin in any genotype (Figure 2A). To determine if the transcription and transcript variants across the genotypes and treatments aligned with our gene expression conclusions, we use IGV to visualize the KNDy neuropeptide transcripts. Figure 2B illustrates that *Tac2* and *Kiss1* transcripts are decreased by E2B in WT and KIKO females, consistent with our gene expression analysis (Figure 2A). In both *Tac2* and *Kiss1,* there are differences between oil- and E2B-treated females across the entire gene. Conversely, we did visualize a change in *Pdyn,* in one region of the gene (on the 5’ end), which contradicts the lack of changes in gene expression in any genotype (Figure 2A).

3.5 Discussion

The results of the current study identify the ARC estrogenome in WT, KIKO, and ERKO females. While E2 is important in many homeostatic processes including reproduction and energy balance, central mechanisms of regulation are largely unknown. In the current study, we identify that many genes involved in calcium balance, metabolism, mitochondrial oxidation, transcription, neurotransmission, and inflammation are all regulated by E2. Interestingly, many of the genes expressing transcription factors that control energy balance and reproduction are regulated by ERE-independent mechanisms, present in WT and KIKO females, but not in ERKO females, suggesting that membrane-mediated responses to E2 are important in the ARC estrogenome. Furthermore, many mitochondrial oxidation genes were regulated by E2B in ERKO females but not WT and KIKO females, suggesting compensatory mechanisms that are ERα-independent may be important for cellular energy production.

3.5.1 The Arcuate Estrogenome is involved in the regulation of a number of functions

In WT females, E2 regulates 132 genes. Of these, 119 genes are regulated by E2B in WT females only, indicating that the transcription is mediated by ERα interacting with ERE. While it is not possible to fully discuss each of these genes, we have characterized these genes into different functions. These functions include: calcium regulation, cell adhesion, cell signaling, chromosome structure, cytoskeleton, detoxification, enzymes, extracellular matrix genes, growth factors, inflammatory genes, ion channels, neurodevelopment, neuropeptides, neurotransmission, protein modification and tracking, receptors, transcription, and translation.

E2 regulated neuropeptides are involved in energy balance and reproduction, processes that are controlled centrally in the ARC. Previous studies suggest that E2

functions in the control of expression of a variety of neuropeptides including pro-opiomelanocortin (POMC), kisspeptin, and dynorphin (Bosch et al., 2012,Brock and Bakker, 2013,Eghlidi and Urbanski, 2015,Gao et al., 1997,Navarro et al., 2011,Pelletier et al., 2007,Qiu et al., 2003,Treiser and Wardlaw, 1992). E2 signaling in energy balance includes regulation of POMC/cocaine- and amphetamine-regulated transcript (CART) and neuropeptide Y (NPY)/agouti-related peptide (AgRP) neurons that are anorectic and orexigenic, respectively (Gao et al., 1997,Pelletier et al., 2007,Roepke et al., 2008,Roepke et al., 2010,Roepke et al., 2011,Smith et al., 2013,Treiser and Wardlaw, 1992). In the present study, *Cartpt,* CART prepropeptide, and *Pomc* were decreased, while *Agrp* was increased by E2B. Previous studies in our lab show that E2B increases *Agrp* expression by qPCR in the ARC and more specificially, in NPY neurons (Yasrebi et al., 2016). E2 differentially regulates these orexigenic neurons through multiple receptor-mediated mechanisms. The nonsteroidal selective ligand for the Gq-mER, STX, functions through an ERE-independent and nuclear receptor-independent pathway to enhance the GABAergic postsynaptic response in NPY neurons leading to a decrease in NPY expression in the ARC (Roepke et al., 2008), which is opposite to the attenuation caused by classical ERα activation. Reproduction is controlled through ERE-dependent and –independent signaling. Differential regulation of *Dyn* (ERE-independent) and *Kiss1* (ERE-dependent) by E2 in KNDy (Kisspeptin-Neurokinin B-Dynorphin) neurons is important for reproduction and energy homeostasis (Gottsch et al., 2009,Mittelman-Smith et al., 2012)

A number of receptors involved in reproduction (*Pgr, Tac3r)* and energy balance (*Cckar, Ghsr)* are regulated by E2B. We previously reported that growth hormone secretagogue receptor (*Ghsr*), the ghrelin receptor, is increased by E2B in the ARC but not in NPY neurons, in which GHSR activation by ghrelin inhibits the M-current (Yasrebi et al., 2016). Ghrelin is a peptide hormone that stimulates food intake through a brain-

gut neural connection. In the current study, *Ghsr* is also increased by E2B only in WT females, which has shown to be due in large part to augmentation in *Tac2* (KNDy) neurons (Yang et al, 2016, *in review*).

Activation of GHSR by ghrelin in NPY neurons involves regulation of calcium homeostasis (Andrews et al., 2008,Cowley et al., 2003). In our study, E2 differentially regulates the ARC expression of two calcium homeostatic genes, matric gla protein *(Mgp)* and stanniocalcin 1 *(Stc1)*, decreasing and increasing expression, respectively. *Mgp* is a calcium binding protein that is important in inhibition of vascular calcification (Luo et al., 1997). To the best of our knowledge, there are no studies on examining E2's regulation of *Mgp* in the brain. *Stc1* is a glycoprotein hormone that is involved in calcium adsorption and phosphate excretion. Overexpression of human STC in mice leads to elevated phosphate levels, altering growth (Varghese et al., 2002). There are few studies on *Stc1* and E2 in peripheral tissues, with one study reporting elevated STC1 protein expression in women with endometriosis, when E2 levels are elevated (Aghajanova et al., 2016). The regulation of these calcium-signaling genes in the ARC by E2 may affect numerous cellular processes and signaling pathways. Furthermore, additional receptors are also regulated in the ARC estrogenome. In addition to changes in receptors involved in energy balance, receptors involved in reproduction including the progesterone receptor (*Pgr)* and tachykinin 3 receptor (*Tac3r)* are increased and decreased with E2B, respectively. These changes in *Pgr* and *Tac3r* by E2B are consistent with previous studies (Yang et al., 2016).

Central regulation of the ARC estrogenome controls many functions and signaling pathways through regulation of cell adhesion, cell signaling, chromosome structure, cytoskeleton, and extracellular matrix genes. While E2 is commonly studied in reproduction, its influence includes the control of cell survival and growth, which is critical to maintaining homeostasis. These novel findings of E2-responsive ARC genes

have generated potential mechanisms for E2 to control neuronal activity and homeostasis. The genes that are regulated by E2B in the WT ARC estrogenome can be found in Table 2.

3.5.2 E2 functions through ERE-independent pathways in the ARC to regulate gene expression

There are a number of genes that are regulated by E2B in both WT and KIKO females, but not in ERKO females, suggesting that ERα-mediated, ERE-independent signaling is important in the control of gene expression in the ARC. These genes include: *Bdnf, C1ql2, Gata2, Kiss1, Pcsk1, Pou2f1, Ptgds, Sox3, Tac2,* and *Tac3r.* Of these genes, all were downregulated by E2B in both WT and KIKO animals with the exception of *Gata2* (decreased in WT, increased in KIKO) and *Pou2f* (increased in WT and KIKO).

A number of these genes have previously been identified in the estrogenome in our previous publication (Yang et al., 2016). Kisspeptin (*Kiss1*) expressing genes in the ARC are often co-expressed in KNDy neurons that act as an important pulse generator in regulation of GnRH neurons and function in E2 negative feedback on the hypothalamic-pituitary-gonadal (HPG) axis (Eghlidi et al., 2010,Goodman et al., 2013). We, as well as others, have shown that *Kiss1* is regulated through ERE-independent mechanisms (Gottsch et al., 2009,Yang et al., 2016). Our previous publication also found that *Tac2* (Neurokinin B) was decreased by E2B only in WT females, unlike in the current study (Yang et al., 2016). However, our previous publication showed a trend to decreased *Tac2* expression with E2B (Yang et al., 2016). The tachykinin 3 receptor, *Tac3r,* is also decreased by E2B in WT and KIKO females, consistent with our previous publication (Yang et al., 2016). While previous studies have examined KNDy-associated neuronal expression through *Kiss1* (ERE-independent) and *Pdyn* (ERE-dependent), it is imperative to consider other genes acting collectively as the pulse generator (Gottsch et

al., 2009). Thus, the KNDy-associated gene expression is regulated through multiple ERα-mediated pathways.

Using cummeRbund visualization of gene expression changes and IGV analysis of differential gene transcription, we analyzed KNDy genes as our control gene group. As previously shown, *Kiss1* is regulated by ERE-independent signaling of E2 (Gottsch et al., 2009,Yang et al., 2016). This is confirmed in the previous study, as E2B decreased *Kiss1* in WT and KIKO females (Figure 2A) and gene transcription between E2B-treated WT and KIKO females is distinct (Figure 2B). *Tac2* is decreased by E2 in WT and KIKO females, suggesting ERE-independent signaling. Lastly, while previous studies have suggested *Dyn* is regulated through ERE-dependent signaling, the comparison does not show differential regulation of *Pdyn* by E2B (Figure 2A), although there are noticeable differences in IGV analysis of *Pdyn* in WT.

Many genes regulated by E2B in WT and KIKO females are classified as transcription factors, including *Gata2, Pou2f1,* and *Sox3. Gata2,* GATA binding protein 2, belongs to the GATA family of transcription factors, which regulate gonadotropin gene expression (LaVoie, 2003). Furthermore, the GATA family of proteins is involved in central regulation of reproduction through the hypothalamus and the pituitary, and GATA-2 is known to be involved in gonadotrope differentiation (LaVoie, 2003). GATA-2 is also important in stimulation of gonadotropin releasing hormone receptor (GnRHR) genes and suppression of GATA-2 in gonadotropes is associated with decreased LH expression (Lo et al., 2011,Steger et al., 1994). Interestingly, in our current studies, *Gata2* was decreased by E2B in WT females and increased by E2B in KIKO females. To the best of our knowledge, there are no studies of *Gata2*, or any proteins in the GATA family, and E2. It is possible that *Gata2* is negatively regulated by E2 through ERE-dependent mechanisms that are compromised without ERE-dependent signaling.

Pou2f1, or *Oct-1*, is a transcription factor in the POU transcription factor family. In our current study, E2B increased *Pou2f1* twofold in WT females and over fourfold in KIKO females. GnRH transcription is tightly controlled by a number of transcription factors, including *Pou2f1* (Clark and Mellon, 1995,Eraly et al., 1998). While there have been no studies to date on the role of *Pou2f1* in the ARC, previous studies in *in vitro* MCF7 cells show that at high E2 concentrations, there is an increase in genes associated with the *Pou2f1* binding regions (Chandrasekharan et al., 2013). While *Pou2f1* interacts with numerous additional transcription factors that both activate and repress GnRH transcription, ERα-mediated, ERE-independent signaling regulation of *Pou2f1* is potentially required for normal HPG axial function.

Sox3, a transcription factor is involved in brain development, is decreased by E2B in WT and KIKO females. SOX3 is important for the formation of the hypothalamic-pituitary axis (Alatzoglou et al., 2009,Rizzoti et al., 2004) as well as neurogenesis (Rogers et al., 2013). To the best of our knowledge, there are no studies that examine *Sox3* in the ARC or its interactions with E2. However, previous studies suggest that other members of the SOX family of transcription factors, including SOX4 and SOX11, are co-expressed in GnRH neurons and thus are important regulators of GnRH mRNA expression, similar to *Gata2* and *Pou2f1* (Kim et al., 2011). The present results suggest that *Sox3* transcription is decreased by E2B through ERα-mediated, ERE-independent mechanisms. This result, combined with the regulation of *Gata2* and Pou2f1 expression, suggests that E2 also controls transcription of ARC reproductive genes (GnRH) through the indirect regulation of specific transcription factors.

Two enzymes are decreased with E2B in WT and KIKO females, *Pcsk1* and *Ptgds*. Proprotein convertase 1, *Pcsk1* or PC1/3*,* is involved in the differential cleavage of pro-opiomelanocortin (POMC) into adrenocorticotropic hormone, ACTH, and another

proprotein conversase, *Pcsk2* (PC2) cleaves the POMC cleavage into α-melanocyte stimulating hormone (α-MSH) (Benjannet et al., 1991). *Pcsk1* suppression through ERE-independent mechanisms shifts the cleavage of the POMC gene product towards α-MSH processing and for subsequent release at downstream MC4 receptor-expression neurons to reduce food intake. Previous studies in ovx, female guinea pigs found that E2B increases *Pomc* expression and STX did not increase *Pomc* expression (Roepke et al., 2008), suggesting that *Pomc* is regulated through nuclear receptor-mediated mechanisms. Consequently, the regulation of *Pomc* through transcriptional and post-translational mechanisms is controlled through ERE-dependent and –independent pathways, respectively. Prostaglandin D2 synthase, *Ptgds,* regulates prostaglandin D2 production, which increases food intake through orexigenic (NPY/AgRP) neurons in the ARC (Ohinata et al., 2008) There are few studies that examine *Ptgds* and E2 in the hypothalamus. Our results suggest E2B regulates *Ptgds* through ERE-independent mechanisms to reduce prostaglandin D2 synthesis, which would suppress food intake. Collectively, these data suggest that E2 either suppresses or activates multiple pathways to regulate ARC homeostatic functions especially energy homeostasis and feeding behaviors.

However, E2 does suppress a gene whose role in energy homeostasis is to reduce food intake and augment energy expenditure, the brain-derived neurotrophic factor gene (Xu et al., 2003). *Bdnf is* involved in multiple processes besides the control of food intake primarily through actions in the VMH including cell growth, proliferation, and synaptic plasticity (Zhu et al., 2013). In the ARC, E2B reduced *Bdnf* through ERα-mediated, ERE-independent mechanisms potentially to reduce localized synaptic plasticity in orexigenic neurons (Liao et al., 2015). In addition to these genes, two other genes, *Stum* and *C1ql2* were increased and decreased, respectively, in WT and KIKO females. *Stum* is a mechanosensory transduction mediator and *C1ql2* is involved in the

complement system of the immune system. There are few studies on *Stum* and *C1ql2* function and no studies on the control these genes by E2 in any tissue.

3.5.3 The electron transport chain is regulated by ERα-independent pathways

In ERKO females, 35 genes were regulated by E2B, suggestive of ERα-independent pathways. A number of these genes are predicted and unidentified to date (data not shown). Furthermore, a number of ribosomal proteins were regulated by E2B in ERKO females. These ribosomal proteins include those that are found in both the small and large ribosomal subunits and all were upregulated by E2B. These data suggest that E2B upregulates ribosomal activity and perhaps downstream events including translation.

We found that the majority of genes regulated by E2B in ERKO females were those involved in mitochondrial respiration and the electron transport chain (ETC). These genes include the following: *Atp5e, Cox5b, Ndufa11, Ndufb10, Ndufb7, Timm13, Uqcr11,* and *Uqcrq.* Interestingly, all these genes were upregulated in ERKO females and were not regulated by E2B in either WT or KIKO females, suggesting that in the absence of ERα, there are compensatory mechanisms in the regulation of the ETC. The ETC is critical to cellular respiration and the production of ATP, involving a number of compounds that function in redox reactions to transfer electrons and to create a proton (H^+ ion) gradient across the mitochondrial membrane.

The compounds that transfer electrons through the ETC are grouped into a number of complexes that span the matrix, inner mitochondrial membrane, and intermembrane space in mitochondria. The genes regulated by E2B in ERKO females are found in a number of these complexes. Complex I, or the nicotinamide adenine dinucleotide (NADH) dehydrogenase complex, transfers electrons from NADH. The NADH dehydrogenase complex is made up of a peripheral and membrane portion (Weiss et al., 1991). Within the peripheral portion of the enzyme complex, there are

subunits α, β, γ, and δ (Weiss et al., 1991). Within the genes regulated by E2B in ERKO females, *Ndufa11* is part of the α subunit, which contains an iron-sulfur cluster that is important in oxidation-reduction during electron transfer (Weiss et al., 1991). Similarly, the β complex contains an iron-sulfur cluster as well (Weiss et al., 1991). *Ndufb10* and *Ndufb7* are both upregulated by E2B, and represent part of the NADH dehydrogenase β subunit. To date, there are no studies suggesting that regulation of these genes is controlled by E2, regardless of receptor subtype or signaling mechanisms.

Complex IV includes cytochrome C oxidase (CcO) and is critical to transfer electrons to the final electron acceptor, O_2. Within the ERKO estrogenome, 3 cytochrome C-associated genes were increased by E2B: *Cox5b, Uqcr11,* and *Uqcrq. Cox5b* is a peripheral subunit of CcO and previous studies indicate that it is critical for CcO activity (Galati et al., 2009). Furthermore, recent studies also report that COX5B is overexpressed in breast cancer, which is highly dependent on E2 levels (Gao et al., 2015). To date, no studies have examined *Cox5b* in the ARC. In addition, to the best of our knowledge, there are no studies that examine *Uqcr11* or *Uqcrq* in the context of E2. Complex V of the ETC includes ATP synthase, which uses the H^+ ion gradient produced during Complexes I-IV to power ATP synthase. Within the ERKO estrogenome, *Atp5e* was increased by E2. *Atep5e* is critical to a functional F1 epsilon subunit in ATP synthase, which represents the catalytic portion of ATP synthase and spans into the mitochondrial matrix (Mayr et al., 2010). In addition to these genes, *Timm13,* a translocase of the inner mitochondrial membrane, was increased by E2B in ERKO females. While it is unclear if there is a role of *Timm13* in the ETC, it functions in import of metabolites from the cytoplasm.

To the best of our knowledge, there are no studies that examine E2 regulation of mitochondrial genes in the hypothalamus and especially in the ARC. Data on peripheral tissue gives us an unclear mechanism and relationship of E2 to ETC. In rat liver, E2

inhibits activity of ATP synthase by uncoupling the enzyme and decreasing oxidative respiration (Moreno et al., 2013). In cardiac tissue, ERβ is important in the upregulation of complex IV (CcO) following trauma, suggesting a protective, ERα-independent effect of E2 (Hsieh et al., 2006). Additional studies also suggest E2 to serve a protective role in maintaining mitochondrial metabolic activity (Ronda et al., 2013). Furthermore, a recent study suggested that in the rat brain, P4 and E2 are important in regulation of mitochondrial oxidative metabolism (Irwin et al., 2008). This study concluded that hormone-treated mitochondria in rats increased expression and activity of CcO (Irwin et al., 2008). Our studies suggest that ERα-independent signaling is critical for maintaining metabolism and the bioenergetics of the ARC. Lastly, this E2-dependent regulation may be critical to the neuroprotective effects of E2.

The ERKO ARC estrogenome functions in ERα-independent signaling. As previously mentioned, in addition to ERα signaling, E2 can function through multiple mechanisms and signaling pathways. While ERβ is not as highly expressed in the ARC as ERα, it is possible that E2 functions to control genes through this classical receptor (Shughrue et al., 1997). Furthermore, in addition the classical receptor, E2 may signal through additional estrogen receptors, including GPER1/GPR30 and the putative membrane estrogen receptor, Gq-mER (Mizukami, 2010,Roepke et al., 2009,Roepke et al., 2010,Smith et al., 2013). Our previous studies suggest that a number of genes are regulated by STX, a selective Gq-mER agonist (Roepke et al., 2008). Furthermore, E2 also functions through regulation of second messenger signaling cascades and protein-protein interactions in rapid signaling of E2 (Malyala et al., 2005,Qiu et al., 2003). Thus, while we have identified the ARC estrogenome in ERKO females, the results of our study require further investigation to determine which E2 signaling pathway is activated.

3.5.4 Comparing gene expression across genotypes using DESeq

Using DESeq, we compared differences in ARC gene expression across genotypes, within treatments. There were few changes present in ERKO vs. KIKO females of both oil- and E2B-treated females, with most changes between WT vs. KIKO and WT vs. ERKO. Among the genes different between WT vs. KIKO and WT vs. ERKO, many differentially regulated genes were significant in both oil- and E2B-treated females. Interestingly, many of these genes overlapped with the previously discussed genes, in relation to genes of the estrogenome. All genes that were different between WT vs. KIKO females were different in both oil- and E2B- treated females (total: 53 genes), sometimes overlapping with WT vs. ERKO females (31 genes). Those genes that were only different between WT vs. KIKO females include enzymes *Pck1* and *Ptgs2,* previous discussed as decreased by E2B in WT and KIKO females. In oil-treated females, *Pck1* and *Ptgs2* were decreased in KIKO compared to WT females. By contrast, in E2B-treated females, *Pck1* and *Ptgs2* were increased in KIKO compared to WT females. These results suggest that both *Pck1* and *Ptgs2* are regulated by E2 through ERα-mediated, ERE-independent signaling. Taken together, these results suggest that *Pck1* and *Ptgs2* are E2-responsive, with KIKO females being less responsive to the E2-mediated decrease. Thus, *Pck1* and *Ptgs2* may be more sensitive to ERα-mediated, ERE-dependent signaling, though it is also regulated by E2 through ERα-mediated ERE-independent signaling.

In the comparison of WT vs. ERKO females, there was only 1 gene that was significantly different in WT vs. ERKO oil-treated females, and not in WT vs. KIKO females. This gene is protein tyrosine phosphatase, non-receptor type 20 (*Ptpn20)*, which to date has an unknown function. Of the remainder of the genes (105 genes) significantly different between WT vs. ERKO females, 31 genes overlap with WT vs. KIKO females. There are 74 genes different between WT vs. ERKO females in both oil- and E2B-treated females. Many neuropeptides are differentially regulated between WT

vs. ERKO females. In KNDy neurons, *Pdyn* and *Tac2* are decreased in ERKO females in oil-treated females and increased in E2B-treated ERKO females. In a previous study using qPCR, we found that *Pdyn* is expressed higher in KIKO than in WT or ERKO females (Yang et al., 2016) (Chapter 2). While not consistent, these data do illustrate that the KNDy genes are differentially expressed in females lacking functional ERα. As previously shown, there is a decrease in *Tac2* expression by E2B in both WT and KIKO females, with no significant change in *Pdyn*. The change observed between *Tac2* and *Pdyn* across genotypes, thus, may be due to WT changes rather than in ERKO changes.

There are 31 common genes that overlap in all of the DESeq comparisons, including *Tac3r,* the *Tac2* receptor, which is decreased in WT vs. KIKO oil compared to WT vs. KIKO E2B and in WT vs. ERKO oil compared to WT vs. ERKO E2B females. While we did not see any changes in *Tac3r* expression by E2B using RNA Seq, our previous publications using quantitative real-time PCR analysis of gene expression show that E2 decreases *Tac3r* through ERα-mediated, ERE-independent signaling ((Ohsugi et al., 1997,Yang et al., 2016). Taken together, these results suggest that *Tac3r* expression may be more responsive to ERE-dependent actions and less so to other ER signaling. In conclusion, our collective data of oil vs. E2B and WT vs. KIKO vs. ERKO females suggests that E2 signaling in the ARC is not based on a single signaling pathway, is dependent on E2 feedback, and is oftentimes a combination of disparate E2 signaling mechanisms. Nonetheless, our data provide an important framework to future studies identifying central signaling of the estrogenome.

3.6 Conclusion

While numerous studies have examined E2's role in controlling many hypothalamic homeostatic processes described in this study, the receptor-mediated

mechanisms are largely unexplored. We show for the first time that ERα-mediated, ERE-independent mechanisms are involved in many transcriptional processes important to reproduction, primarily through transcription factors that control GnRH expression and the HPG axis downstream. These studies enable the design of future studies exploring these transcription factors especially GATA2, Pou2f1, and Sox3 pathways.. In addition to reproduction, many of the genes and pathways impacted by E2, through both ERE-dependent and –independent pathways, are involved in the control energy expenditure and feeding behavior. Finally, regulation of mitochondrial oxidation genes in ERKO females, but not in WT and in KIKO females, suggests that ERα-independent pathways may compensate to maintain energy production, when ERα is lacking. These results suggest that ERα-independent pathways, such as ERβ, GPR30, or the putative Gq-mER, are important in regulating mitochondrial functions in the arcuate nucleus. In conclusion, these pathways may be important not only in independent processes and pathways regulating reproduction and energy balance, but also the interaction of the two. This interaction is critical to understand, as additional problems in both overnutrition and undernutrition impact reproduction (Bray, 1997,Helge, 2001,Jacobs, 1982,Moran et al., 2011). Our investigation of the ARC estrogenome is an important step in understanding molecular and cellular pathways impacted by E2 and on provides novel mechanisms for future studies.

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Tables

Table 1. Verification table for qPCR and RNA sequencing

Genes	WT		KIKO		ERKO	
	qPCR (Ch. 2)	RNA Seq (Ch. 3)	qPCR (Ch. 2)	RNA Seq (Ch. 3)	qPCR (Ch. 2)	RNA Seq (Ch. 3)
Kiss1	↓	↓	↓	↓	-	-
Tac2	↓	↓	-	↓	-	-
Pdyn	↓	-	-	-	-	-
Tac3r	↓	-	↓	-	-	-
Kiss1r	↑	-	↑	-	↑	-
Pomc	-	↓	-	-	-	-
Npy	-	-	-	-	-	-
Cartpt	N/A	↓	N/A	-	N/A	-
Cart	↓	-	↓	-	↑	-
Ghsr	↑	↑	-	-	-	-
Esr1	↓	-	-	-	-	-
Esr2	↑	-	↑	-	-	-
Pgr	↑	↑	-	-	-	-

Verification table showing direction of change in gene expression with E2B, compared to oil. The table represents select genes of interest and compares similarities and differences in gene expression using qPCR and RNA sequencing.

Table 2. Genes regulated by E2B in WT females

Gene	Fold Change	p-value	Function	Gene	Fold Change	p-value	Function
Mgp	0.49	5.00E-05	calcium regulation	*Accn4*	4.10	5.00E-05	ion channel
Stc1	1.99	0.00035	calcium regulation	*Fxyd2*	1.99	0.00065	ion channel
Mfge8	0.63	5.00E-05	cell adhesion	*Grik1*	1.98	5.00E-05	ion channel
Pcdh20	1.81	5.00E-05	cell adhesion	*Kcnk10*	0.47	2.00E-04	ion channel
Pvrl1	1.78	5.00E-05	cell adhesion	*C1ql2*	0.60	5.00E-05	neurodevelopment
Crabp1	1.83	5.00E-05	cell signaling	*Cbln2*	1.74	1.00E-04	neurodevelopment
Dock5	0.65	0.00055	cell signaling	*Fam5b*	1.52	5.00E-05	neurodevelopment
Efs	0.63	1.00E-04	cell signaling	*Prok2*	2.02	5.00E-05	neurodevelopment
Enpp1	0.39	1.00E-04	cell signaling	*Robo1*	0.63	0.00015	neurodevelopment
Gbp6	0.50	5.00E-05	cell signaling	*Agrp*	1.56	5.00E-05	neuropeptide
Grap	3.67	5.00E-05	cell signaling	*Cartpt*	0.52	5.00E-05	neuropeptide
Jak3	0.56	5.00E-04	cell signaling	*Cck*	0.49	5.00E-05	neuropeptide
Map2k1	1.55	5.00E-05	cell signaling	*Kiss1*	0.08	5.00E-05	neuropeptide
Net1	1.78	5.00E-05	cell signaling	*Nms*	0.40	5.00E-05	neuropeptide
Pcp4	1.85	5.00E-05	cell signaling	*Nts*	2.18	5.00E-05	neuropeptide
Pcp4l1	1.58	5.00E-05	cell signaling	*Pdyn*	0.49	5.00E-05	neuropeptide
Pim3	1.63	5.00E-05	cell signaling	*Pomc*	0.64	5.00E-05	neuropeptide
Ptprg	1.51	0.00065	cell signaling	*Scg2*	1.50	5.00E-05	neuropeptide
Rab37	5.51	5.00E-05	cell signaling	*Tac2*	0.43	5.00E-05	neuropeptide
Rasd1	2.69	5.00E-05	cell signaling	*Vgf*	0.36	5.00E-05	neuropeptide
Rps6ka6	1.76	5.00E-05	cell signaling	*Vip*	1.67	5.00E-05	neuropeptide
Rrad	0.53	5.00E-05	cell signaling	*Chga*	0.66	5.00E-05	neurotransmission
Rybp	1.64	5.00E-05	cell signaling	*Chgb*	0.63	5.00E-05	neurotransmission
Trp53i11	1.82	5.00E-05	cell signaling	*Nxph3*	0.29	5.00E-05	neurotransmission
Tyro3	0.65	5.00E-04	cell signaling	*Slc6a3*	0.39	5.00E-05	neurotransmission
H2afj	0.58	5.00E-05	chromosome structure	*Sv2b*	1.68	5.00E-05	neurotransmission
Mad2l1	5.19	5.00E-05	chromosome structure	*Syt2*	2.17	5.00E-05	neurotransmission
Rec8	0.26	5.00E-05	chromosome structure	*Syt6*	2.18	5.00E-05	neurotransmission
Arc	0.54	5.00E-05	cytoskeleton	*Gfra1*	2.93	5.00E-05	neurotrophic factors
Ezr	1.75	5.00E-05	cytoskeleton	*Ret*	1.58	5.00E-05	neurotrophic factors
Gfap	0.57	5.00E-05	cytoskeleton	*Pcsk1*	0.45	5.00E-05	protein modification
Inf2	0.66	2.00E-04	cytoskeleton	*Pcsk1n*	0.66	5.00E-05	protein modification
Myoc	0.44	5.00E-05	cytoskeleton	*Pcsk6*	1.88	5.00E-05	protein modification
Spnb4	0.68	6.00E-04	cytoskeleton	*Galnt6*	2.22	5.00E-05	protein trafficking
Gstm5	1.70	5.00E-05	enzyme	*Galntl4*	1.53	1.00E-04	protein trafficking

Table 2. Genes regulated by E2B in WT females (continued)

Gene	Fold Change	p-value	Function	Gene	Fold Change	p-value	Function
Pter	1.57	4.00E-04	enzyme	*Cckar*	1.80	5.00E-05	receptor
Aldh1a2	0.43	0.00035	enzyme	*Ghsr*	3.18	5.00E-05	receptor
Chst11	1.54	5.00E-05	enzyme	*Gpr88*	2.04	5.00E-05	receptor
Ptgds	0.57	5.00E-05	enzyme	*Oxtr*	3.19	5.00E-05	receptor
Tiparp	1.54	2.00E-04	enzyme	*Pgr*	2.63	5.00E-05	receptor
Bgn	0.61	2.00E-04	extracellular matrix	*Tacr3*	0.20	5.00E-05	receptor
Col1a1	0.39	5.00E-05	extracellular matrix	*Unc5b*	1.75	5.00E-05	receptor
Col2a1	2.17	5.00E-05	extracellular matrix	*Phf21b*	5.13	1.00E-04	transcription factor
Col3a1	0.47	5.00E-05	extracellular matrix	*Ccdc36*	3.16	0.00025	transcription factor
Fmod	0.46	0.00055	extracellular matrix	*Ccdc85b*	0.65	0.00035	transcription factor
Lamb1	3.32	5.00E-05	extracellular matrix	*Egr4*	0.32	5.00E-05	transcription factor
Lamb3	3.22	5.00E-05	extracellular matrix	*Ets2*	1.61	5.00E-05	transcription factor
Lor	0.53	0.00015	extracellular matrix	*Fosl2*	1.60	5.00E-05	transcription factor
Lum	0.40	5.00E-05	extracellular matrix	*Gata2*	0.57	0.00055	transcription factor
Sdc1	0.32	5.00E-05	extracellular matrix	*Greb1*	9.70	5.00E-05	transcription factor
Smoc2	4.96	5.00E-05	extracellular matrix	*Lcorl*	1.58	5.00E-05	transcription factor
Tnxb	3.88	5.00E-05	extracellular matrix	*Mamld1*	1.62	5.00E-05	transcription factor
Bdnf	0.29	5.00E-05	growth factor	*Msx1*	0.50	2.00E-04	transcription factor
Igfbp2	0.18	5.00E-05	growth factor	*Myc*	2.47	5.00E-05	transcription factor
Igfbp3	0.49	5.00E-05	growth factor	*Mycn*	2.92	5.00E-05	transcription factor
Alox5ap	0.24	0.00045	immune/inflammation	*Nr4a2*	5.13	5.00E-05	transcription factor
H2-Aa	0.33	1.00E-04	immune/inflammation	*Nr5a2*	1.58	0.00055	transcription factor
H2-Q2	1.58	5.00E-05	immune/inflammation	*Olig2*	0.60	5.00E-05	transcription factor
Hs3st5	2.45	5.00E-05	immune/inflammation	*Pou2f1*	1.95	3.00E-04	transcription factor
Icam5	0.59	5.00E-05	immune/inflammation	*Pou3f1*	2.51	5.00E-05	transcription factor
Islr	0.64	1.00E-04	immune/inflammation	*Sox3*	0.36	5.00E-05	transcription factor
Mrc2	0.54	0.00045	immune/inflammation	*Nup62cl*	4.89	3.00E-04	translation
Procr	0.22	5.00E-04	immune/inflammation	*Rbms3*	1.79	5.00E-05	translation
Spsb1	1.84	0.00025	immune/inflammation	*Zfp804a*	1.70	2.00E-04	translation
Tmem90a	2.23	5.00E-05	immune/inflammation	*Sfrp5*	0.56	5.00E-05	WNT signaling
Tnfrsf11b	0.33	5.00E-05	immune/inflammation	*Wnt4*	1.86	5.00E-05	WNT signaling

Genes listed are significantly regulated by E2B in WT females, which have a p-value < 0.05 and FPKM values > 1. Genes in table represent increases and decreases in gene expression by a fold-change (FC) of 0.67 < FC < 1.5.

Table 3. Genes regulated by E2B in KIKO females

Gene	Fold Change	p-value	Function
Cabp7	1.64	5.00E-05	calcium regulation
Cd74	1.74	5.00E-05	cancer
Epcam	2.40	5.00E-05	cell adhesion
Cdkn1c	1.99	5.00E-05	cell proliferation
Cyp2f2	1.90	5.00E-05	enzyme
Inmt	2.05	5.00E-05	enzyme
Ptgds	0.46	5.00E-05	enzyme
Bdnf	0.53	5.00E-05	growth factor
Igf2	0.50	5.00E-05	growth factor
Kcnh3	2.42	5.00E-05	ion channel
C1ql2	0.65	5.00E-05	neurodevelopment
Gldn	3.93	5.00E-05	neurodevelopment
Kiss1	0.53	5.00E-05	neuropeptide
Pnoc	0.66	5.00E-05	neuropeptide
Tac2	0.66	5.00E-05	neuropeptide
Pcsk1	0.63	5.00E-05	protein modification
Tacr3	0.54	5.00E-05	receptor
Gata2	1.80	5.00E-05	transcription factor
Pitx1	1.88	0.0001	transcription factor
Pou2f1	3.49	5.00E-05	transcription factor
Sox3	0.58	5.00E-05	transcription factor
Slc17a7	3.39	5.00E-05	transporter

Genes listed are significantly regulated by E2B in KIKO females, which have a p-value < 0.05 and FPKM values > 1. Genes in table represent increases and decreases in gene expression by a fold-change (FC) of $0.67 < FC < 1.5$.

Table 4. Genes regulated by E2B in ERKO females

Gene	Fold Change	p-value	Function	Gene	Fold Change	p-value	Function
C1qtnf4	2.19	5.00E-05	cancer	*Tac1*	1.91	5.00E-05	neuropeptide
Ccdc85b	2.15	5.00E-05	cell growth	*Vip*	0.42	5.00E-05	neuropeptide
Nenf	2.02	1.00E-04	cell growth	*Npy6r*	0.31	5.00E-05	receptor
Katnal1	2.72	5.00E-05	cytoskeleton	*Mrpl52*	2.09	5.00E-05	ribosomes
Tmsb10	1.91	1.00E-04	cytoskeleton	*Rpl18a*	1.91	0.00015	ribosomes
Alox5ap	0.23	5.00E-05	immune/inflammation	*Rpl36*	2.06	5.00E-05	ribosomes
Fkbp2	1.94	1.00E-04	immune/inflammation	*Rpl36a*	2.22	5.00E-05	ribosomes
Iigp1	0.31	5.00E-05	immune/inflammation	*Rpl36al*	1.85	1.00E-04	ribosomes
Atp5e	2.17	5.00E-05	mitochondrial oxidation	*Rpl37*	2.00	1.00E-04	ribosomes
Cox5b	1.86	0.00015	mitochondrial oxidation	*Rpl38*	1.98	1.00E-04	ribosomes
Ndufa11	1.88	1.00E-04	mitochondrial oxidation	*Rpl41*	2.22	1.00E-04	ribosomes
Ndufb10	2.05	5.00E-05	mitochondrial oxidation	*Rps24*	2.12	1.00E-04	ribosomes
Ndufb7	1.95	0.00015	mitochondrial oxidation	*Med29*	1.96	1.00E-04	transcript factor
Uqcr11	1.98	0.00015	mitochondrial oxidation	*Scand1*	3.15	5.00E-05	transcript factor
Uqcrq	2.13	5.00E-05	mitochondrial oxidation	*Atox1*	2.11	5.00E-05	transporter
Avp	3.64	5.00E-05	neuropeptide	*Hba-a1*	2.30	5.00E-05	transporter
Oxt	3.03	5.00E-05	neuropeptide	*Timm13*	1.96	5.00E-05	transporter

Genes listed are significantly regulated by E2B in ERKO females, which have a p-value < 0.05 and FPKM values > 1.

Genes in table represent increases and decreases in gene expression by a fold-change (FC) of 0.67 < FC < 1.5.

Table 5. Top 10 Canonical Pathways regulated by E2B in WT, KIKO, and ERKO females

Top Canonical Pathways	p-value	Genes
WT		
Intrinsic Prothrombin Activation Pathway	0.0002	*Col1a1, Col2a1, Col3a1*
Dendritic Cell Maturation	0.0004	*Col1a1, Col2a1, Hla-Dqa1, Tnfrsf11b, Col3a1*
Thyroid Cancer Signaling	0.0005	*Myc, Cdh1, Bdnf*
Hepatic Fibrosis / Hepatic Stellate Cell Activation	0.0008	*Col1a1, Igfbp3, Col2a1, Tnfrsf11b, Col3a1*
Neuroprotective Role of THOP1 in Alzheimer's Disease	0.0091	*Pdyn, Nts*
Atherosclerosis Signaling	0.0107	*Col1a1, Col2a1, Col3a1*
Endometrial Cancer Signaling	0.0195	*Myc, Cdh1*
T Helper Cell Differentiation	0.0302	*Hla-Dqa1, Tnfrsf11b*
Wnt/β-catenin Signaling	0.0309	*Myc, Cdh1, Sox3*
PDGF Signaling	0.0398	*Myc, Sphk1*
KIKO		
Prostanoid Biosynthesis	0.004	*Ptgds*
Role of JAK1, JAK2 and TYK2 in Interferon Signaling	0.0091	*Cga*
Autoimmune Thyroid Disease Signaling	0.0158	*Cga*
Nicotine Degradation II	0.0234	*Inmt*
Glutamate Receptor Signaling	0.0245	*Slc17a7*
Eicosanoid Signaling	0.0263	*Ptgds*
Role of BRCA1 in DNA Damage Response	0.0339	*Pou2f1*
PKCθ Signaling in T Lymphocytes	0.0479	*Pou2f1*
Ovarian Cancer Signaling	0.055	*Cga*
Glucocorticoid Receptor Signaling	0.1102	*Pou2f1*
ERKO		
Oxidative Phosphorylation	0.0003	*Uqcrq, Ndufb10, Atp5e*
Circadian Rhythm Signaling	0.0009	*Avp, Vip*
Mitochondrial Dysfunction	0.0013	*Uqcrq, Ndufb10, Atp5e*
EIF2 Signaling	0.0015	*Rpl36a, Rpl41, Rps24*
Retinoic acid Mediated Apoptosis Signaling	0.0603	*Tnfsf10*
nNOS Signaling in Neurons	0.0603	*Capn11*
Amyloid Processing	0.0661	*Capn11*
Regulation of Cellular Mechanics by Calpain Protease	0.0724	*Capn11*
Eicosanoid Signaling	0.0813	*Alox5p*
GPCR-Mediated Integration of Enteroendocrine Signaling Exemplified by an L Cell	0.0891	*Vip*

Top 10 canonical pathways as analyzed using Ingenuity Pathway Analysis (IPA). Genes listed following each canonical pathway represents either an increase of decrease in gene expression.

Supplementary Table 1. Genes regulated across oil-treated WT, KIKO, and ERKO females

Function	Gene expression	WT vs. KIKO	WT vs. ERKO
Cancer	Up	*Ph21b*	*Adamts8, Phf21b, Tspan32*
	Down	-	*Cyb5r2, Ecm1*
Cell growth/proliferation	Up	*Mad2l1*	*Bub1b, Kif15, Mad2l1, Nlrp1a, Orc1*
	Down	*Palb2*	*Upk1a*
Cell signaling	Up	*Rab37, Rasd1*	*Rab37, Rasd1*
	Down	*Gldn*	*Rrad, Lgals3, Lrrtm1*
Channels	Up	*Aqp5, Asic4, Grik1*	*Aqp5, Asic4*
	Down	-	*Kcnk12*
Chaperone protein	Up	-	-
	Down	-	*Hspa1a, Hspa1b, Hspb1*
Cytoskeleton	Up	-	-
	Down	*Fermt3*	*Fermt3, Gfap*
Enzyme	Up	*Tpte, Cyp2s1, Dbh, Map3k15, Pfkfb1*	*Acsm5, Mastl, Cyp2s1, Map3k15, Tph2*
	Down	*Car3, Hck, Pck1, Ptgs2, Tgm3*	*Hpse, Pck1, Pcsk1n, Ptgs2*
Extracellular matrix	Up	*Col2a1, Dmp1, Lamb3, Smoc2, Tnxa, Tnxb*	*Dmp1, Smoc2, Tnxa, Tnxb*
	Down	-	*Lor*
Growth factor	Up	*Btc*	*Angptl2, Btc*
	Down	*Igfbp2*	*Bdnf, Igfbp2*
Hormone	Up	*Fam132b*	*Fam132b*
	Down	-	*Avp*
Immune/inflammation	Up	-	*Cd46, Hs3st5, Mme*
	Down	*Ccl12, Oas1b, Procr, Slfn9, Timp1, Tlr5*	*C1qtnf4, C4b, Ccdc88b, Cd200r2, Cd52, Ifitm1*
Neuro -development and -signaling	Up	-	*Cplx3, Gfra1*
	Down	*Slc6a3*	*C1ql2*
Neuropeptide	Up	*Rln3*	*Rln3*
	Down	-	*Cartpt, Pdyn, Pomc, Tac2, Vgf*
Nucleosome structure	Up	-	-
	Down	-	*H2afj, Hist1h2al, Hist2h2ac, Hist2h3c2*
Receptor	Up	*Nr4a2, Oxtr*	*Ghsr, Nr4a2, Oxtr, Pgr, Rxfp2*
	Down	*Ephb3, Gpr139, Tac3r*	*F2rl2, Tac3r, Nxph3*
Transcription factor	Up	*Ccdc36, Greb1, Mycn*	*Ccdc36, Emx2, Greb1, Mycn, Nrip1, Pou3f1*
	Down	*Trim29*	*Ccdc85b, Egr4, Osr1, Sox3*
WNT signaling	Up	-	-
	Down	-	*Wnt11*
Zinc finger protein	Up	-	*Snai2*
	Down	-	*Scand1*

Genes differentially regulated between WT and KIKO and WT and ERKO females. Genes were normalized to WT oil values. Genes expression change “up” represents increased expression from WT to KIKO and from WT to ERKO females. Genes

expression change “down” represents decreased expression from WT to KIKO and from WT to ERKO females

Supplementary Table 2. Genes regulated across E2B-treated WT, KIKO, and ERKO females

Function	Gene expression	WT vs. KIKO	WT vs. ERKO
Cancer	Up	-	-
	Down	*Dmp1, Phf21b*	*Adamts8, Cplx3, Dmp1, Phf21b, Tspan32*
Cell growth/proliferation	Up	*Palb2*	*Upk1a*
	Down	*Mad2l1*	*Bub1b, Kif15, Mad2l1, Nlrp1a, Orc1*
Cell signaling	Up	*Gldn*	*Rrad, Lgals3, Lrrtm1*
	Down	*Rab37, Rasd1*	*Rab37, Rasd1*
Channels	Up	-	*Kcnk12*
	Down	*Aqp5, Asic4, Grik1*	*Aqp5, Asic4*
Chaperone protein	Up	-	*Hspa1a, Hspa1b, Hspb1*
	Down	-	-
Cytoskeleton	Up	*Fermt3*	*Fermt3, Gfap*
	Down	-	-
Enzyme	Up	*Car3, Hck, Pck1, Ptgs2, Tgm3*	*Hpse, Pck1, Pcsk1n, Ptgs2, Cyb5r2*
	Down	*Tpte, Cyp2s1, Map3k15, Pfkfb1*	*Acsm5, Mastl, Cyp2s1, Map3k15, Tph2*
Extracellular matrix	Up	-	*Lor*
	Down	*Col24a1, Dbh, Lam3, Smoc2, Tnxa, Tnxb*	*Col24a1, Smoc2, Tnxa, Tnxb*
Growth factor	Up	*Igfbp2*	*Bdnf, Igfbp2*
	Down	*Btc*	*Angptl2, Btc*
Hormone	Up	-	*Avp, F2rl2, Fam132b*
	Down	*Fam132b*	-
Immune/inflammation	Up	*Ccl12, Oas1b, Procr, Slfn9, Timp1, Tlr5*	*C1qtnf4, C4b, Ccdc88b, Cd200r2, Cd52, Ifitm1, Procr, Sprr1a, Timp1*
	Down	-	*Cd46, Hs3st5, Mme*
Neuro - development and - signaling	Up	*Slc6a3*	*C1ql2*
	Down	*Col2a1*	*Gfra1*
Neuropeptide	Up	-	*Cartpt, Pdyn, Pomc, Tac2, Vgf*
	Down	*Rln3*	*Rln3*
Nucleosome structure	Up	-	*H2afj, Hist1h2al, Hist2h2ac, Hist2h3c2*
	Down	-	-
Receptor	Up	*Ephb3, Gpr139, Tacr3*	*Tacr3, Nxph3*
	Down	*Nr4a2, Oxtr*	*Emx2, Ghsr, Nr4a2, Oxtr, Pgr, Rxfp2*
Transcription factor	Up	*Trim29*	*Ccdc85b, Ecm1, Egr4, Osr1, Sox3*
	Down	*Ccdc36, Greb1, Mycn*	*Ccdc36, Greb1, Mycn, Nrip1, Pou3f1*
WNT signaling	Up	-	*Wnt11*
	Down	-	-
Zinc finger protein	Up	-	*Scand1*
	Down	-	*Snai2*

Genes differentially regulated between WT and KIKO and WT and ERKO females. Genes were normalized to WT oil values. Genes expression change “up” represents increased expression from WT to KIKO and from WT to ERKO females. Genes expression change “down” represents decreased expression from WT to KIKO and from WT to ERKO females.

Figures

Figure 1. Number of genes regulated by E2B in WT, KIKO, and ERKO females. (A) Flowchart of the number of genes regulated by E2B in each genotype and overlap of genes. All differentially regulated genes have FPKM > 1 and p-values < 0.05. (B) Principle component analysis (PCA) of each treatment, showing clustering of replicates. (C) Volcano plots showing number of genes in samples and total genes regulated. Red dots signify genes that are differentially regulated. Black dots represent genes that are not significantly regulated. (D) Venn diagram showing overlap of genes regulated by E2B in WT, KIKO, and ERKO females.

Figure 2. KNDy genes are regulated through differential E2 signaling. (A) Bar graphs of the KNDy neuropeptide genes (*Kiss1, Tac2,* and *Pdyn*) in WT, KIKO, and ERKO females. FPKM represents fragments per kilobase of transcript per million mapped reads and is a measurement of relative gene expression. Data are expressed as mean ± SEM. (B) Transcript analysis of KNDy neuropeptide genes using Integrative Genomics Viewer (IGV).

Figure 3. Overlap of differential gene expression in WT, KIKO, and ERKO females. Venn diagram of overlapping genes expressed differentially in WT, KIKO, and ERKO females. Genes were compared to WT females and represent either significantly increased or decreased gene expression. Genes represent FC > 1.5 and p < 0.05. All genes differentially expressed by WT vs. KIKO and WT vs. ERKO females were different in both oil- and E2B-treated groups, with the exception of *Ptpn20,* which was different between WT vs. ERKO oil, but not E2B females.

Figure 1

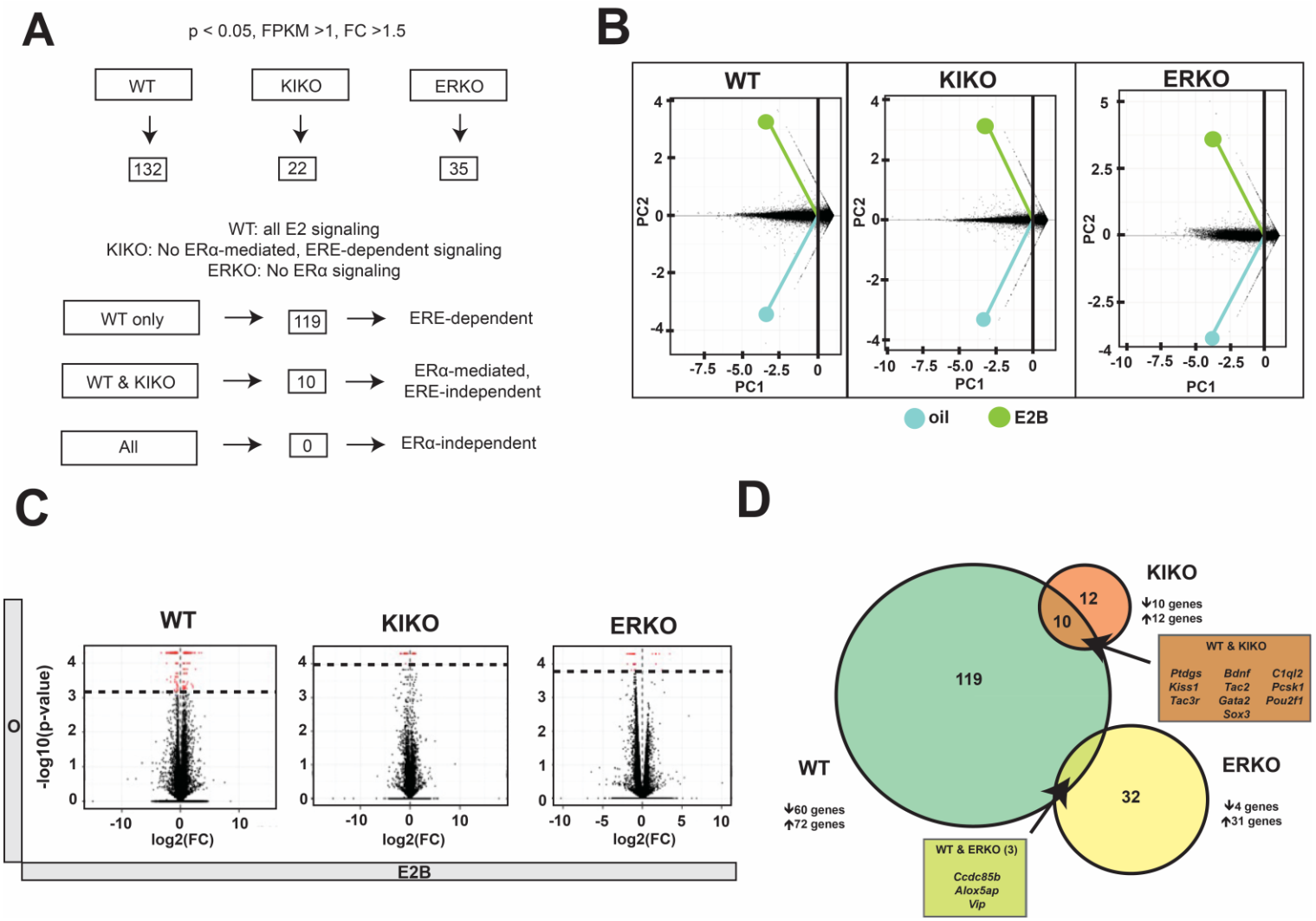

A
p < 0.05, FPKM >1, FC >1.5
WT
KIKO
ERKO
132
22
35
WT: all E2 signaling
KIKO: No ERα-mediated, ERE-dependent signaling
ERKO: No ERα signaling
WT only
119
ERE-dependent
WT & KIKO
10
ERα-mediated, ERE-independent
All
0
ERα-independent
B
WT
KIKO
ERKO
PC1
PC2
oil
E2B
C
WT
KIKO
ERKO
-log10(p-value)
log2(FC)
E2B
D
KIKO
↓10 genes
↑12 genes
12
10
119
WT & KIKO
Ptdgs Kiss1 Tac3r
Bdnf Tac2 Gata2 Sox3
C1ql2 Pcsk1 Pou2f1
WT
↓60 genes
↑72 genes
32
ERKO
↓4 genes
↑31 genes
WT & ERKO (3)
Ccdc85b
Alox5ap
Vip

Figure 2

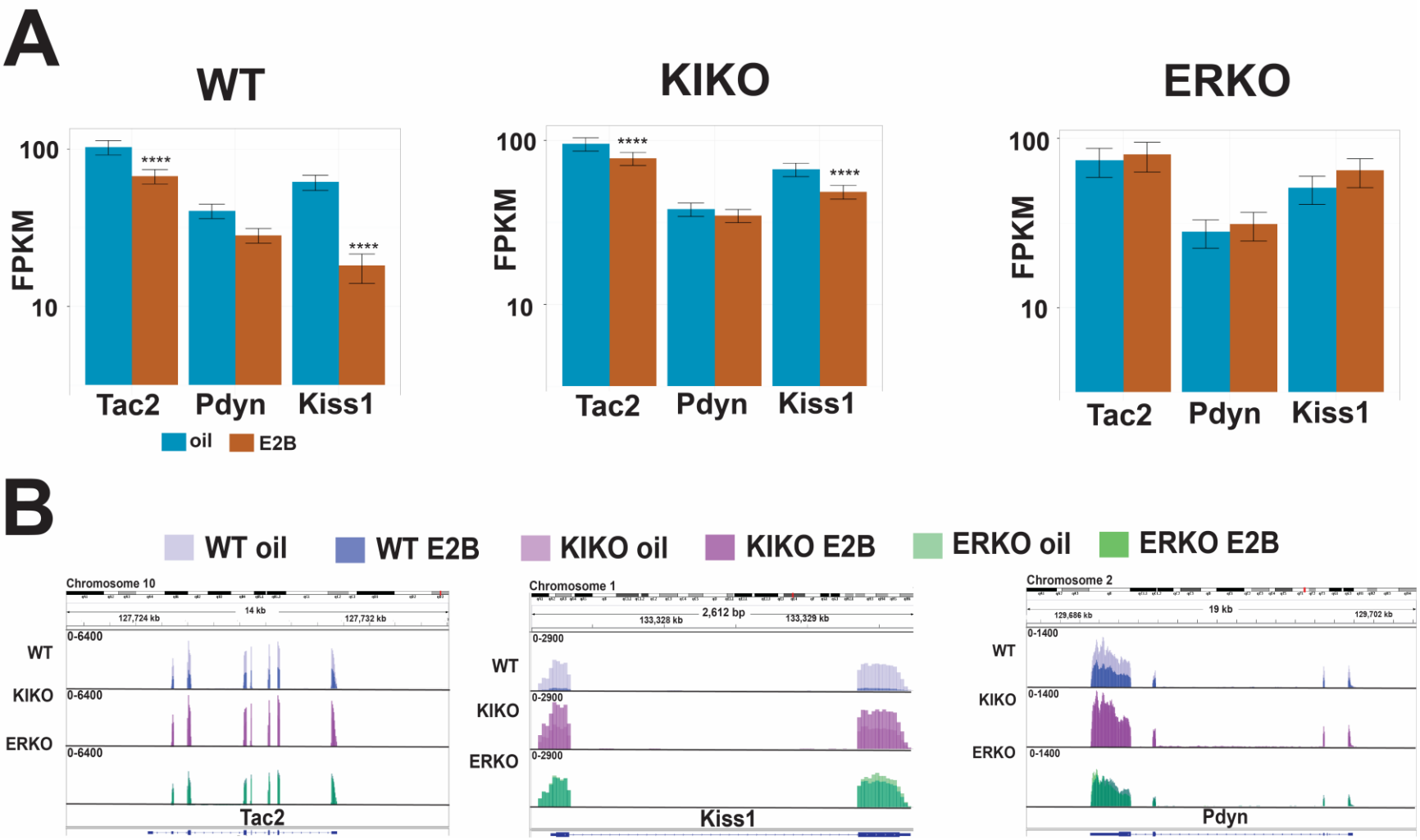
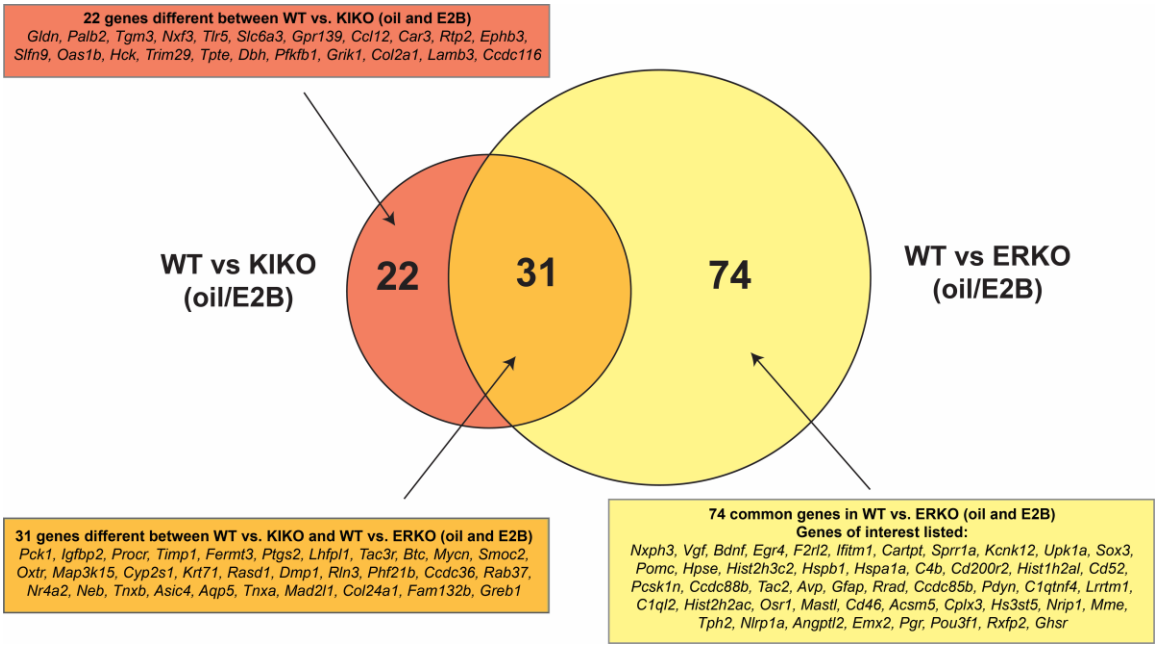

A
WT
KIKO
ERKO
FPKM
100
10
****
Tac2
Pdyn
Kiss1
oil
E2B
B
WT oil
WT E2B
KIKO oil
KIKO E2B
ERKO oil
ERKO E2B
Chromosome 10
127,724 kb
14 kb
127,732 kb
0-6400
WT
KIKO
ERKO
Tac2
Chromosome 1
133,328 kb
2,612 bp
133,329 kb
0-2900
Kiss1
Chromosome 2
129,686 kb
19 kb
129,702 kb
0-1400
Pdyn

Figure 3


22 genes different between WT vs. KIKO (oil and E2B)
Gldn, Palb2, Tgm3, Nxf3, Tlr5, Slc6a3, Gpr139, Ccl12, Car3, Rtp2, Ephb3, Slfn9, Oas1b, Hck, Trim29, Tpte, Dbh, Pfkfb1, Grik1, Col2a1, Lamb3, Ccdc116
WT vs KIKO (oil/E2B)
22
31
74
WT vs ERKO (oil/E2B)
31 genes different between WT vs. KIKO and WT vs. ERKO (oil and E2B)
Pck1, Igfbp2, Procr, Timp1, Fermt3, Ptgs2, Lhfpl1, Tac3r, Btc, Mycn, Smoc2, Oxtr, Map3k15, Cyp2s1, Krt71, Rasd1, Dmp1, Rln3, Phf21b, Ccdc36, Rab37, Nr4a2, Neb, Tnxb, Asic4, Aqp5, Tnxa, Mad2l1, Col24a1, Fam132b, Greb1
74 common genes in WT vs. ERKO (oil and E2B)
Genes of interest listed:
Nxph3, Vgf, Bdnf, Egr4, F2rl2, Ifitm1, Cartpt, Sprr1a, Kcnk12, Upk1a, Sox3, Pomc, Hpse, Hist2h3c2, Hspb1, Hspa1a, C4b, Cd200r2, Hist1h2al, Cd52, Pcsk1n, Ccdc88b, Tac2, Avp, Gfap, Rrad, Ccdc85b, Pdyn, C1qtnf4, Lrrtm1, C1ql2, Hist2h2ac, Osr1, Mastl, Cd46, Acsm5, Cplx3, Hs3st5, Nrip1, Mme, Tph2, Nlrp1a, Angptl2, Emx2, Pgr, Pou3f1, Rxfp2, Ghsr

CHAPTER 4: THE INTERACTION OF FASTING, CALORIC RESTRICTION, AND DIET-INDUCED OBESITY WITH 17β-ESTRADIOL ON THE EXPRESSION OF KNDY NEUROPEPTIDES AND THEIR RECEPTORS IN THE FEMALE MOUSE

4. The interaction of fasting, caloric restriction, and diet-induced obesity with 17β-estradiol on the expression of KNDy neuropeptides and their receptors in the female mouse

4.1 Abstract

Arcuate neurons that coexpress kisspeptin (*Kiss1)*, neurokinin B *(Tac2),* and dynorphin (*Pdyn)* mediate negative feedback of 17β-estradiol (E2) on the HPG axis. Previous studies report that fasting and caloric restriction reduce *Kiss1* expression. The objective of this study was to determine the interactions of E2 with fasting, caloric restriction, and diet-induced obesity on KNDy gene and receptor expression. Ovariectomized female mice were separated into control and estradiol benzoate (E2B)-treated groups. E2B decreased *Kiss1* and the tachykinin 2 receptor, *Tac3r,* in ARC tissue and *Tac2* in *Tac2* neurons. Diet-induced obesity decreased *Kiss1* in oil-treated animals and the kisspeptin receptor, *Kiss1r* and *Tac3r* in the ARC of E2B-treated animals. Chronic caloric (30%) restriction reduced all three neuropeptides in oil-treated females and *Kiss1r* by E2B in CR animals. Taken together, our experiments suggest that steroidal environment and energy state negatively regulate KNDy gene expression in both ARC and *Tac2* neurons.

4.2 Introduction

The growing obesity epidemic in the United States suggests a 70% increase in obesity within the past decade (Andreyeva et al., 2008). While obesity has been linked to decreased life expectancy, coronary heart disease, and type 2 diabetes mellitus (T2DM) (Fontaine et al., 2003), it also impacts reproduction. In women, obesity leads to irregular menses, infertility, and miscarriages, among other complications (Moran et al., 2011,Norman and Clark, 1998). These problems in reproductive parameters extend to

individuals suffering from undernutrition. Amenorrhea is prominent in athletes and anorexia nervosa patients, largely due to inadequate energy, as represented by their decreased body mass index (Jacobs, 1982,Sundgot-Borgen, 1994). These perturbations in energy balance can lead to problems in the hypothalamic pituitary gonadal (HPG) axis, though the mechanisms remain unclear.

Energy balance and reproduction are centrally regulated processes that are controlled, in part, by neurons in the arcuate nucleus (ARC). Regulation of reproduction in the ARC is controlled by negative feedback mechanisms of 17β-estradiol (E2) on the HPG axis mediated, in part, by neurons expressing the neuropeptide, kisspeptin. There are two main groups of kisspeptin neurons located in the rodent brain (Brock and Bakker, 2013). Kisspeptin in the anteroventral periventricular nucleus (AVPV) regulates the surge of luteinizing hormone (LH) leading to ovulation in females (Brock and Bakker, 2013). Kisspeptin is also expressed in the ARC in KNDy neurons that co-express kisspeptin (*Kiss1)*, neurokinin B (*Tac2)*, and dynorphin (*Dyn*). These neurons contribute to negative feedback of E2 on the HPG axis (Navarro and Tena-Sempere, 2011,Oakley et al., 2009). Both AVPV and ARC kisspeptin regulate the HPG axis by binding to their receptor, G protein-coupled receptor 54 (GPR54), or *Kiss1r,* expressed on gonadotropin releasing hormone (GnRH) neurons (Bosch et al., 2013,Oakley et al., 2009). In ARC KNDy neurons, the two neuropeptides neurokinin B and dynorphin act as positive and negative autoregulators of KNDy neuronal excitability, respectively (de Croft et al., 2013,Ruka et al., 2016,Uenoyama et al., 2014,Weems et al., 2016). Together, the three KNDy neuropeptides act as a pulse generator for GnRH release (Goodman et al., 2013).

Recent evidence suggests that KNDy neurons are also involved in the regulation of negative and positive energy balance. Ablation of KNDy neurons abrogates the post-ovariectomy weight gain associated with E2 in rats (Mittelman-Smith et al., 2012), suggesting that KNDy neurons mediate, in part, the anorectic effects of E2. In male

mice, acute caloric restriction (12, 24, and 48 h fasting) decreases *Kiss1* mRNA in the whole hypothalamus, although it is unclear which hypothalamic kisspeptin population is affected (Luque et al., 2007). In female rats, *Kiss1* gene expression in the ARC is unchanged in a 48 h fast (Kalamatianos et al., 2008). The inconsistency between these studies is potentially due to differences in species, sexes, hypothalamic tissue, and duration of fasting. Chronic caloric restriction also suppresses ARC *Kiss1* as well as *Tac2* and *Pdyn* expression (True et al., 2011). Positive energy balance similarly disrupts ARC kisspeptin. Diet-induced obesity (DIO) reduces hypothalamic kisspeptin expression in mice, though the mechanism is unclear (Quennell et al., 2011). Furthermore, DIO suppresses E2-regulated, hypothalamic gene expression (Balasubramanian et al., 2012). Taken together, it is important to note that most of these studies fail to examine the expression of the KNDy receptors or the receptors involved in gonadal steroid negative feedback, ERα (*Esr1*) and PR (*Pgr*). Therefore, this study examines expression of the KNDy neuropeptides and receptors during positive energy balance (DIO) and negative energy balance (acute 24 h fast and chronic caloric restriction), with or without E2.

The mechanisms that link reproduction and energy balance are not well understood. One hormone of interest is the stomach-derived hormone ghrelin, which functions to increase feeding behavior and weight gain. Ghrelin is thought to function in the ARC through its cognate receptor, growth hormone secretagogue receptor (GHSR) to stimulate NPY neurons, while simultaneously suppressing POMC neurons through inhibitory γ-aminobutyric acid (GABA)-ergic inputs (Andrews, 2011). Ghrelin's role in reproduction is the suppression of LH pulse frequency (Forbes et al., 2009). Thus, control of ghrelin/GHSR signaling may influence both reproduction and energy balance. While ghrelin signaling has been characterized in NPY neurons, there are only a few studies examining the role of ghrelin in KNDy neuronal signaling. In one study, *Kiss1*

mRNA expression in the ARC was unchanged with administration of ghrelin in a fed or fasted state (Forbes et al., 2009). In another, ghrelin depolarized more KNDy neurons in E2-treated females than in oil-treated females, which was due to an increase *Ghsr* expression in the ARC by E2 (Frazao et al., 2014,Yasrebi et al., 2016).

We hypothesize that negative and positive energy balance disrupt KNDy-associated gene expression in both ARC and *Tac2* (KNDy) neurons leading to disruption of the HPG axis. To address this, we examined KNDy neuropeptide and receptor gene expression under the following paradigms: 1) oil vs. estradiol benzoate (E2B); 2) fed vs. 24 h fast; 3) DIO in ovx females; and 4) 30% caloric restriction. These experiments were conducted in wild type (WT) females to examine ARC tissue gene expression. Experiments 1-3 were repeated in *Tac2*-EGFP animals to examine gene expression change in *Tac2* neurons. Our results indicate that both positive and negative energy balance impact KNDy neuropeptides and their receptors with and without E2 replacement.

4.3 Methods

4.3.1 Animal care and experimental design

All animal procedures were completed in compliance with institutional guidelines based on National Institutes of Health standards and were performed with Institutional Animal Care and Use Committee approval at Rutgers University. Adult mice were housed under constant photoperiod conditions (12/12 h light/dark cycle), with lights on/off at 700 h and 1900 h, and maintained at a controlled temperature (25°C). Animals were given food (LabDiet 5V75) and water *ad libitum,* unless otherwise noted. Animals were weaned at postnatal day 21 (PD21). Two different strains of mice, C57/BL and Swiss Webster (SW), were used for experiments and were purchased from Jackson

Laboratory. SW *Tac2*-EGFP were used for single-cell harvesting experiments. The strain used for each experiment is noted in Table 2.

All experimental design timelines can be viewed in Figure 1. In Experiment #1, we used a 24 h fast prior to sacrifice to determine if acute negative energy balance regulates ARC KNDy neuropeptide and receptor gene expression. To elucidate the interactions of E2 and fasting on gene expression, adult females were bilaterally ovx under isoflurane anaesthesia using sterile no-touch technique according to the NIH Guidelines for Survival Rodent Surgery. Animals were given a dose of analgesic [4 mg/kg carprofen (Rimadyl®)] one day following surgery for pain management. Animals typically lost 1-2 grams of weight one day after surgery. Following ovx, C57/BL females were separated into 2 treatments – oil and estradiol benzoate (E2B) – and two feeding states – fed and 24 h fasted (n = 5-6, per group). An E2B injection protocol was used that has been shown to alter gene expression in the hypothalamus (Bosch et al., 2013). Animals were injected subcutaneously (s.c.) at 1000 h on post-ovx day 5 with either 0.25 μg of E2B or oil. On post-ovx day 6, a 1.5 μg dose of E2B or oil was injected at 1000 h. Females in the fed groups were allowed to feed *ad libitum* and females in the fasted group were food restricted 24 h prior to sacrifice at 1000 h on post-ovx day 7. Animals were sedated with ketamine (100 μl of 100 mg/ml stock, intraperitoneal [i.p.]) and rapidly decapitated. Brains were removed and rinsed in ice-cold Sorensen's Phosphate Buffer (0.2 M sodium phosphate, dibasic and 0.2 M sodium phosphate, monobasic) for 30-60 sec. The basal hypothalamus (BH) was cut using a brain slice matrix (Ted Pella, Inc., Redding, CA, USA) into 1-mm thick coronal rostral and caudal blocks corresponding to Plates 42 to 47 and Plates 48 to 53, respectively, from The Mouse Brain in Stereotaxic Coordinates (Franklin and Paxinos, 2008). The slices were transferred to a 50/50 RNA*later*®/Pyrogard water solution and fixed overnight at 4°C. The ARC was found in

two slices and identified based on The Mouse Brain Stereotaxic Coordinates (Franklin and Paxinos, 2008). ARC nuclei were microdissected using a dissecting microscope, following our previous publications (Bosch et al., 2009,Franklin and Paxinos, 2008,Mamounis et al., 2013,Roepke et al., 2008). The microdissected sections represent the entirety of the ARC tissue. Dissected tissue was stored at –80°C until RNA extraction. Trunk blood was collected at sacrifice to measure plasma E2, LH, and FSH levels. Uteri were removed and wet weight was recorded. Uterine weights (mg) are expressed as wet weight and also as wet weight relative to body weight (g), as indicated in Table 2.

In Experiment #2, we used a diet-induced obesity (DIO) model to determine if chronic positive energy balance regulates ARC gene expression. C57/BL females were fed either a control LFD (10% fat, D12450B, Research Diets, New Brunswick, NJ) or a HFD (45% fat, D12451, Research Diets) diet. Following ovx, females were separated into four groups: 1) LFD oil, 2) LFD E2B, 3) HFD oil or 4) HFD E2B (n = 8-10). Animals were orally dosed with oil or E2B (300 μg/kg body weight) in peanut butter daily at 900 h, starting on the day of ovx. Animals were pair housed and fed either LFD or HFD *ad libitum* for 8 weeks post-ovx. Body weights and energy intake were measured each week. To determine feeding efficiency, we used the formula: body weight gained/kCal food consumed. Prior to sacrifice, body composition (lean mass and fat mass) was measured twice using an EchoMRI (Houston, TX, USA). Data for fat and lean mass were averaged for each animal. Animals were sacrificed at 1000 h and the ARC was microdissected. Trunk blood was collected to measure plasma E2, LH, and FSH levels. Uteri were removed to determine wet weight.

To determine if *Tac2* neurons exhibit similar gene expression changes as whole ARC, we repeated our experiments in *Tac2*-EGFP mice, which have a Swiss Webster (SW) backbone. In Experiment #3, we wanted to confirm the effects of E2B on KNDy

expression in the ARC from SW females. Following ovx, SW wild-type females were separated into a control sesame oil-treated group and an E2B-treated group and were sacrificed on post-ovx day 7 at 1100 h, n = 5-8/treatment. The E2B injection protocol for Experiment #3 is the same as Experiment #1. The ARC was microdissected for gene expression studies. Uteri were collected to determine wet weight. Trunk blood was collected to determine plasma E2 and LH concentrations. In Experiment #4, we repeated Experiment #1 in *Tac2*-EGFP animals (n=5-6, per treatment) and harvested single *Tac2* neurons for single cell PCR co-localization in oil- and E2B-treated, fed females. Pools of 5 *Tac2* neurons were also harvested from oil- and E2B-treated, fed and fasted females. Single-cell harvesting of *Tac2* neurons and pools is outlined below. In Experiment #5, we repeated Experiment #2 in *Tac2*-EGFP animals (n=6-8, per treatment) to determine if DIO regulates gene expression in *Tac2* neurons.

To determine if chronic negative energy balance regulates ARC KNDy gene expression, we conducted a caloric restriction study (Experiment #6). Wild-type SW females were separated into four groups (n = 7-8, per group): 1) oil *ad libitum,* 2) oil calorie restricted (CR), 3) E2B *ad libitum,* and 4) E2B CR. Animals were ovx prior to the experiment (Week 0) and recovered for one week. All animals were fed *ad libitum* for the first four weeks, while orally dosed with oil or E2B (300 μg/kg body weight) in powdered, fat-free peanut butter (peanut oil removed) daily. The caloric restriction phase followed this *ad libitum* feeding stage, lasting for 6 weeks. Females were dosed with oil or E2B (continuing the same treatment as Weeks 1-4) at 1700 h to minimize disruption of circadian rhythmicity. The two CR groups (oil and E2B) were fed 70% of the daily average (± 0.02 g) of each of the *ad libitum* groups within steroid treatment. Body weights and energy intake were measured each week. Body composition was measured using an MRI (EchoMRI) prior to and following (pre- and post-) caloric restriction. Pre-MRI was conducted on Week 4 and post-MRI was conducted on Week 9. Animals were

sacrificed at 1000 h and the ARC was microdissected following the procedures described in Experiment #1.

4.3.2 Drugs

Estradiol benzoate (E2B) was purchased from Sigma-Aldrich and dissolved in ethanol (1mg/ml) prior to mixing in sesame oil (Sigma-Aldrich). Ketamine was purchased from Henry Schein Animal Health (Melville, NY, USA) and was used for sedation prior to sacrifice.

4.3.3 Blood preparation and hormone assays

Whole trunk blood was subjected to centrifugation (4°C at 1300 rpm for 30 min). The supernatant was subjected to an additional 15 min of centrifugation (4°C at 1300 rpm) and the plasma supernatant was transferred and stored in a cryotube at –80°C until E2 analysis. E2 was analyzed using the Mouse Calbiotech ELISA (ES180S-100) at the University of Virginia Center for Research in Reproduction Ligand Assay and Analysis Core for Experiment #3 (Haisenleder et al., 2011). For the remaining experiments, E2 ELISA was run in house using the Mouse Calbiotech ELISA (ES180S-100). The Calbiotech ELISA is specific to detect 17β-estradiol and has a standard range of 3-300 pg/ml and an analytical sensitivity of 3 pg/ml. For Experiment #3, the University of Virginia Center for Research in Reproduction Ligand Assay and Analysis Core measured LH. For Experiment #1, 2, and 6, LH and FSH levels were measured using a Millipore Luminex MAGPIX plate (#MPTMAG-49K). The assay has a minimum detectable concentration of 9.5 pg/mL (FSH) and 1.9 pg/mL (LH), an intraassay coefficienct of varaiance of <15% (both), and an interassay coefficient of variance of <20% (both).

4.3.4 Cell harvesting of dispersed Tac2 neurons

In Experiments #4-5, we collected *Tac2* neurons to determine cell-type specific changes in gene expression similar to our previous publication (Yasrebi et al., 2016).

Briefly, animals were sedated with ketamine (100 µl of 100 mg/ml stock, i.p.) and decapitated. Brains were transferred to a vibratome containing cold, oxygenated aCSF and sliced into 250µM thick basal hypothalamic (BH) slices. BH slices were transferred to an auxiliary chamber (~1 h) containing oxygenated aCSF. The ARC was microdissected and incubated in a protease solution (15 min at 37°C) and washed with low calcium aCSF followed by regular aCSF. The ARC was titurated using flame-polished Glass Pasteur pipettes to disperse cells, which were placed on a glass-bottomed Petri dish (60 mm) and perfused with aCSF for the duration of the experiment (2 ml/min). *Tac2* cells were visualized using a Leica DM-IL fluorescent microscope, patched, and harvested by applying low negative pressure to the pipette using the Xenoworks manipulator system (Sutter Instruments, Novato, CA). Positive pressure was used to expel the contents of the pipette into a siliconized microcentrifuge tube containing: 1 µl 5x Superscript III Buffer (Life Technologies), 15 U Rnasin (Promega), 0.5 µl 100 mM DTT, and DEPC-treated water in 8 µl total volume. *Tac2* neurons were harvested both individually as single cells or collected into 10-15 pools of 5 *Tac2* neurons from each animal.

Harvested single cells and pools were reverse transcribed as previously described (Bosch et al., 2013,Roepke et al., 2011). In brief, tubes of harvested cells and a positive control (25 ng of total hypothalamic RNA in 1 µl) were denatured for 5 min at 65°C and cooled on ice for 5 min. Reverse transcription was conducted by adding 50 U Superscript III RT, 3 µl 5x Superscript Buffer, 5 mM $MgCl_2$, 0.625 mM dNTPs (Clontech), 15 U Rnasin, 400 ng anchored oligo(d)T (Life Technologies), 100 ng random hexamers (Promega), 10 mM DTT in DEPC-water in a total volume of 25 µl. One single cell and one tissue RNA tube were used as negative controls, processed without RT. aCSF was collected every 2-3 pools or 10 single cells to analyze for contamination. Reverse transcription protocol is as follows: 5 min at 25°C, 60 min at 50°C, 15 min at 70°C.

For single cell colocalization experiment, *Tac2* neurons were analyzed using standard PCR protocols and gel electrophoresis as previously described (Roepke et al., 2011,Xu et al., 2008). Primers for single cell PCR are those used with ARC tissue qPCR (Table 1), with the exception of *Tac2:* F: 5'-TCTGGAAGGATTGCTGAAAGTG-3'; R: 5'-GTAGGGAAGGGAGCCAACAG-3'. Each reaction was amplified for 50 cycles using a C1000 Thermal Cycle (Bio-Rad, Hercules, CA) at an annealing temperature of 60°C. Negative (cell and tissue samples without RT), aCSF, and positive tissue controls were analyzed with each PCR run.

4.3.5 RNA extraction of ARC tissue

RNA was extracted from ARC using Ambion RNAqueous® Micro Kits (Life Technologies, Carlsbad, CA, USA) according to the manufacturer's protocol, followed by DNase-I treatment to remove contamination by genomic DNA (Life Technologies). RNA samples were run on a NanoDrop™ ND-2000 spectrophotometer (ThermoFisher, Inc., Waltham, MA, USA) to assess quantity, followed by an Agilent 2100 Bioanalyzer analysis using the RNA 6000 Nano Kit (Agilent Technologies, Inc., Santa Clara, CA, USA) to assess quality. Samples with a RNA integrity number (RIN) > 8 were used for quantitative real-time PCR (qPCR).

4.3.6 Quantitative real-time PCR

For ARC, cDNA was synthesized from 200 ng of total RNA, following our previous publication (Yasrebi et al., 2016). A 1:20 dilution of the cDNA was produced using nuclease-free water (Gene Mate) for a final cDNA concentration of 0.5 ng/µl and stored at –20°C. Primers for qPCR were designed to span exon-exon junctions and were synthesized by Life Technologies using Clone Manager 5 software (Sci Ed Software, Cary, NC, USA). We used 4 µl of cDNA (equivalent to 2 ng of total RNA) amplified with SsoAdvanced™ SYBR Green (BioRad, Hercules, CA, USA) on CFX-Connect Real-time PCR Instrument (BioRad). A standard curve was generated for each primer pair using

serial dilutions of BH cDNA in triplicate. Efficiencies were calculated as a percent efficiency and are approximately equal (90%-110% or one doubling per cycle). Amplification protocol for genes was as follows: initial denaturing 95°C for 3 min followed by 40 cycles of amplification at 94°C for 10 sec (denaturing), 60°C for 45 sec (annealing), and completed with a dissociation step for melting point analysis with 60 cycles of 95°C for 10 sec, 65°C to 95°C (in increments of 0.5°C) for 5 sec and 95°C for 5 sec. The reference genes used were *Gapdh, Hprt, and Actb.* Positive, negative and water blank controls were included in the qPCR plate design. See Table 1 for a list of all primers used for qPCR.

Analysis of qPCR was done using the comparative C_q method using a 1:20 diluted BH cDNA (equivalent to 2 ng of RNA) sample from a male as the calibrator (Livak and Schmittgen, 2001,Pfaffl, 2001). All values were normalized and are expressed as relative mRNA expression. In all plates, we maintained a consistent threshold level, set at the lowest but steepest slope of the exponential curve. We calculated the linear quantity of target genes using the formula $2^{-\Delta\Delta Cq}$. The *n*-fold difference was used for statistical analysis.

4.3.7 Data analysis

All data are expressed as mean ± SEM. All data were analyzed using GraphPad® Prism software (GraphPad Software, La Jolla, CA, USA) except when multi-factorial and repeated measures ANOVA were conducted using Statistica (Dell Statistica, Tulsa, OK, USA; Figure 3C, 5C, 5E). In all experiments, a $p < 0.05$ was considered to be significant. In Experiments #1-2 and 4-6, data were analyzed with a two-way ANOVA followed by Newman-Keuls comparison within steroid, across diet, and within diet, across steroid. In Experiment #3, data were analyzed with a *t*-test comparing oil and E2B samples within each gene (Figure 4A, 4B). Data for cumulative weight gain and weekly energy intake

were analyzed using a repeated measures, multi-factorial ANOVA (time x diet x steroid) over time, followed by Newman-Keuls's multiple comparison tests.

4.4 Results

4.4.1 Effects of a 24-hour fast on ARC KNDy gene expression and circulating reproductive hormones (Experiment #1)

Acute caloric restriction (a 24 h fast) regulated ARC KNDy gene expression. For *Kiss1* expression, there was a steroid (Figure 2A; $F(1, 20) = 48.10$, $p < 0.0001$) and fasting*steroid ($F(1, 20) = 4.442$, $p < 0.05$) effect. E2B suppressed *Kiss1* in both fed ($p < 0.01$) and fasted ($p < 0.0001$) females and fasting increased *Kiss1* in oil-treated animals ($p < 0.01$). For the receptors, there was a steroid effect on *Kiss1r* expression (Figure 2B; $F(1, 20) = 6.749$, $p < 0.05$), and a fasting effect on *Kor* (Figure 2B; $F(1, 20) = 7.053$, $p < 0.05$). Both *Kiss1r* ($p < 0.05$) and *Kor* ($p < 0.05$) were reduced by fasting in E2B (*Kiss1r)* or oil (*Kor*)-treated females and *Kor* was decreased by E2B only in fed-animals ($p < 0.05$; Figure 2B). *Tac3r* was reduced by E2B in fed ($p < 0.05$) and fasted ($p < 0.01$) animals (Figure 2B; steroid: $F(1, 20) = 20.22$, $p < 0.001$). *Esr1* expression did not change by steroid or fasting, but there was a steroid effect on *Pgr* expression (Figure 2C; $F(1, 20) = 41.69$, $p < 0.0001$), which was augmented by E2B treatment in both fed ($p < 0.001$) and fasted ($p < 0.001$) females. For gonadotropins, there were effects of diet, (Figure 2D; $F(1, 17) = 12.96$, $p < 0.01$), steroid ($F(1, 17) = 12.26$, $p < 0.01$), and diet*steroid ($F(1, 17) = 10.70$, $p < 0.01$) on plasma LH levels. In fed animals, LH levels were significantly decreased by E2B ($p < 0.001$). In addition, fasting decreased LH levels in oil-treated animals ($p < 0.001$). Plasma FSH levels were not changed by steroid or diet treatment (Figure 2E).

There was no significant difference in body weight by diet or steroid among females at sacrifice (Table 2), although fasted females lost 2.7 ± 0.3 g in oil and 2.7 ±

0.2 g in E2B. Uterine weights were elevated by E2B in both fed ($p < 0.001$) and fasted animals ($p < 0.0001$) (Table 2; steroid: $F(1, 20) = 54.58$, $p < 0.0001$). Uterine weight relative to body weight was increased in E2B-treated compared to oil-treated animals in both fed ($p < 0.01$) and fasted ($p < 0.01$) states (Table 2; steroid: $F(1, 20) = 28.63$, $p < 0.0001$). E2 levels were elevated in E2B-treated compared to oil-treated animals, within both fed ($p < 0.05$) and fasted ($p < 0.05$) animals (Table 2; steroid: $F(1, 16) = 7.922$, $p < 0.05$).

4.4.2 Diet-induced obesity effects on body composition and ARC KNDy gene expression (Experiment #2)

DIO affects body composition and ARC gene expression. To measure the changes in body composition, animals were placed into an MRI at the conclusion of the study. For lean mass, there were diet (Figure 3A; $F(1, 28) = 13.34$, $p < 0.01$) and steroid ($F(1, 28) = 20.98$, $p < 0.0001$) effects. Lean mass was higher in LFD-fed than HFD-fed animals, within both oil and E2B treatment ($p < 0.01$, both). For fat mass, there were diet (Figure 3B; $F(1, 28) = 15.17$, $p < 0.001$) and steroid ($F(1, 28) = 22.35$, $p < 0.0001$) effects. Fat mass was lower in LFD-fed than HFD-fed animals within both oil and E2B treatment ($p < 0.01$, both). Cumulative weekly body weight gain was affected by steroid (Figure 3C; $F(1, 36) = 24.524$, $p < 0.0001$), diet ($F(1, 36) = 36.344$, $p < 0.0001$), time ($F(8, 288) = 137.196$, $p < 0.0001$), time*steroid ($F(8, 288) = 14.266$, $p < 0.0001$), and time*diet ($F(8, 288,) = 15.781$, $p < 0.0001$). Beginning on week 4, cumulative body weight gain in HFD oil was greater than in LFD oil animals. From weeks 5-9, body weight gain was higher in HFD-fed vs. LFD-fed, E2B-treated females. Beginning week 7, body weight gain in LFD-fed, E2B-treated females was less than their oil-treated counterparts. Beginning week 5, body weight gain in HFD-fed, E2B-treated females was less than their oil-treated counterparts. There was an effect of steroid (Figure 3D; $F(1, 28) = 11.45$, $p < 0.01$) on feeding efficiency and effect of diet on energy intake (Figure

3E; $F(1, 28) = 69.51$, $p < 0.0001$). Energy intake was higher in HFD-fed females in both steroid treatments ($p < 0.0001$, both).

For KNDy neuropeptide expression, there were diet (Figure 3F; $F(1, 28) = 6.648$, $p < 0.05$) and steroid ($F(1, 28) = 20.78$, $p < 0.0001$) effects on *Kiss1,* which was decreased five-fold by E2B in LFD-fed animals ($p < 0.001$) and by two-fold in HFD-fed, oil-treated animals ($p < 0.01$). *Tac2* decreased by E2B in both LFD ($p < 0.05$) and HFD ($p < 0.05$) fed females (Figure 3F; steroid: $F(1, 28) = 14.01$; $p < 0.001$). For *Kiss1r*, there was a diet effect (Figure 3G; $F(1, 27) = 6.796$, $p < 0.05$) with *Kiss1r* being suppressed two-fold by HFD in E2B-treated females ($p < 0.05$). Similarly, there was a diet effect (Figure 3G; $F(1, 20) = 11.58$, $p < 0.01$) on *Tac3r* expression, which was decreased by HFD in E2B-treated animals ($p < 0.05$). There were no changes in *Pdyn, Kor, Esr1,* or *Pgr* expression.

Within HFD, plasma LH levels were suppressed by E2B ($p < 0.05$; Figure 3I; steroid: $F(1, 31) = 9.398$, $p < 0.01$). There were no differences in plasma FSH levels (Figure 3J). Uterine weight was increased by E2B treatment in both LFD- ($p < 0.0001$) and HFD- ($p < 0.0001$) fed animals (Table 2; steroid: $F(1, 36) = 55.13$, $p < 0.0001$). Uterine weight relative to body weight was increased in E2B- compared to oil-treated animals in both LFD- and HFD-fed animals, as we have previously reported (Yasrebi et al., 2016). Plasma E2 concentrations were elevated in E2B-treated animals, within both diets (Yasrebi et al., 2016).

4.4.3 Estradiol benzoate effects on ARC KNDy gene expression in SW females (Experiment #3)

We next characterized E2B regulation of ARC gene expression in SW mice. *Kiss1* and *Tac2* were reduced by E2B (Figure 4A: $p < 0.01$, both), while *Pgr* expression was increased by E2B (Figure 4A: $p < 0.001$). There were no changes in expression of *Pdyn, Kiss1r, Tac3r, Kor,* and *Esr1.* Animals did not have any differences in body weight.

Uterine weight (Table 2; steroid: $F(1, 28) = 97.33$, $p < 0.0001$) and uterine weight relative to body weight (Table 2; steroid: $F(1, 28) = 157.6$, $p < 0.0001$) were increased by E2B ($p < 0.0001$, both). E2 levels were elevated in E2B-treated females ($p < 0.001$; Table 2; steroid: $F(1, 27) = 18.98$, $p < 0.001$). There was no difference in plasma LH between oil- and E2B-treated females (oil: 1.40 ± 0.33 ng/ml vs. E2B: 2.20 ± 0.45 ng/ml).

4.4.4 Single cell colocalization and Tac2 neuronal gene expression changes by fasting and HFD (Experiments #4-5)

Single *Tac2* neurons were harvested from oil- and E2B-treated females to determine co-expression patterns in individual neurons. E2B decreased colocalization of *Tac2* with *Kiss1* (Figure 4B; $p < 0.001$) and of *Tac2* with *Pdyn* (Figure 4B; $p < 0.05$) and subsequently decreased KNDy co-localization by ~50% ($p < 0.0001$). E2B had no effect on *Tac3r* or *Esr1* but did increase *Pgr* in *Tac2* neurons (Figure 4B; $p < 0.05$). Colocalization patterns are shown in a representative gel (Figure 4C). The KNDy neuropeptides (*Kiss1, Tac2, Pdyn*) are boxed in panels A-C and the representative gels illustrate gene expression colocalization patterns within the same 10 cells. Panels D-F are the receptors examined and are not representative of colocalization patterns within the same 10 cells, but show expression patterns within 10 positive *Tac2* neurons. We did not detect *Kor* or *Kiss1r* in single *Tac2* neurons or pools of 5 *Tac2* neurons.

To determine if fasting regulates receptor gene expression in *Tac2* neurons similar to ARC, Experiment #1 was repeated and pools of 5 *Tac2* neurons were harvested to use for qPCR on the following genes: *Tac2, Tac3r, Esr1,* and *Pgr*. *Tac2* was decreased by E2B in fed-animals ($p < 0.05$), with a similar trend in fasted animals (Figure 4D; steroid: $F(1, 20) = 14.09$, $p < 0.01$). Progesterone receptor, *Pgr* was increased by E2B three-fold in both fed ($p < 0.01$) and fasted ($p < 0.001$) animals (Figure 4D; steroid: $F(1, 60) = 25.83$, $p < 0.0001$). There were no changes in *Tac3r* or *Esr1.* Uterine weights were increased by E2B (Table 2; $F(1, 19) = 73.93$, $p < 0.0001$) in fed (p

< 0.0001) and fasted ($p < 0.0001$) females. E2B increased uterine weight relative to body weight in fed ($p < 0.001$) and fasted ($p < 0.001$) females (Table 2; steroid: $F(1, 19) = 43.30$, $p < 0.0001$).

To determine effect of DIO on *Tac2* neurons, Experiment #2 was repeated in *Tac2*-EGFP animals and pools of 5 neurons were collected. There was no difference in cumulative body weight gain across treatments, but there was a general trend of E2B-treated animals gaining less than their oil-treated controls, within LFD and HFD (Figure 4E). *Tac2* ($p < 0.01$) was decreased ~75% by E2B in HFD fed animals (Figure 4F; steroid: $F(1, 20) = 14.77$, $p < 0.01$). *Pgr* was augmented by E2B in both diet treatments (Figure 4F; LFD: $p < 0.001$; HFD: $p < 0.0001$; steroid: $F(1, 61) = 46.41$, $p < 0.001$). There was no difference in body weight at sacrifice, unlike in C57/BL animals from Experiment #2 (Table 2). This is potentially due to differences in the response to DIO (HFD) between C57/BL and SW with C57/BL being more susceptible to DIO (Winzell and Ahren, 2004). E2 levels were elevated in E2B-treated females ($p < 0.05$; Table 2; steroid: $F(1, 24) = 10.85$, $p < 0.01$). Finally, there were steroid effects on both uterine weight (Table 2; $F(1, 27) = 217.5$, $p < 0.0001$) and uterine weight relative to body weight (Table 2; $F(1, 27) = 257.0$, $p < 0.0001$), with E2 increasing both, in LFD and HFD females ($p < 0.0001$; all).

4.4.5 Caloric restriction effects on body composition and ARC KNDy gene expression (Experiment #6)

To determine the effects of chronic caloric restriction on body composition and KNDy-associated gene expression, ovx females were orally dosed with oil or E2B and separated into *ad libitum* and 30% CR groups. Using an MRI, there were no changes in lean mass between pre- and post-treatment, within steroid or diet. In addition, there were steroid (Figure 5B; $F(1, 28) = 18.64$, $p < 0.001$), diet ($F(1, 28) = 8.25$, $p < 0.01$), and time*diet ($F(1, 28) = 38.89$, $p < 0.0001$) effects on percent fat mass, which decreased in oil CR animals ($p < 0.01$). Cumulative body weight gain was measured; animals were fed

ad lib for the first 4 weeks before they were maintained on *ad lib* or changed to CR. For the initial 5 weeks, there was a steroid (Figure 5C; $F(1, 20) = 31.09$, $p < 0.0001$), time ($F(4, 120) = 37.818$, $p < 0.0001$) and steroid*time ($F(4, 120) = 6.457$, $p < 0.0001$) effect on cumulative body weight gain. During these initial weeks, oil-treated females gained more weight than E2B-treated females from week 2-5. Following diet change, there was a steroid (Figure 5C; $F(1, 27) = 14.650$, $p < 0.001$), diet ($F(1, 27) = 77.898$, $p < 0.0001$), time ($F(5, 135) = 12.599$, $p < 0.0001$), and time*diet ($F(5, 135) = 45.023$, $p < 00001$) effect on cumulative body weight gain. Both CR groups (oil- and E2B-treated) lost body weight compared to *ad lib* animals in weeks 7-10. From week 6-8, there was a decrease in cumulative body weight gain in E2B CR animals compared to oil CR animals.

Feeding efficiency (g gained/kCal consumed) was also affected by CR with diet (Figure 5D; $F(1, 58) = 27.79$, $p < 0.0001$), time ($F(1, 58) = 40.07$, $p < 0.0001$), and time*diet ($F(1, 58) = 22.83$, $p < 0.0001$) effects. Feeding efficiency decreased in CR groups within both oil- ($p < 0.0001$) and E2B-treated ($p < 0.001$) females. For energy intake, there were steroid (Figure 5E; $F(1, 10) = 72.302$, $p < 0.0001$), diet ($F(1, 10) = 251.131$, $p < 0.0001$), time ($F(4, 40) = 8.905$, $p < 0.0001$), and time*steroid ($F(4, 40) = 9.492$, $p < 0.0001$) effects within weeks 6-9. Energy intake decreased during weeks 6-9 by CR in E2B-treated females. Average energy intake also was effected by steroid (Figure 5E; $F(1, 57) = 30.73$, $p < 0.0001$), diet ($F(1, 57) = 61.72$, $p < 0.0001$), steroid*diet ($F(1, 57) = 8.61$, $p < 0.01$), time ($F(1, 57) = 146.6$, $p < 0.0001$), and time*diet ($F(1, 37) = 59.32$, $p < 0.0001$). Average energy intake in CR females was lower in post-CR compared to pre-CR in both oil- ($p < 0.0001$) and E2B- ($p < 0.0001$) treated animals (Figure 5F; steroid: $F(3, 132) = 46.11$, $p < 0.0001$; time: $F(1, 132) = 174.7$, $p < 0.0001$; steroid*time: $F(3, 132) = 27.77$, $p < 0.0001$).

All relative mRNA expression were normalized to oil *ad lib* animals within each gene. For *Kiss1*, there was an effect of diet (Figure 5G; $F(1, 28) = 42.26$, $p < 0.0001$),

steroid ($F(1, 28) = 132.1$, $p < 0.0001$), and diet*steroid ($F(1, 28) = 32.39$, $p < 0.0001$) and *Kiss1 was* decreased by E2B (*ad lib:* $p < 0.0001$, CR: $p < 0.001$) and by CR, in oil-treated animals ($p < 0.0001$). For *Tac2* expression, there was an effect of diet (Figure 5G; $F(1, 28) = 16.60$, $p < 0.001$), steroid ($F(1, 28) = 46.31$, $p < 0.0001$), and diet*steroid ($F(1, 28) = 11.55$, $p < 0.01$). *Tac2* expression was suppressed by E2B in *ad lib* animals ($p < 0.0001$) and by CR in oil-treated females ($p < 0.0001$). For *Pdyn*, there was a diet (Figure 5G; $F(1, 28) = 10.40$, $p < 0.01$), steroid ($F(1, 28) = 7.118$, $p < 0.05$), and diet*steroid ($F(1, 28) = 42.12$, $p < 0.0001$) effect. *Pdyn* was suppressed by E2B in *ad lib* animals ($p < 0.0001$) and by CR in oil-treated females ($p < 0.0001$) and increased by both E2B in CR animals ($p < 0.05$) and by CR in E2B-treated animals ($p < 0.05$).

For the KNDy receptors, there was a diet*steroid effect on *Kiss1r* expression (Figure 5H; $F(1, 28) = 12.58$, $p < 0.01$), which was augmented by CR in oil-treated animals ($p < 0.01$) and decreased by E2B in CR animals ($p < 0.05$). There were no other changes observed in KNDy-associated receptors. *Pgr* was increased by E2B (Figure 5I; *ad lib:* $p < 0.001$, CR: $p < 0.05$; steroid: $F(1, 28) = 23.61$, $p < 0.0001$), with no change observed for *Esr1.* Because we did not observe any effects of energy states on the receptors, we did not repeat the chronic caloric restriction experiment in pooled *Tac2* neurons.

For the gonadotropins, there were diet (Fig 5J; $F(1, 28) = 10.10$, < 0.01), steroid ($F(1, 28) = 56.44$, $p < 0.0001$), and diet*steroid ($F(1, 28) = 13.78$, $p < 0.001$) effects on plasma LH levels. LH levels were increased by CR in oil-treated animals ($p < 0.0001$) and by E2B in *ad lib* ($p < 0.05$) and CR ($p < 0.0001$) animals. Within CR animals, FSH levels were decreased by E2B ($p < 0.05$) compared to oil (Figure 5K; diet*steroid: $F(1, 28) = 5.218$, $p < 0.05$). For uterine weights, there were diet (Table 2; $F(1, 28) = 44.69$, $p < 0.0001$), steroid ($F(1, 28) = 232.4$, $p < 0.001$), and diet*steroid ($F(1, 28) = 38.40$, $p < 0.0001$) effects. Uterine weight were decreased by CR in E2B-treated females ($p <$

0.0001) and increased by E2B in both *ad lib* and CR females ($p < 0.0001$, both). There were diet (Table 2; $F(1, 28) = 15.92$, $p < 0.001$), steroid ($F(1, 28) = 256.9$, $p < 0.0001$), and diet*steroid ($F(1, 28) = 14.03$) effects on uterine weights/body weight (mg/g). E2B increased uterine weights/body weight in both *ad lib* ($p < 0.0001$) and CR ($p < 0.0001$) groups and decreased by CR within E2B treated animals ($p < 00001$). E2 levels were affected by diet (Table 2; $F(1,24) = 12.54$, $p < 0.01$), steroid ($F(1, 24) = 23.01$, $p < 0.0001$), and diet*steroid ($F(1, 24) = 12.79$, $p < 0.01$). E2 were elevated by E2B in *ad lib*-fed animals only ($p < 0.0001$) and were reduced in E2B CR animals compared to E2B *ad lib* animals ($p < 0.0001$).

4.4.6 Expression of genes involved in the ghrelin signaling cascade in Tac2 neurons

To determine if ghrelin signaling is involved in *Tac2* neurons, we selected key genes involved in the ghrelin signaling cascade to conduct single cell PCR and qPCR analysis. Activation of GHSR initiates the CaMKK-AMPK-UCP2 pathway, which upregulates carnitine palmitoyl transferase 1 (CPT1) to increase fatty acid oxidation. Subsequently, uncoupling protein 2 (UCP2), which is involved in decreasing reactive oxygen species (ROS) that are produced during oxidation, is activated (Andrews, 2011). Individual *Tac2* neurons were harvested and single cell PCR was conducted to determine expression of the ghrelin's receptor, growth hormone secretagogue receptor (*Ghsr)*. In Figure 6A, there was a nearly ~55% increase in colocalization of *Tac2* with *Ghsr* by E2B with products confirmed through gel electrophoresis (Figure 6B). To determine if ghrelin signaling genes are changed in fed-fasted and DIO animals, we conducted qPCR on *Tac2* pooled cells collected in Experiments #4 and #5. In the fed-fasted experiment, there were no changes in gene expression of *Ghsr, Ucp2,* or *Cpt1c* by fasting (Figure 6C). In *Tac2* pools, *Ghsr* expression was augmented by 6- to 8-fold in E2B-treated females in both fed ($p < 0.01$) and fasted ($p < 0.01$) groups (steroid: $F(1, 20)$

= 5.948, $p < 0.05$). In diet-induced obesity animals, there were no change in any ghrelin signaling gene by steroid or diet (Figure 6D).

4.5 Discussion

Disturbances in energy balance (positive and negative) are linked to reproductive problems, though the mechanisms are unclear. For example, hypogonadism secondary to obesity is common, with bariatric surgery correcting a significant percentage of infertility (Calderon et al., 2015,Milone et al., 2015). Similarly, patients with eating disorders have increased spontaneous abortions and miscarriages (Linna et al., 2013). In the present study using a mouse model, we show that there are disruptions in KNDy neuropeptide gene expression, both in the ARC and in *Tac2* neuronal pools, and in KNDy receptor gene expression in the ARC by negative (anorexia) and positive (obesity) energy balance. The steroid receptors, ERα and PR, were impervious to any change in energy balance. A summary of the differential regulation of KNDy-associated neuropeptides and receptors is presented in Table 3.

Disruption of energy balance may challenge E2 and P4 actions in the hypothalamus and disrupt reproduction. Previously, kisspeptin has been identified as a key regulator in reproduction and energy balance (Goodman et al., 2013,Mittelman-Smith et al., 2012,Uenoyama et al., 2014), but these studies focused primarily on AVPV kisspeptin. Of those studies that examined KNDy neurons, the effects of these physiological states on KNDy receptors and the steroid receptors, ERα and PR, which mediate negative feedback on the HPG axis (Eghlidi et al., 2010,Lehman et al., 2010), were not examined. NKB *(Tac2)* acts as a positive autoregulator to kisspeptin production through the NKB receptor (*Tac3r*) (Lehman et al., 2010) and dynorphin (DYN/*Pdyn*) is thought to act as a negative autoregulator, via an unidentified interneuronal network,

through the κ-opioid receptor (KOR/*Kor*) (de Croft et al., 2013,Lehman et al., 2010,Ruka et al., 2016,Weems et al., 2016) Therefore, ERα, PR, and the KNDy-associated receptors, *Kiss1r, Tac3r,* and *Kor,* are essential players in the pulse generator and are potential targets for negative and positive energy balance.

4.5.1 The effects of energy deficiency in the ARC

Previous studies suggest that energy deficiency leads to decreased kisspeptin gene expression (Castellano et al., 2005,Kalamatianos et al., 2008,Luque et al., 2007,Matsuzaki et al., 2011,Polkowska et al., 2015,Roa et al., 2009,True et al., 2011,Wahab et al., 2008). In the present study, a 24 h fast increased ARC *Kiss1* and decreased *Kor* in oil-treated females and decreased *Kiss1r* in E2B-treated females. Chronic caloric restriction decreased expression of all KNDy neuropeptides and increased *Kiss1r* in the ARC of oil-treated females. Clearly, *Kiss1* expression is suppressed by negative energy balance states. However, many of the previous studies do not consider the regulatory role of E2 in modulating kisspeptin regulation, which could explain differences between our results for fasting and previous experiments.

The differences in KNDy neuropeptide and receptor gene expression between a 24 h fast and 30% CR suggest that the endocrine and neurological mechanisms controlling gene expression differ between acute and chronic negative energy balance and that the duration of caloric restriction and severity are important. Two factors may influence the apparent duration-dependent differences: age and change in body weight. Previous studies report that in the pubertal female rat ARC, kisspeptin-IR neurons decrease by a 48 h fast, but not in adult females. In the same study, change in body weight (a body weight reduction of 24% pubertal vs. 12% adult) was proposed to be important (Castellano et al., 2010). In our study, females lost <10% of body weight after a 24 h fast and lost ~20% of body weight in the CR experiment (after Week 5). In

addition, the decreases in body weight are due to changes in lean vs. fat mass. In our study, CR reduced overall fat mass and did not change lean mass in oil-treated females. These changes in body weight composition may dictate kisspeptin gene expression independent of leptin (True et al., 2011). Differences in energy deficiency (undernutrition vs. elevated energy expenditure) are also important to consider; however, in our present study, we did not examine energy expenditure in the 24 h fast or the 30% CR. Collectively, considering the importance of KNDy neurons on GnRH pulsatility (Goodman et al., 2013), these data expand on our understanding of the impact of anorexia, cachexia, and other states of negative energy homeostasis on the neuroendocrine control of reproduction.

Another interesting finding in our study is the differential regulation of *Kiss1* by fasting (increased) and CR (decreased) in oil-treated females, which corresponds to differential regulation of plasma LH levels by fasting (decreased) and CR (increased). These differences between the acute and chronic energy deficiency extend to the other KNDy neuropeptides, *Tac2* and *Pdyn*, which were decreased by CR and not altered after 24 h fasting, and to *Kiss1r* expression (reduced by a 24 h fast and augmented by 30% CR). While the main function of *Kiss1r* is the activation of GnRH neurons to regulate the HPG axis, recent studies have characterized *Kiss1r* expression in other ARC neurons (Fu and van den Pol, 2010,Higo et al., 2016). One function of non-GnRH *Kiss1r* is the control of feeding behavior through the direct excitation of ARC POMC neurons by kisspeptin (Fu and van den Pol, 2010). Perhaps, a suppression of *Kiss1r* in POMC neurons would reduce the *Kiss1*-induced activation of POMC and thus increase feeding in fasting animals.

The interaction of E2 and caloric restriction on the KNDy circuitry and HPG disruption are illustrated in Figure 7. In E2B-treated animals, fasting does not augment KNDy-associated gene expression with the exception of *Kiss1r* (Fig. 7). During chronic

restriction with E2, expression of all of the KNDy genes and *Kiss1r* are altered leading to a suppression of both LH and FSH. Suppression of *Kiss1* and *Tac2* (trending suppression of *Tac2*) and augmentation of *Dyn* expression potentially represses the HPG axis by blunting the pulse generator and reducing the regulation of GnRH neuronal excitability and pulsatility. Our data suggest that chronic CR leads to a total disruption of the HPG axis, which would slow folliculogenesis and block ovulation (similar to amenorrhea).

4.5.2 The effects of diet-induced obesity in the ARC

The obesity epidemic has fueled the need to study reproductive problems in obese populations. Factors such as inflammation, leptin, and E2 may contribute to the dysregulation of the hypothalamic KISS1 system in obese human males (George et al., 2010). This hypothesis has been supported by rodent studies, which suggest that positive energy balance decreases hypothalamic *Kiss1* mRNA (Castellano et al., 2006,Iwasa et al., 2015). *Kiss1* is also expressed in peripheral tissues (Dudek et al., 2016) and its expression is regulated by positive energy balance in the ovary (Zhou et al., 2014) and the testes (Dudek et al., 2016). In our study, DIO decreased *Kiss1* in oil-treated females and *Kiss1r* in E2B-treated females in the ARC, which did not correlate with changes in either LH nor FSH plasma levels. Therefore, steroid treatement is important to consider when evaluating steroid hormone feedback under the influence of diet or energy state.

We hypothesize that chronic DIO leads to a major disruption in the HPG axis (Figure 7). Collectively, the decrease in *Kiss1, Tac2*, *Kiss1r,* and *Tac3r* expression in E2B-treated females by DIO reduces positive autoregulatory function of *Tac2* and the activity of the pulse generator. Subsequently, negative feedback of E2 on the KNDy system is augmented, decreasing LH output. Suppression of LH will inhibit the late stages of folliculogenesis and ovulation, compromising reproduction in females.

Nonetheless, it is important to note that our studies are conducted on ovx females supplemented with E2B. Future studies will be expanded by examining changes in energy balance in intact, cycling females.

It is unclear what central or peripheral mechanisms regulate this interaction between positive energy balance and KNDy neurons. One potential peripheral signal is leptin, which is produced by adipocyties. Leptin receptor, LepR, is expressed in >40% of ARC *Kiss1* neurons (Hill et al., 2008), and the decrease in ARC KiSS-1 in male *ob/ob* mice is restored by leptin (Smith et al., 2006). In both oil- and E2B-treated animals, DIO increased fat mass; thus, the increase in leptin production by excess adipose tissue may be important in the disruption of KNDy neuronal functions including neuropeptide and receptor expression, unlike the *Kiss1* disruption associated with caloric restriction. In our analyses, we did not consider differences across the interaction of steroid and diet (that is, differences between LFD oil vs. HFD E2B and LFD E2B vs. HFD oil). Interestingly, body composition between these groups are similar, while the effects on gene expression are distinct, suggesting that body composition may be important only to a degree in regulation of gene expression.

4.5.3 The effects of steroid and energy balance on Tac2 neurons

To confirm that ARC KNDy neuropeptide and receptor gene expression changes by negative and positive energy balance occur at the single cell level, we harvested single *Tac2* neurons for single cell PCR amplification and pools of *Tac2* neurons. First, to determine if there are strain differences between C57/BL and SW (*Tac2*-EGFP) females, we repeated Experiment #1 in the SW wild-type females. As expected, *Kiss1* decreased with E2B, while *Pgr* increased with E2B, which has been previously reported (Bosch et al., 2012,Frazao et al., 2014,Gottsch et al., 2009,Zuloaga et al., 2012). Due to the availability of transgenic animals, mice used in the ARC studies (C57/BL) and the

single cell studies (SW) are of a different strain, which is important factor to consider in interpreting our data.

To determine coexpression patterns in *Tac2* neurons, we conducted single cell amplification of harvested *Tac2* cells. Coexpression of the KNDy neuropeptides decreases with E2B administration. Similar coexpression patterns exist in the male mice, as testosterone has been shown to decrease coexpression of KNDy neuropeptides (Navarro et al., 2011,Ruka et al., 2013). These studies report between 80-90% colocalization of both *Kiss1* with *Pdyn* or *Tac2* (gonadoectomized males) and of *Tac2* with *Kiss1* or *Pdyn* (intact males) (Navarro et al., 2011,Ruka et al., 2013). The similar colocalization percentages between our study in female *Tac2*-EGFP mice and in previous studies using both *Tac2*-EGFP and *Kiss1*-creGFP male mice suggests that the main driver underlying KNDy coexpression is gonadal steroids suppressing *Kiss1* expression.

To determine if fasting or DIO had any effects on *Tac3r* or the steroid receptors specifically in KNDy neurons, we repeated those experiments and collected pools of *Tac2* neurons. Like in the ARC, there were no changes in *Tac3r* or steroid receptor gene expression caused by fasting or DIO in *Tac2* neurons. However, *Pgr* expression in *Tac2* neurons was increased by E2B, regardless of energy state. Therefore, the increase in ARC *Pgr* expression by E2B is due, in part, to the striking increase in KNDy *Pgr* expression. These data also suggest that E2's priming of KNDy neurons for the negative feedback of P4 during the transition of pro/estrus is not disrupted by changes in energy balance (Eghlidi and Urbanski, 2015).

4.5.4 Estradiol benzoate increases Ghsr in Tac2 neurons

To date, the hormonal mechanisms connecting energy balance and KNDy neurons are largly uncharacterized, although peripheral peptide hormones have been considered as candidates regulating these changes (True et al., 2011). We hypothesize

that another brain-gut peptide, ghrelin, may be involved in mediating the interaction of energy balance and KNDy-associated reproduction. Ghrelin is a stomach-derived hormone that increases feeding behavior, weight gain, and other aspects of energy homeostasis. In the ARC, ghrelin is thought to directly stimulate NPY neurons and indirectly suppress POMC neurons through inhibitory GABAergic inputs (Andrews, 2011). Ghrelin also controls reproduction by suppressing LH pulse frequency (Forbes et al., 2009). There are few studies examining ghrelin signaling in KNDy neurons. In one recent study, ghrelin depolarized ARC kisspetin (KNDy) neurons in an E2-dependent manner with these neurons being more sensitive to ghrelin from ovx+E2 females (Frazao et al., 2014). Nonetheless, the interaction of ghrelin, E2, and KNDy neurons is largely unexplored.

In our study, E2B increased the percentage of *Ghsr*-expressing *Tac2* neurons and increased *Ghsr* expression in *Tac2* pools by 6-fold, regardless of fed state. We have previously shown that in both fed and fasted females, E2B increases *Ghsr* expression in the ARC, but not in NPY neurons (Yasrebi et al., 2016). Therefore, the E2B-induced *Ghsr* expression in the ARC is due, in large part, to *Ghsr* expression in *Tac2* neurons. Unlike in the ARC or in NPY neurons (Yasrebi et al., 2016), there were no changes in genes of the ghrelin signaling cascade by fasting or by DIO in *Tac2* neurons. We hypothesize that E2 augments ghrelin sensitivity in KNDy neurons in its role as an anorectic steroid in females. We formulate this hypothesis because ablation of KNDy neurons suppressed post-ovariectomy body weight gain and its attenuation by E2 replacement (Mittelman-Smith et al., 2012) and because KNDy neurons simultaneously excite POMC neurons and suppress NPY/AgRP neurons through both kisspeptin (Fu and van den Pol, 2010) and the pluripotent actions of glutamate (Nestor et al., 2016). By increasing *Ghsr* expression specifically in KNDy neurons and thus their sensitivity to either circulating or local ghrelin, E2 indirectly augments the activity of these neurons,

which eventually leads to a suppression of food intake in females and counteracts the effects of ghrelin on feeding.

4.6 Conclusion

Collectively, our data suggest that negative (24 h fast and 30% CR) and positive (DIO) states of energy balance differentially impact the expression of ARC KNDy neuropeptides and their receptors to alter the activity of the HPG axis. While the link between energy balance and reproduction is not clear, we demonstrate that E2 can both augment and oppose the effects of positive or negative energy states on KNDy neuropeptides and receptors, potentially leading to a disruption to the HPG axis. We see a progression in severity of HPG disruption from acute fasting to chronic DIO and CR. These disruptions in HPG disruption are elucidated through changes in LH and FSH levels, which may produce downstream problems with reproductive functions. Furthermore, the E2-induced increase in *Ghsr* expression in *Tac2* neurons suggests that steroid and peripheral peptides interact in the ARC to control both energy balance and reproduction. Future experiments will use electrophysiology to examine the E2B-regulated increase in *Ghsr* expression in *Tac2* neurons. In addition, it is important to note that peripheral tissues express *Kiss1* and *Kiss1r* suggesting that the interaction of central and peripheral signals is crucial to understanding this complicated system (Dudek et al., 2016,Song et al., 2014).

Infertility due to poor or excess nutrition may continue to worsen with the rise of the obesity epidemic. According to a study published by the Centers for Disease Control (CDC) in 2013, 6% of women and 12% of men are infertile (Chandra et al., 2013). Hypothalamic amenorrhea is common in both underweight and overweight females. While new technologies are making progress in addressing the number of infertile individuals, it is imperative to consider additional mechanisms of action that impact

reproduction. Multiple factors lead to reproductive problems including genetics, which are equally important in the regulation of energy homeostasis. For many individuals experiencing infertility problems, energy balance dysfunction may be an underlying factor that is unexplored and not considered when evaluating potential solutions. Therefore, understanding the effects and causal mechanisms are critical to the development of reproductive therapy.

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Tables

Table 1. Primer sequences for qPCR and single cell PCR

Gene Name	Product length	% Eff	Primer sequence	Base pair #	Accession #
Kiss1	154	91	F: TGATCTCAATGGCTTCTTGGCAGC	40-63	NM_178260
			R: CTCTCTGCATACCGCGATTCCTTT	170-193	
Kiss1r	138	100	F: CCTTCACCGCACTCCTCTAC	1993-2012	NM_03244
			R: CATACCAGCGGTCCACACTC	2111-2130	
Pdyn	133	105	F: AGCTTGCCTCCTCGTGATG	335-353	NM_018863
			R: GGCACTCCAGGGAGCAAAT	441-459	
Pgr	191	104	F: TGAAAGAGCGTCATTCTTAC	2980-2999	NM_008829
			R: CAATTCGCGGATATAGCTTG	3151-3170	
Tac2	220	103	F: CGTGACATGCACGACTTC	505-522	NM_001199971
			R: CCAACAGGAGGACCTTAC	707-724	
Tac3r	124	99	F: TACACCATCGTTGGAATTAC	1026-1045	NM_021382
			R: ATGTCACCACCACAATAATC	1130-1149	
ERα	107	96	F: GCGCAAGTGTTACGAAGTG	919-937	NM_007956
			R: TTCGGCCTTCCAAGTCATC	1007-1025	
β-actin	63	100	F: GCCCTGAGGCTCTTTTCCA	849–867	NM_007393
			R: TAGTTTCATGGATGCCACAGGA	890-911	
Hprt	85	117	F: GCAGTACAGCCCCAAAATGG	599-618	NM_013556
			R: AACAAAGTCTGGCCTGTATCCA	662-683	
Gapdh	98	93	F:TGACGTGCCGCCTGGAGAAA	778-797	NM_008084.2
			R:AGTGTAGCCCAAGATGCCCTTCAG	852-875	
Kor	237	110	F: TCCTTGGAGGCACCAAAGTCAGGG	799-822	NM_001204371
			R: TGGTGATGCGGCGGAGATTTCG	1014-1035	
Ghsr	122	111	F: CAGGGACCAGAACCACAAAC	1003-1022	NM_177330
			R: AGCCAGGCTCGAAAGACT	1107-1124	
Ucp2	194	105	F: CATTGGCCTCTACGACTC	668-685	NM_011671
			R: CGACAGTGCTCTGGTATC	844-861	
Cpt1c	191	96	F: GGCTGGCATTGGTCAGAATC	719-738	NM_153679.2
			R: CGTGCAACCTCAGGAAGTC	892-910	

Forward primer (F) is listed first with the reverse primer (R) second. *Kiss1,* kisspeptin; *Kiss1r,* kisspeptin receptor; *Pdyn,* prodynorphin; *Pgr*, progesterone receptor; *Tac2,* tachykinin 2; *Tac3r,* tachykinin 3 receptor; *ERα*, estrogen receptor alpha; *β-actin,* beta-actin; *Hprt,* hypoxanthine-guanine phosphoribosyltransferase; *Gapdh,* glyceraldehyde-3-phosphate dehydrogenase; *Kor,* kappa opioid receptor; *Ghsr,* growth hormone secretagogue receptor; *Ucp2,* uncoupling protein 2*; Cpt1c,* carnitine palmitoyltransferase 1c.

Table 2. Summary of animal body and uterine weights and serum E2 levels

Exp #	Strain	Treatment	Body weight (g) at sacrifice	Uterine weight (mg)	Uterine weight (mg)/Body weight (g)	E2 (pg/µl)
1	C57/BL	Oil fed	23.2 ± 0.5	30.4 ± 1.8	1.3 ± 0.1	12.3 ± 1.5
		E2B fed	24.4 ± 0.3	114.6 ± 8.0 **c**	4.7 ± 0.4 **b**	32.8 ± 6.8 **a**
		Oil fasted	20.4 ± 1.4	39.5 ± 10.9	2.1 ± 0.8	11.7 ± 1.0
		E2B fasted	21.6 ± 0.7	134.9 ± 20.1 **d**	6.4 ± 1.2 **b**	86.5 ± 42.2 **a**
2	C57/BL	LFD oil	28.2 ± 1.4	11.7 ± 1.2	0.4 ± 0.0	10.0 ± 0.4
		LFD E2B	25.3 ± 0.8	108.5 ± 24.6 **d**	4.3 ± 1.0 **d**	22.6 ± 1.5 **a**
		HFD oil	36.6 ± 1.6 **B**	14.6 ± 2.1	0.4 ± 0.1	11.1 ± 1.1
		HFD E2B	31.7 ± 2.0 **a A**	119.6 ± 11.3 **d**	3.9 ± 0.5 **d**	25.0 ± 5.9 **a**
3	Swiss Webster	Oil	41.0 ± 2.2	55.7 ± 3.9	1.4 ± 0.2	6.6 ± 0.7
		E2B	44.0 ± 2.5	146.2 ± 11.2 **d**	3.3 ± 0.2 **d**	36.4 ± 7.9 **c**
4	Swiss Webster	Oil fed	36.8 ± 2.7	81.4 ± 3.9	2.2 ± 0.1	3.7 ± 0.5
		E2B fed	40.7 ± 3.2	105.8 ± 22.7 **d**	2.6 ± 0.9 **c**	37.8 ± 5.4 **d**
		Oil fasted	38.0 ± 3.9	76.5 ± 5.9	2.0 ± 0.1	3.5 ± 0.3
		E2B fasted	40.7 ± 4.8	119.7 ± 15.1 **d**	2.9 ± 0.3 **c**	24.4 ± 6.2 **b A**
5	Swiss Webster	LFD oil	37.5 ± 1.5	16.8 ± 1.6	0.5 ± 0.0	2.3 ± 0.3
		LFD E2B	33.3 ± 1.0	177.4 ± 13.6 **d**	5.4 ± 0.4 **d**	26.3 ± 6.0 **a**
		HFD oil	38.4 ± 1.7	23.6 ± 3.8	0.6 ± 0.1	1.8 ± 0.3
		HFD E2B	36.4 ± 1.6	187.3 ± 17.2 **d**	5.2 ± 0.44 **d**	18.7 ± 5.6 **a**
6	Swiss Webster	Oil *ad lib*	38.2 ± 1.4	24.1 ± 1.6	0.6 ± 0.0	3.9 ± 0.2
		E2B *ad lib*	40.0 ± 2.1	239.8 ± 16.2 **d**	6.0 ± 0.3 **d**	224.8 ± 41.0 **d**
		Oil CR	33.8 ± 0.8 **A**	19.2 ± 1.6	0.6 ± 0.0	4.8 ± 0.4
		E2B CR	28.7 ± 1.3 **a D**	110.2 ± 11.7 **d D**	3.9 ± 0.5 **d D**	37.0 ± 19.1 **D**

Data were analyzed differently in different experiments based on experimental paradigm. See materials and methods for comprehensive statistical method outline. Lowercase letters signify differences between oil- vs. E2B-treated females, within the same treatment (different according to experiment: time of sacrifice, energy balance state, diet). Uppercase letters signify differences within steroid, across treatment. (a/A = $p < 0.05$; b/B = $p < 0.01$; c/C = $p < 0.001$; d/D = $p < 0.0001$)

Table 3. Summary table of KNDy-associated gene expression changes in ARC and *Tac2* pools.

Gene Name	Fed vs. Fasted* Exp. #1 (ARC) & 4 (pools)				LFD vs. HFD* Exp. #2 (ARC) & 5 (pools)				SW Oil vs. + E2B	Ad lib vs. CR Exp. #6			
	Fed vs. Fasted		Oil vs. E2B		LFD vs. HFD		Oil vs. E2B			Ad lib vs. CR		Oil vs. E2B	
	Oil	E2B	Fed	Fasted	Oil	E2B	LFD	HFD	Exp. #3	Oil	E2B	Ad lib	CR
Kiss1	↑ ARC		↓ ARC	↓ ARC	↓ ARC		↓ ARC		↓ ARC	↓ ARC		↓ ARC	↓ ARC
*Tac2**			↓ pools				↓ ARC	↓ ARC ↓ pools	↓ ARC	↓ ARC		↓ ARC	
Pdyn										↓ ARC	↑ ARC	↓ ARC	↑ ARC
Kiss1r		↓ ARC				↓ ARC				↑ ARC			↓ ARC
*Tac3r**			↓ ARC	↓ ARC		↓ ARC							
Kor	↓ ARC		↓ ARC										
*Esr1**													
*Pgr**			↑ ARC ↑ pools	↑ ARC ↑ pools			↑ pools	↑ pools	↑ ARC			↑ ARC	↑ ARC
Figure	**ARC: Figure 2; pools: Figure 4D**				**ARC: Figure 3; pools: Figure 4F**				**Figure 4A**	**Figure 5**			

Experiments and genes with a * indicate qPCR analysis of ARC and *Tac2* pools. Experiments without * were only analyzed in ARC tissue. For all experiments except oil vs E2B (+), data were analyzed with using a two-way ANOVA followed by post-hoc

Newman-Keuls. For oil vs. E2B experiment, data were analyzed with a Student's t-test Corresponding graphs of each gene expression change is noted in the "Figure" row. Arrows indicate the direction of gene expression (↑= upregulated and ↓ = downregulated). For example, in the ARC, *Kiss1* is increased by fasting in oil-treated animals (Fig. 2A) and decreased by E2B in both fed ans fasted animals (Fig. 2A).

Figures

Figure 1. Experimental design timelines. Experimental numbers correspond to Table 2 experimental numbers as well as those written in the text. Abbreviations: ovx, ovariectomy; LFD, low fat diet; HFD, high fat diet; CR, calorie restricted

Figure 2. Fasting (24 h) regulates KNDy-associated gene expression in the ARC (Experiment #1). (A) KNDy genes: *Kiss1, Tac2,* and *Pdyn*; (B) KNDy-associated receptors: *Kiss1r, Tac3r,* and *Kor;* and (C) steroid hormone receptors: *Esr1* and *Pgr.* D) Plasma LH levels and (E) Plasma FSH levels. For all, n = 5 - 6. Gene expression data were normalized to oil fed controls for each gene. All data were analyzed using a two-way ANOVA (fasting x steroid) followed by post-hoc Newman-Keuls analysis (a = $p < 0.05$; b = $p < 0.01$; c = $p < 0.001$; d = $p < 0.0001$).

Figure 3. Diet-induced obesity disrupts KNDy-associated gene expression in the ARC (Experiment #2). (A) Total lean mass percentage. (B) Total fat mass percentage. (C) Cumulative body weight gain. (D) Feeding efficiency. (E) Weekly energy intake (kCal). (F) KNDy genes: *Kiss1, Tac2,* and *Pdyn*; (G) KNDy-associated receptors: *Kiss1r, Tac3r,* and *Kor*; and (H) Steroid hormone receptors: *Esr1* and *Pgr.* Gene expression data were normalized to ND-oil controls. (I) Plasma LH and (J) FSH levels. For all data, n = 8 – 10. (A-B, D-J) data were analyzed using a two-way ANOVA. (C) Data were analyzed by a repeated-measures, multifactorial ANOVA. All *post hoc* comparisons were Newman-Keuls multiple comparison tests. For (A-B), (D-J), a = $p < 0.05$; b = $p < 0.01$; c = $p < 0.001$; d = $p < 0.0001$). For (C), lowercase letters denote differences between steroid, within diet and uppercase letters denote differences between diet, within steroid (a/A = $p < 0.05$; b/B = $p < 0.01$; c/C= $p < 0.001$; d/D = $p < 0.0001$).

Figure 4. Differential gene expression in *Tac2* neurons by E2B, fasting, and diet-induced obesity (Experiments #3-5). Figure 4A-C: Experiment #3; Figure 4D: Experiment #4; Figure 4E-F: Experiment #5. (A) ARC KNDy neuropeptide and receptor gene expression in Swiss Webster females (n = 5 – 8). Data were normalized to oil controls, within each gene. Data were analyzed with Student's t-test for each gene. (B) Percent of *Tac2* cells coexpressing KNDy neuropeptides and receptors. Data were analyzed using a Student's t-test, within each gene. (C) Representative gel of single-cell PCR amplification products. Panels A-C are the KNDy neuropeptides and gels represent colocalization patterns in the same 10 *Tac2* neurons. Panels D-F are the receptors we analyzed for single cell PCR and represent expression patterns within gene and do not show colocalization patterns across the same 10 *Tac2* neurons. (D) Gene expression in *Tac2* neurons of fed-fasted females (n = 5 – 6). Data were normalized to oil fed controls, within each gene. Data were analyzed using a two-way ANOVA (fasting x steroid) followed by post-hoc Newman-Keuls analysis. (E) Cumulative body weight gain in Swiss Webster females following either *ad lib* LFD or HFD, orally dosed with E2B (n = 6 – 8). Data were analyzed by a repeated measures multifactorial ANOVA followed by post-hoc Newman-Keuls. (F) Gene expression in *Tac2* neurons. (n = 6 – 8) Data were normalized to LFD oil controls, within each gene and analyzed using a two-way ANOVA (diet x steroid), followed by Newman-Keuls multiple comparison test. For all graphs, a = $p < 0.05$; b = $p < 0.01$; c = $p < 0.001$; d = $p < 0.0001$.

Figure 5. Caloric restriction disrupts KNDy-associated ARC gene expression (Experiment #6). (A) Total lean mass percentage. (B) Total fat mass percentage. (C) Cumulative body weight gain (g). At week 5, animals were either fed *ad libitum* or put on a caloric restriction diet. (D) Weekly feeding efficiency pre- and post-diet treatment. (E)

Weekly energy intake (kCal). (F) Overall average energy intake (kCal) pre- and post-diet treatment. (G) KNDy genes: *Kiss1, Tac2,* and *Pdyn*; (H) KNDy-associated receptors: *Kiss1r, Tac3r,* and *Kor*; and (I) Steroid hormone receptors: *Esr1* and *Pgr.* Expression was normalized to oil-*ad lib* samples. (J) Plasma LH and (K) FSH levels. For all data, n = 7 – 8. (A-B, D, F) Data were analyzed by a repeated-measures, multifactorial ANOVA. (C, E) Data were analyzed by a repeated-measures, two-way ANOVA. (G-K) Data were analyzed using a two-way ANOVA. All *post hoc* comparisons were Newman-Keuls multiple comparison tests. For (A-B), (D), (F-K): a = $p < 0.05$; b = $p < 0.01$; c = $p < 0.001$; d = $p < 0.0001$. For (C) and (E), lowercase letters signify differences across steroid, within diet and uppercase letters represent differences across diet, within steroid (a/A = $p < 0.05$; b/B = $p < 0.01$; c/C = $p < 0.001$; d/D = $p < 0.0001$).

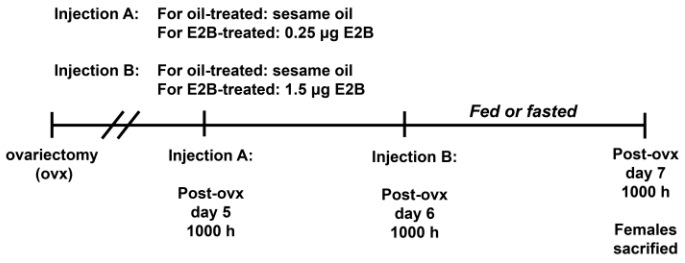
Figure 6. Ghrelin signaling in *Tac2* neurons is regulated by steroid but not fasting or diet-induced obesity.(A) Percent of *Tac2* cells coexpressing *Ghsr*. Data were analyzed using a Student's t-test (c = $p < 0.001$). (B) Representative gel of single-cell PCR amplification products in oil- and E2B-treated *Tac2* neurons. (C) Gene expression in *Tac2* neurons of fed-fasted females. Data were normalized to oil fed controls, within each gene. Data were analyzed using a two-way ANOVA (fasting x steroid) followed by post-hoc Newman-Keuls analysis (b = $p < 0.01$). (D) Gene expression in *Tac2* neurons. Data in C & D were normalized to LFD oil controls except for D: *Ghsr* which was normalized to LFD-E2B and analyzed within each gene using a two-way ANOVA (diet x steroid), followed by Newman-Keuls multiple comparison test.

Figure 7. Chronic caloric overnutrition (DIO) and undernutrition (30% CR) augments the negative feedback effects of E2 leading to disruption of the HPG axis. K = Kisspeptin; N = Neurokinin B (Tac2); D = Dynorphin. Black arrows indicate the

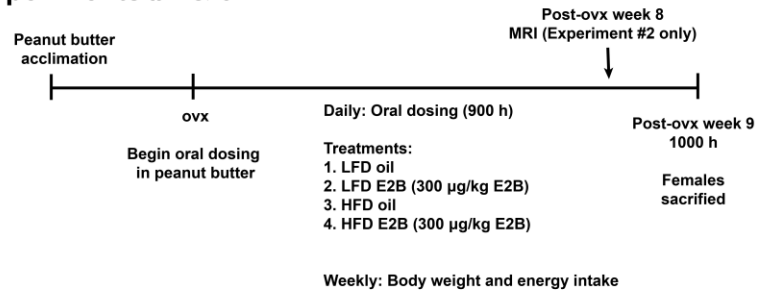
direction of gene expression (↑= upregulated and ↓ = downregulated) in E2B-treated females. Blue arrows indicate the suppression of gonadotropins by diet condition in oil-treated and red arrows indicate suppression by E2B within that diet condition.

Figure 1

Experiments #1 & 4



Experiments #2 & 5



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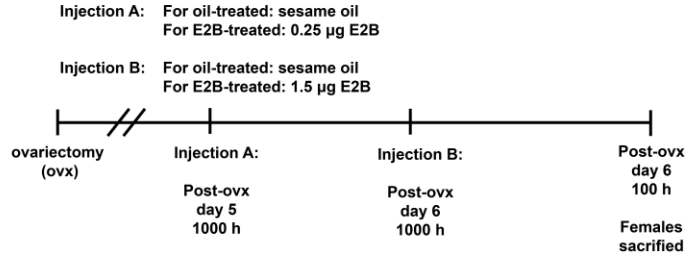


Experiment #6

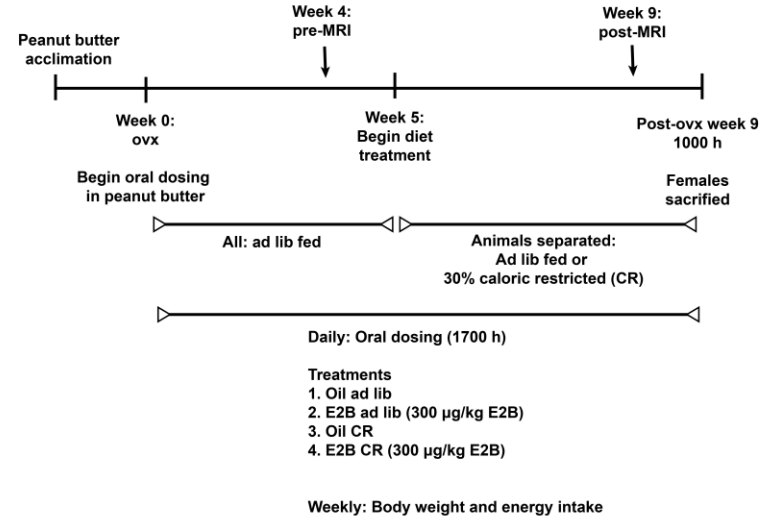


Figure 2

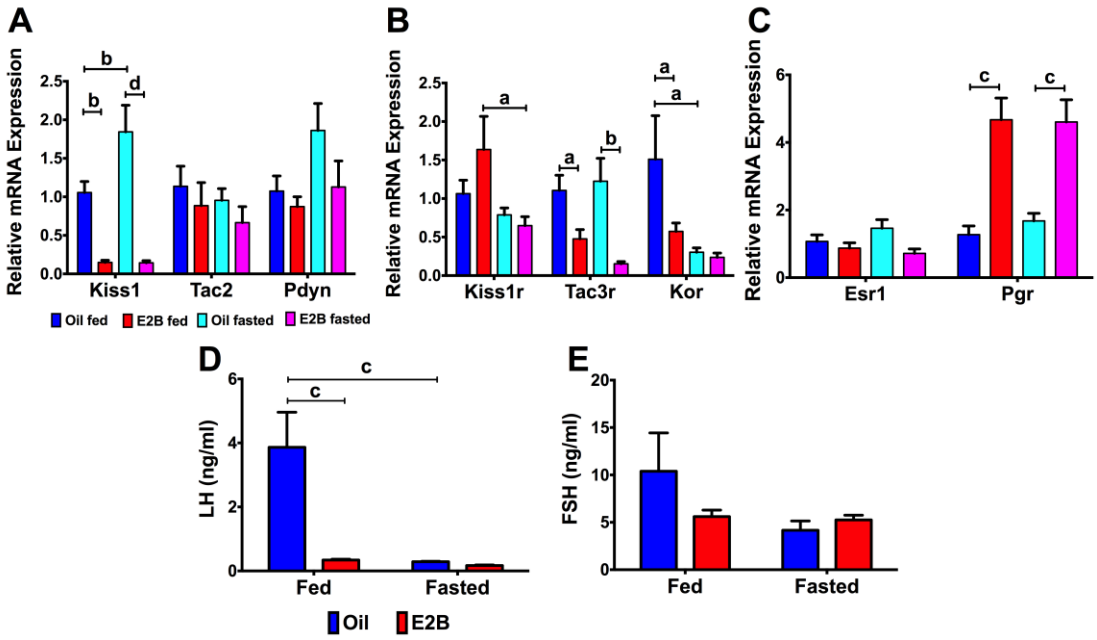

A
Relative mRNA Expression
Kiss1
Tac2
Pdyn
Oil fed
E2B fed
Oil fasted
E2B fasted
B
Kiss1r
Tac3r
Kor
C
Esr1
Pgr
D
LH (ng/ml)
Fed
Fasted
Oil
E2B
E
FSH (ng/ml)
Fed
Fasted

Figure 3

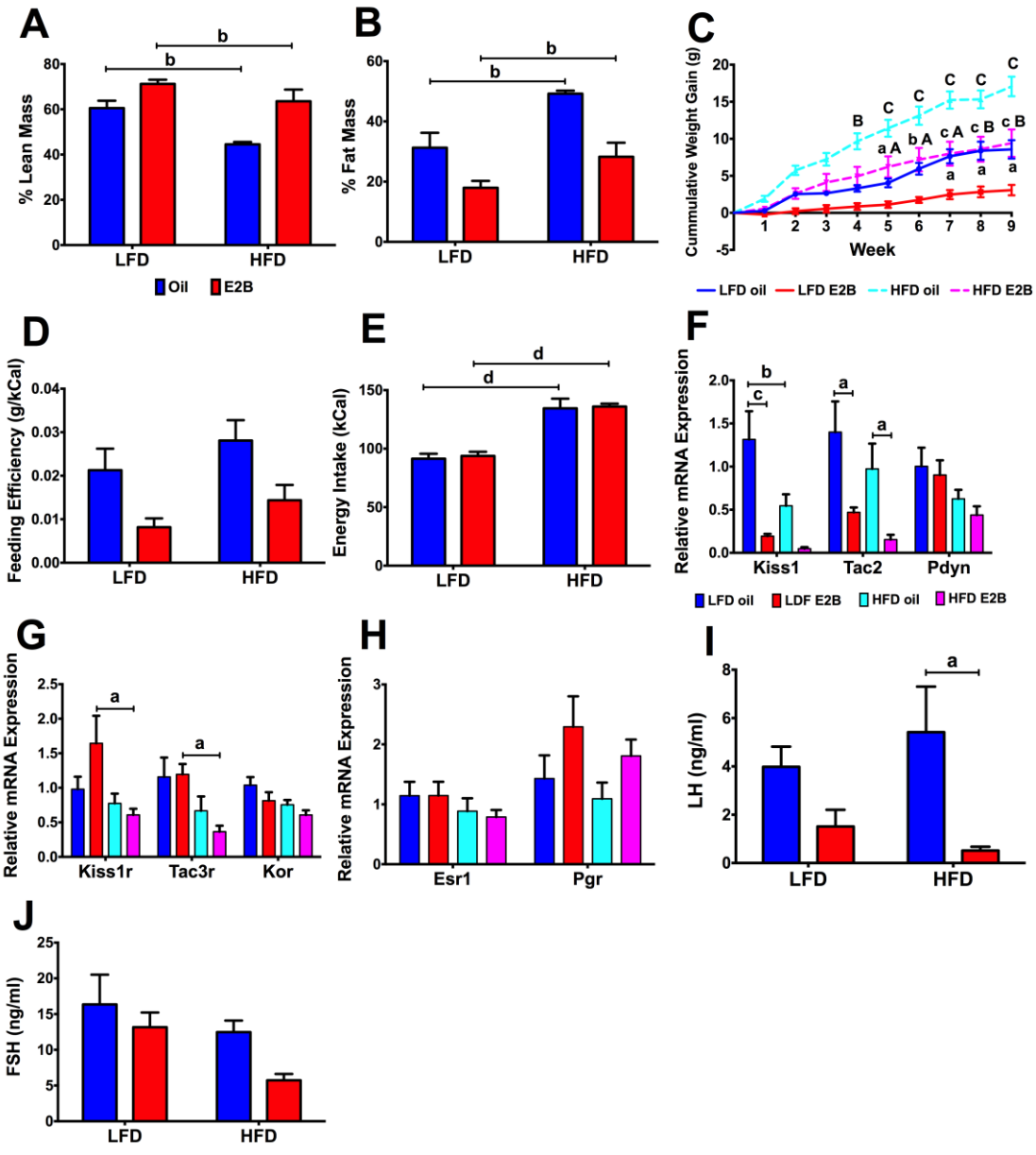

A
% Lean Mass
LFD
HFD
Oil
E2B
B
% Fat Mass
C
Cummulative Weight Gain (g)
Week
LFD oil
LFD E2B
HFD oil
HFD E2B
D
Feeding Efficiency (g/kCal)
E
Energy Intake (kCal)
F
Relative mRNA Expression
Kiss1
Tac2
Pdyn
LFD oil
LDF E2B
HFD oil
HFD E2B
G
Relative mRNA Expression
Kiss1r
Tac3r
Kor
H
Relative mRNA Expression
Esr1
Pgr
I
LH (ng/ml)
J
FSH (ng/ml)

Figure 4

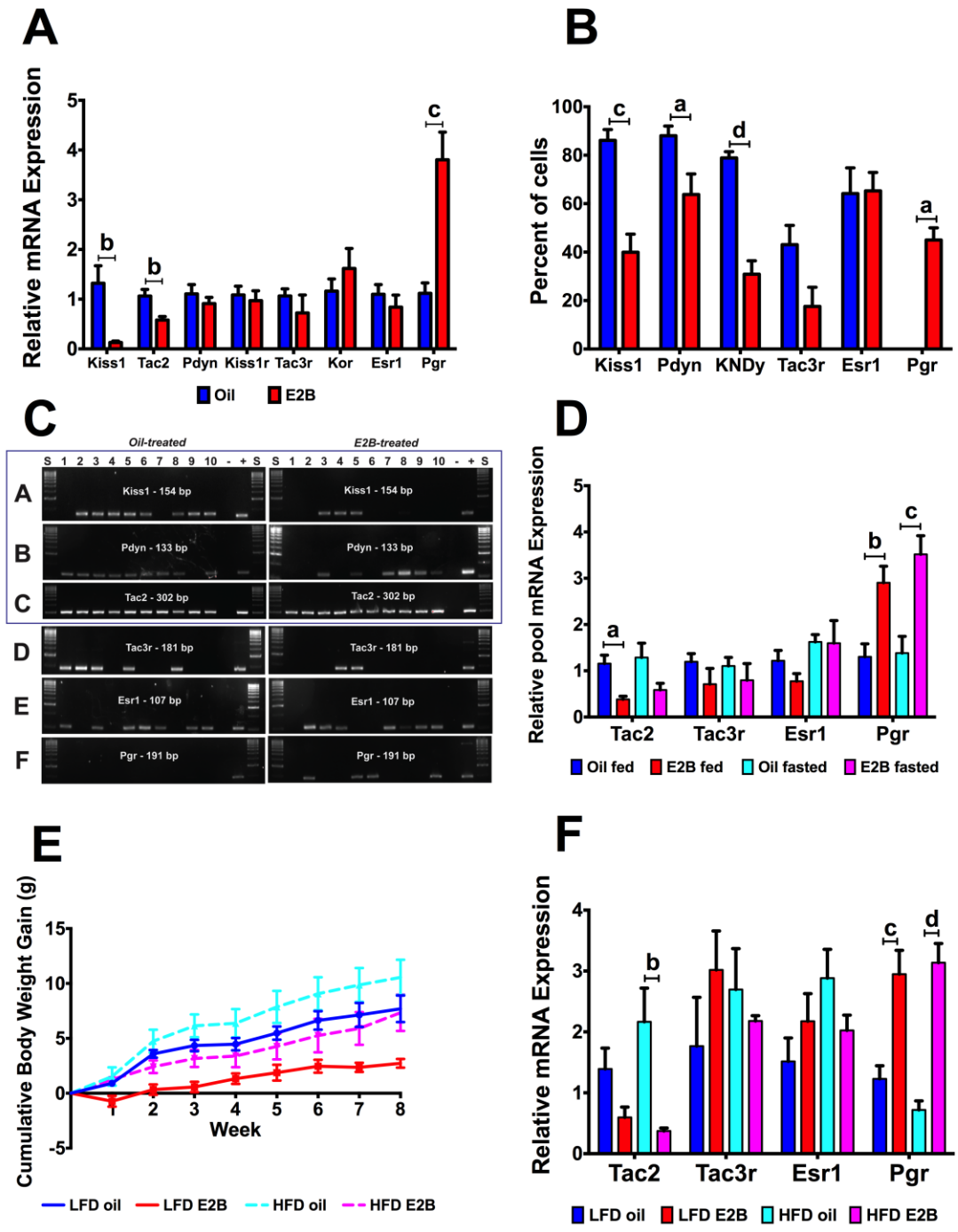

A
Relative mRNA Expression
Kiss1
Tac2
Pdyn
Kiss1r
Tac3r
Kor
Esr1
Pgr
Oil
E2B
B
Percent of cells
Kiss1
Pdyn
KNDy
Tac3r
Esr1
Pgr
C
Oil-treated
E2B-treated
Kiss1 - 154 bp
Pdyn - 133 bp
Tac2 - 302 bp
Tac3r - 181 bp
Esr1 - 107 bp
Pgr - 191 bp
D
Relative pool mRNA Expression
Tac2
Tac3r
Esr1
Pgr
Oil fed
E2B fed
Oil fasted
E2B fasted
E
Cumulative Body Weight Gain (g)
Week
LFD oil
LFD E2B
HFD oil
HFD E2B
F
Relative mRNA Expression
Tac2
Tac3r
Esr1
Pgr
LFD oil
LFD E2B
HFD oil
HFD E2B

Figure 5

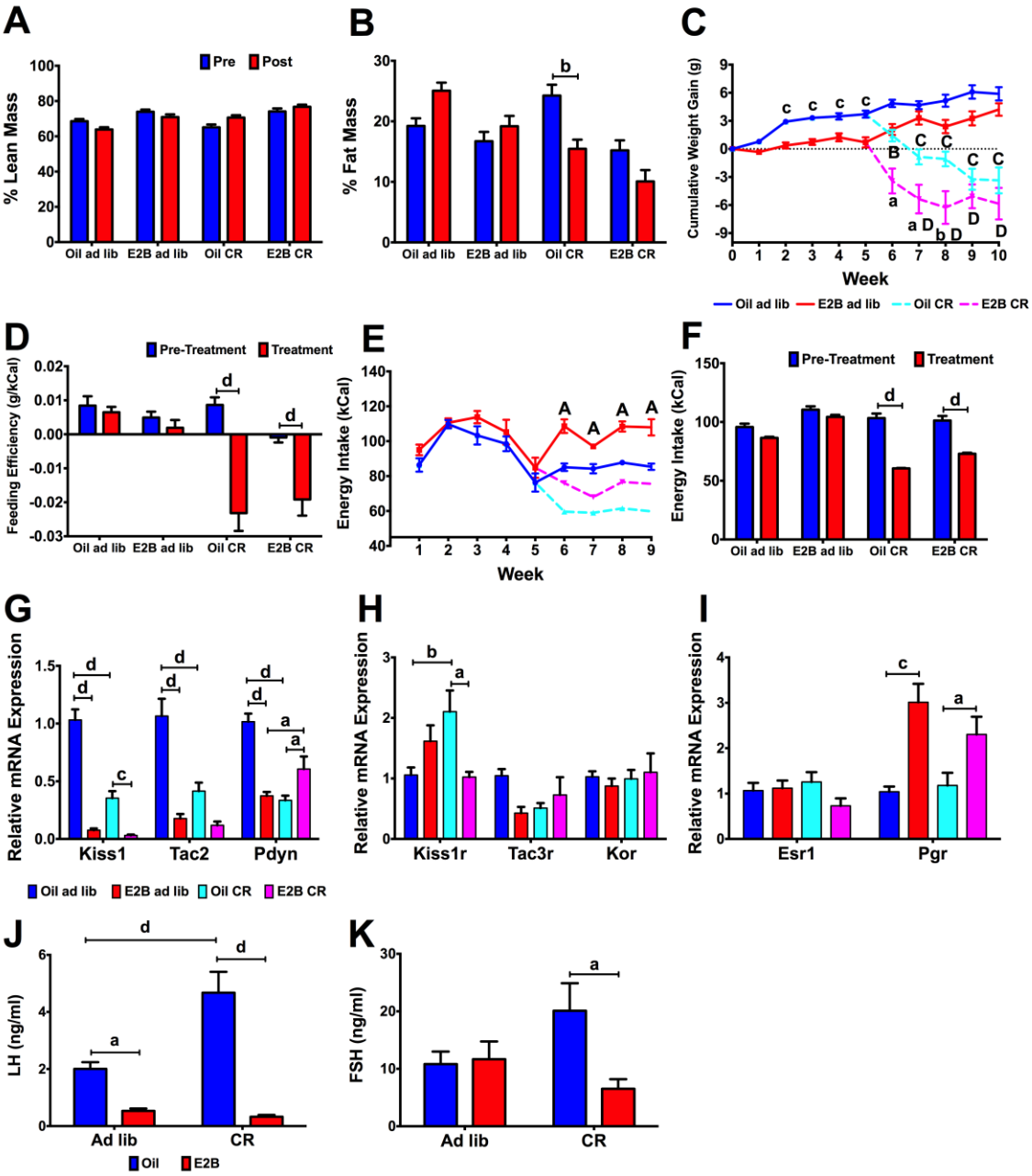

A
% Lean Mass
Pre
Post
Oil ad lib
E2B ad lib
Oil CR
E2B CR
B
% Fat Mass
C
Cumulative Weight Gain (g)
Week
Oil ad lib
E2B ad lib
Oil CR
E2B CR
D
Feeding Efficiency (g/kCal)
Pre-Treatment
Treatment
E
Energy Intake (kCal)
Week
F
Energy Intake (kCal)
Pre-Treatment
Treatment
G
Relative mRNA Expression
Kiss1
Tac2
Pdyn
Oil ad lib
E2B ad lib
Oil CR
E2B CR
H
Relative mRNA Expression
Kiss1r
Tac3r
Kor
I
Relative mRNA Expression
Esr1
Pgr
J
LH (ng/ml)
Ad lib
CR
Oil
E2B
K
FSH (ng/ml)
Ad lib
CR

Figure 6

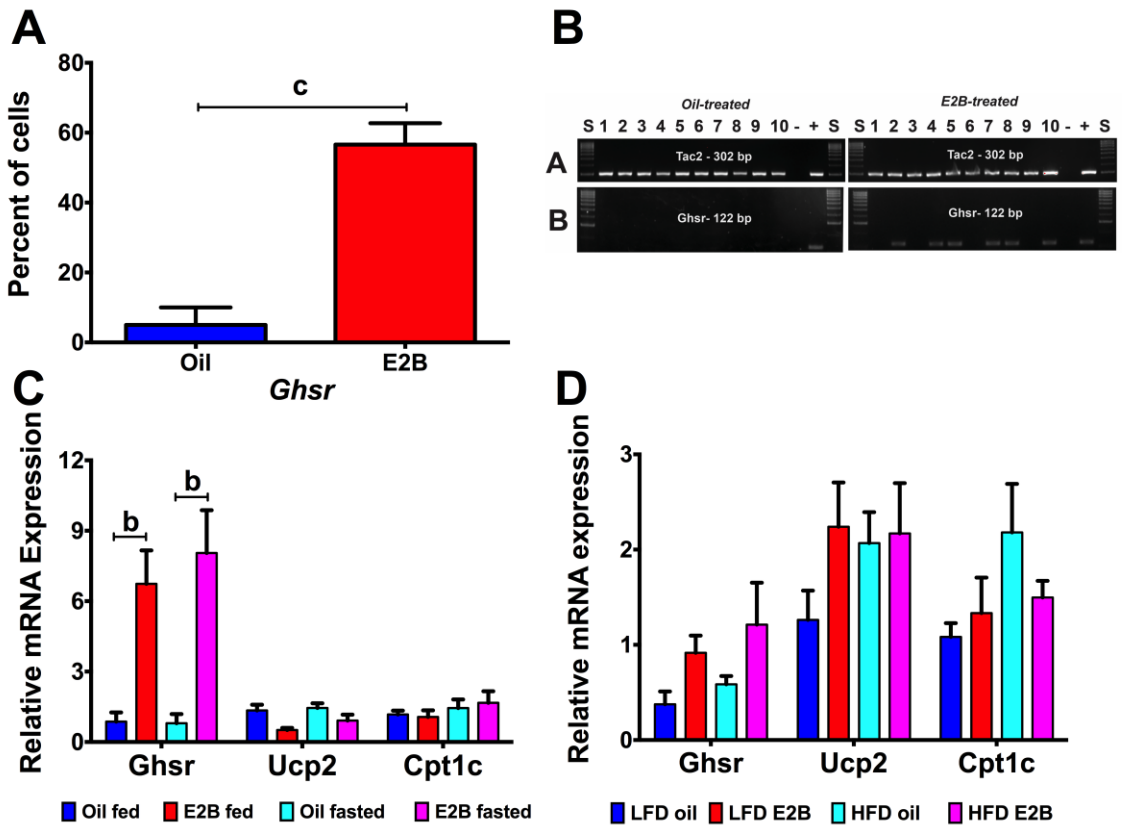
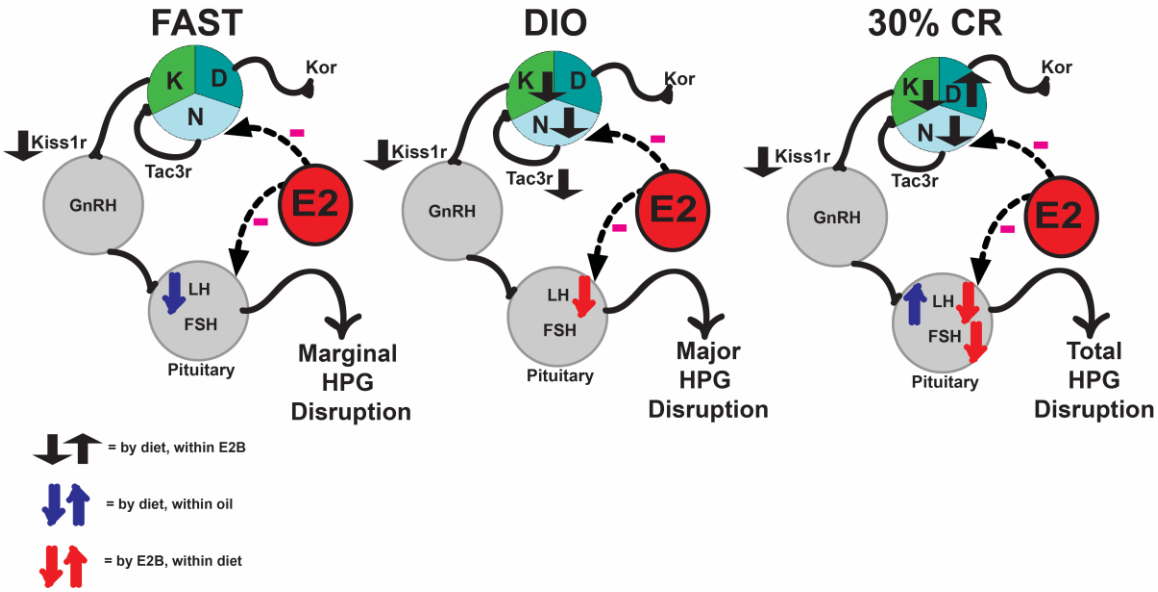

A
Percent of cells
80
60
40
20
0
c
Oil
E2B
Ghsr
B
Oil-treated
E2B-treated
S 1 2 3 4 5 6 7 8 9 10 - + S
S 1 2 3 4 5 6 7 8 9 10 - + S
A
B
Tac2 - 302 bp
Tac2 - 302 bp
Ghsr- 122 bp
Ghsr- 122 bp
C
Relative mRNA Expression
12
9
6
3
0
b
b
Ghsr
Ucp2
Cpt1c
Oil fed
E2B fed
Oil fasted
E2B fasted
D
Relative mRNA expression
3
2
1
0
Ghsr
Ucp2
Cpt1c
LFD oil
LFD E2B
HFD oil
HFD E2B

Figure 7


FAST
DIO
30% CR
K
D
N
Kor
Kiss1r
Tac3r
GnRH
E2
LH
FSH
Pituitary
Marginal HPG Disruption
Major HPG Disruption
Total HPG Disruption
= by diet, within E2B
= by diet, within oil
= by E2B, within diet

CHAPTER 5: SUMMARY

5. Summary

Estrogen signaling is critical in the central regulation of many processes including energy balance and reproduction. In the current studies, we elucidate the differential pathways of E2 signaling and the role of E2 feedback on central regulation of energy homeostasis. We show that in the ARC, E2 signals through multiple mechanisms including ERE-dependent and ERE-independent signaling. Thus, in addition to classical signaling through ERα and ERβ, other pathways including activation of second messenger signaling cascades and regulation through GPR30/GPER1 and the putative Gq-mER, are also critical in central regulation of energy homeostasis. E2 was shown to regulate ARC genes that function as cation channels, receptors for hormones and neurotransmitters, and neuropeptides. Cation channels regulated by E2 include voltage-dependent T-type calcium channels and calcium-activated potassium channels, which may be involved in neurotransmitter release and neuronal excitability. In addition, genes for multiple receptors involved in reproduction, including *Esr1, Esr2,* and *Pgr* were also regulated by E2.

Interestingly, distinctive E2 signaling pathways may be active at different E2 concentrations, which fluctuates throughout the ovarian cycle in females. In the HPG axis of females, E2 functions in both negative and positive feedback to control GnRH neuronal excitability and pulsatility. While in the ARC, negative feedback of E2 is the primary driver in regulation of the HPG axis, we did notice a difference between gene expression changes at a “high” E2 dose (Chapter 2) and a “low” E2 dose (Appendix 6.1). Thus, in future studies, it is imperative to consider the doses of E2 given and how changes in these doses may lead to activation of different E2 signaling pathways. Lastly, we define the ARC estrogenome as the subset of genes regulated by E2 in the ARC, through all of the previously described pathways.

During our preliminary experiments in WT, KIKO, and ERKO females (Chapter 2), we noticed multiple genes that were regulated by E2, through different pathways. Many of these genes are neuropeptides and receptors that are coexpressed in KNDy neurons of the ARC. KNDy neurons coexpress **K**isspeptin (*Kiss1)*, **N**eurokinin B (*Tac2)*, and **Dy**norphin (*Pdyn)*, all of which were regulated by E2 in Chapter 2. *Kiss1* was decreased by E2 through ERE-dependent signaling, which has previously been shown (Gottsch et al., 2009). Furthermore, *Tac2* and *Pdyn* were decreased by E2 through ERE-independent signaling. Interestingly, the associated receptors, *Kiss1r* and *Tac3r* were also regulated by E2. Thus, we chose to examine ARC KNDy neurons to determine the role that E2 functions in KNDy-associated gene expression.

In the ARC, kisspeptin is primarily responsible for negative feedback of E2 on the HPG axis. However, increasing evidence suggests that kisspeptin functions in other processes of energy homeostasis outside of reproduction. For instance, kisspeptin is suggested to be involved in the regulation of core body temperature (i.e. hot flushes during menopause) and body weight gain during hypoestrogenic states, both of which are dependent on E2 signaling (Mittelman-Smith et al., 2012,Mittelman-Smith et al., 2012). As reviewed in De Bond and Smith, many studies now suggest that kisspeptin is an important link between reproduction and energy balance (De Bond and Smith, 2013). Physiologically, this makes sense, as individuals suffering from undernutrition, such as with anorexia nervosa patients and young athletes, and overnutrition, such as with T2DM and obese individuals, also suffer from reproductive reprocussions. In both populations, reproduction is compromised in many forms including hypogonadotropic hypogonadism and infertility (Bulik et al., 1999,Fernandez-Fernandez et al., 2006,Linna et al., 2013,Moran et al., 2011,Norman and Clark, 1998). Furthermore, in both negative and positive energy balance, kisspeptin expression and signaling are disrupted (Castellano et al., 2010,Sanchez-Garrido et al., 2014,Wahab et al., 2008).

In our studies, we examined the role that negative (acute 24 h fast, chronic caloric restriction) and positive (DIO through a high fat diet) energy balance had on ARC KNDy-associated gene expression. In the ARC, we saw a disruption in KNDy-associated gene expression in all cases, although most severe in caloric restricted animals. This is expected and supports multiple studies that have shown similar changes (Castellano et al., 2010,Sanchez-Garrido et al., 2014,Wahab et al., 2008). Next, we wanted to determine the mechanism that targets kisspeptin as a link between reproduction and energy balance. Previous studies have identified potential roles for leptin and less so for insulin, though few studies have examined the role of ghrelin, a brain-gut peptide that stimulates NPY neurons and inhibits POMC neurons (Andrews, 2011).

There are few studies that identify ghrelin signaling in kisspeptin neurons. While one study reported that ghrelin administration had no effect on *Kiss1* mRNA, additional studies suggest that ghrelin depolarizes KNDy neurons to increase neuronal excitability (Forbes et al., 2009,Frazao et al., 2014). Experiments in our lab also suggest that ghrelin signaling is controlled, at least in part, by E2, which increases *Ghsr* expression in the ARC, but not in NPY neurons (Yasrebi et al., 2016). This study illustrates the importance of targeting disctint nuclei and cells in examining gene expression changes, as changes in one nucleus (ARC) may not correspond to changes in distinct cell populations (NPY). In the present studies, we show that the E2 mediated increase in *Ghsr* is primarly due to augmentation of *Kiss1* expression in *Tac2* neurons. However, neither fasting nor DIO had any effect on *Tac2, Tac3r,* or the ghrelin signaling (*Ghsr* signaling pathway) genes. This suggests that ghrelin is not a critical signal in the regulation of energy balance and reproduction in *Tac2* neurons. Nonetheless, E2 signaling may be important to consider in these neurons. Thus, future studies in our lab will examine ghrelin signaling through the *Ghsr* signaling pathway in *Tac2* neurons.

In conclusion, our studies are important in elucidating mechanisms regulating energy balance and reproduction. These studies provide the foundation to understand E2 signaling mechanisms in the central regulation of processes such as energy balance, reproduction, core body temperature, and stress, among others. In the ARC, kisspeptin signaling is important in regulation of GnRH neurons and therefore the HPG axis. Our studies show that changes in energy balance disrupt *Tac2* neurons, which are involved in reproduction downstream of the ARC. We show for the first time that ghrelin signaling in *Tac2* neurons is regulated by E2, perhaps acting as an important mediator in energy balance and reproduction through E2. Taken together, these studies indicate that understanding the mechanisms of energy balance dysfunction may be important to elucidate mechanisms of other problems in human health such as cardiovascular disease and infertility.

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CHAPTER 6: APPENDICES

6. Appendices

Appendices (6.1 and 6.2) are supplemental data for Chapters 2 and 3, respectively. Data in appendices are analyzed similar to respective chapter.

6.1 A low E2 dose regulates arcuate gene expression through ERE-dependent and ERE-independent signaling

6.1.1 Introduction

Appendix 6.1 is supplemental data to Chapter 2 and was removed from the publication due to reviewer criticism of plasma E2 levels.

Feedback of E2 is important in regulation of gene expression patterns. Because circulating levels of endogenous E2 changes throughout the estrous cycle, we choose to examine gene expression in WT, KIKO, and ERKO mice using: 1) a high E2B injection paradigm (Chapter 2) and 2) a low E2B injection paradigm (Appendix 6.1) We hypothesize that the E2-induced regulation of homeostatic genes in mice occurs through ERE-dependent gene expression in a dose-dependent pattern.

6.1.2 Methods

6.1.2.3 Animals

All animal procedures are in compliance with institutional guidelines based on National Institutes of Health standards, and were performed with Institutional Animal Care and Use Committee approval at Rutgers University. Adult C57BL/6 mice (WT, KIKO, and ERKO) were housed under constant photoperiod conditions (12/12 h light/dark cycle) and genotyped, as previously described (Chapter 2) to be used for gene expression studies.

6.1.2.4 Drugs

E2B was purchased from Steraloids (Newport, RI, USA) and dissolved in ethanol 1mg/ml prior to mixing in sesame oil (Sigma-Aldrich). Ketamine was purchased from Henry Schein Animal Health (Melville, NY, USA) and used for sedation prior to sacrifice.

6.1.2.5 Ovariectomy

Adult females were bilaterally ovx under isofluorane anesthesia 7 days prior to sacrifice using sterile no-touch technique according to the NIH Guidelines for Survival Rodent Surgery. Animals were given a dose of analgesic [4 mg/kg carprofen (Rimadyl®)] one day following surgery for pain management. Females were monitored daily and allowed to recover for 5 days prior to the first injection of E2B or oil.

6.1.2.6 Experimental Design

Twelve females of each genotype (WT, KIKO, ERKO) were ovx. Within each genotype, females were separated into a control sesame oil-treated group (n=6 per genotype) and an E2B-treated group (n=6 per genotype). Animals were injected s.c. at 1000 h on post-ovx day 5 with either 0.25 µg of E2B or sesame oil and on post-ovx day 6 at 1000 h. Animals were sacrificed on post-ovx day 7 at 10:00 h. Brains were removed for ARC microdissection, trunk blood collected at sacrifice for plasma E2 and uteri collected to be weighed.

6.1.2.7 Tissue extraction

RNA was extracted from ARC using Ambion RNAqueous® Micro Kits followed by DNase-I treatment, as previously described. RNA samples were run on a NanoDrop™ ND-2000 spectrophotometer and on an Agilent 2100 Bioanalyzer run using the RNA 6000 Nano Kit Samples with a RNA integrity number (RIN) > 6 were used for qPCR.

6.1.2.8 Blood preparation

Whole trunk blood was subjected to centrifugation (1300 rpm at 4°C for 30 min). The supernatant was subjected to an additional 15 minutes of centrifugation (4°C at 1300 rpm), then the plasma supernatant was removed and stored at -20°C until E2 was

analyzed using Mouse Calbiotech ELISA at the University of Virginia Center for Research in Reproduction Ligand Assay and Analysis Core.

6.1.2.9 Quantitative real-time PCR

cDNA was synthesized from 200 ng of total RNA, as previously described (Chapter 2). BH tissue RNA, which contains the ARC from a male mouse was used as a positive control. A negative tissue control (BH with no reverse transcriptase) was also used. A Taqman® Low-Density Array (TLDA) was used to analyze ARC gene expression in WT females,. We analyzed genes that were significantly regulated in KIKO and ERKO females of Chapter 2. qPCR was performed on a StepOnePlus™ Real-Time PCR System (Life Technologies, Inc.) using Taqman® Gene Expression Master Mix. For qPCR, we used 4 µg of cDNA (equivalent to 2 ng of total RNA). The amplification protocol for all genes was as follows: a holding stage consisted of 2 min at 50°C and 95°C for 10 min, followed by a cycling stage of 95°C for 15 sec (denaturing) and at 60°C (annealing) for 1 min for 40 cycles.

In addition to the genes analyzed in the TLDA, we also analyzed 4 additional genes: *Adra1b, Cart, Ghsr* and *Chrm1*. For qPCR of these 4 genes, we used 4 µg of cDNA (equivalent to 2 ng of total RNA) amplified with either PowerSYBR® Green Master Mix (*Adra1b, Chrm1;* Life Technologies, Inc.) or SsoAdvanced™ SYBR Green (*Cart, Ghsr;* BioRad, Inc., Hercules, CA, USA) on CFX-Connect Real-time PCR Instrument (BioRad, Inc.). Amplification protocol for table these genes was as follows: initial denaturing 95°C for 10 min (PowerSYBR®) or 3 min (SsoAdvanced™) followed by 40 cycles of amplification at 94°C for 10 sec (denaturing), 60°C for 45 sec (annealing), and completed with a dissociation step for melting point analysis with 60 cycles of 95°C for 10 sec, 65°C to 95°C (in increments of 0.5°C) for 5 sec and 95°C for 5 sec. The reference genes used were *β-actin* (PowerSYBR®) or *Gapdh* (SsoAdvanced™). Positive, negative and water blank controls were included in the qPCR plate design.

Analysis of qPCR was done using the comparative C_q method using a 1:20 diluted BH cDNA (equivalent to 2 ng of RNA) sample from a male as the calibrator (Livak and Schmittgen, 2001,Pfaffl, 2001). All values were normalized to oil controls and are expressed as relative mRNA expression. In all plates, we maintained a consistent threshold level, set at the lowest but steepest slope of the exponential curve. We calculated the linear quantity of target genes using the formula $2^{-\Delta\Delta Cq}$. Data are expressed as *n*-fold difference from the calibrator, normalized to oil controls. The *n*-fold difference was used for statistical analysis.

6.1.2.10 Identifying ERE promoter sequences

To determine if genes contained an ERE pattern in the promoter regions to permit ERE-dependent regulation, we analyzed all the genes using the Dragon Estrogen Response Element Finder, version 2 (Bajic et al., 2003). This program utilizes a probabilistic model to determine if a given sequence contains ERE patterns, using the consensus ERE 5′-GGTCAnnnTGACC-3′, where n is any nucleotide. Additional information on the program can be found online at http://datam.i2r.a-star.edu.sg/ereV3/.

Statistical analyses

All statistical analyses were performed using GraphPad® Prism software (GraphPad Software, Inc., La Jolla, CA, USA). Data are expressed as mean ± SEM. Data were analyzed by a two-way ANOVA (genotype x treatment), followed by a *post-hoc* Bonferroni's multiple comparison test between oil- and E2B-treated groups, within each genotype (WT, KIKO, ERKO). Uterine weights were analyzed using a two-way ANOVA (genotype x treatment) followed by Bonferroni's multiple comparison test between oil- and E2B-treated groups, within each genotype (WT, KIKO, ERKO). Plasma E2 levels of all three genotypes were pooled together for oil vs. EB treatment analysis, as there was no genotype effect (data not shown). An unpaired Student's t-test was used to analyze plasma E2 levels in the oil- vs. EB-treated groups within each

experiment. In all instances, a $p < 0.05$ was considered to be significant. Furthermore, data were reanalyzed using Data Assist® software (Life Technologies, Inc.) to determine that appropriate statistical analyses were conducted given our qPCR plate design. This software is used to import raw data from TLDAs to calculate and normalize data across reference genes.

6.1.3 Results

6.1.3.1 Uterine weights and plasma E2 levels

Following sacrifice, we dissected out the uterus of each female to confirm the hypertrophic actions of E2, an ERα-mediated process (Kurita et al., 2001). Uterine weights were significantly increased in WT animals treated with E2B (Table 1; ANOVA; $F(2,27) = 20.43$; $p < 0.0001$). Similar to a high E2B dose, a low E2B had no effect on KIKO and ERKO genotypes. There was no significant difference in body weight at sacrifice for E2B-treated females in all genotypes.

In our E2B injection regimen, we used a high E2B dose (Chapter 2) and a low E2B dose. These doses are based on previously published work (Bosch et al., 2013). We pooled plasma E2 data across genotypes (WT, KIKO, ERKO), within each experiment because there was no genotype effect observed (data not shown). Plasma E2 was significantly increased from oil (9.9 ± 1.4 pg/ml) to E2B (24.6 ± 5.1 pg/ml) (Student's t-test: $p < 0.01$).

6.1.3.2 Estrogen Response Element sequence identification

As shown in our experiments, E2B regulated ARC gene expression through both ERE-dependent and ERE-independent mechanisms. We used the Dragon Estrogen Response Element Finder to determine if any of the E2B regulated genes have ERE sequences associated with the gene analyzed. Table 2 includes a list of all analyzed genes in the present study and the number of ERE patterns located in the forward and

reverse strands. The genes with the greatest number of ERE patterns were *Abcc8* (15), *Bcl2* (4), *Kiss1* (7), *Mtor* (4) and *Pgr* (5) while the remaining genes had 0-2 ERE patterns in the promoter sequence. It is important to note that not all of the genes with ERE patterns were regulated by our E2B treatment paradigm.

6.1.3.3 A low E2B dose regulates ARC gene expression

A low dose E2B treatment did not regulate as many genes as a high dose E2B treatment. However, identical neuropeptides and receptors were regulated by E2B. The two neuropeptides that were regulated by a low E2B dose were *Kiss1* and *Tac2,* which are often coexpressed in KNDy neurons of the ARC. There was an eleven-fold decrease in *Kiss1* in WT females and a threefold decrease in *Kiss1* with E2B in KIKO females (Figure 1A: ANOVA; $F(2, 34) = 8.132$; $p = 0.0013$). There was a two-fold decrease in *Tac2* by EB in only WT females (Figure 1B: ANOVA; $F(2,30) = 7.269$; $p = 0.0027$). Three receptors genes were also regulated by a low EB dose. *Ghsr* (Figure 2A: ANOVA; $F(2,30) = 3.723$; $p = 0.0359$) and *Pgr* (Figure 2B: ANOVA; $F(2,30) = 6.031$; $p = 0.0063$) were increased two-fold in WT females while *Tac3r* expression was significantly decreased in both WT and KIKO females (Figure 2C: ANOVA; $F(2,30) = 4.723$; $p = 0.0165$).

6.1.4 Discussion

To study the effect of different E2B doses on gene expression changes in the ARC, we used a low and high E2B dose to differentiate ERE-dependent and -independent signaling within WT, KIKO, and ERKO females. Interestingly, in many genes, the type of E2 signaling pathway differed, depending on E2 concentration. These data suggest that feedback of steroid hormones is important to consider when analyzing gene expression studies. Future studies should include identifying differences across the estrous cycle, though this may be difficult to control as ERKO animals have disrupted

ovarian cyclicity. Nonetheless, it is important to consider hormone effects when analyzing future gene expression studies involving WT, KIKO, and ERKO animals.

Using the Dragon Estrogen Response Element Finder, we were able to identify which genes have associated, putative ERE sequences. It is of interest to note that *Kiss1r, Tac2, Tac3r, Ghsr* and *Cart* showed no ERE patterns, yet three of those genes (*Tac2*, *Ghsr* and *Cart*) are only regulated by E2 in WT suggesting in *in vivo* experiments, ERE-dependent signaling is, at least, involved if not necessary. Further analysis of these genes and their promoters are required before we can state with confidence that ERE-dependent signaling is the primary transcriptional mechanism.

As discussed in Chapter 3, we have expanded on these studies by examining the ARC estrigenome using RNA sequencing, containing genes differentially regulated by E2 during the “high” dose E2B injection protocol (see Chapter 2). Additionally, future studies should examine the ARC estrogenome usin RNA seqnecing in the “low” E2B injection protocol. Feedback of E2 is important to consider in these studies, as E2 in low and high concentrations can lead to negative and positive feedback, respectively. Furthermore, because we know that ERE-independent signaling is rapid and occur within minutes, the changes from negative to positive feedback may have pronounced effects on the type of E2 signaling involved. Lastly, changes throughout the ovarian cycle may be important to consider. In Appendix 6.2, we examined the differences in select genes across a 24 h circadian cycle (every 3 hrs) in ovx animals. Future studies that examine the differences in gene expression across a 24 h circadian cycle may be important to consider as well. This includes differences in E2 signaling mechanism across WT, KIKO, and ERKO females. In conclusion, while our studies of differential E2 signaling in “low” and “high” E2B signaling can serve as preliminary in identifying central regulation of E2 signaling, additional studies should expand on time (circadian rhythm) and E2 concentration.

6.2 Circadian rhythm regulation of arcuate KNDy-gene expression

6.2.1 Introduction

Appendix 6.2 is supplemental data to Chapter 4 and was removed from our manuscript submission since time was not a variable that was considered in the remainder of our studies.

Identifying the role of circadian rhythms on KNDy-associated gene expression is critical to understanding reproduction, especially in rodents and seasonally breeding animals. To date, there have been no studies examining circadian rhythm of KNDy gene expression, with limited data on AVPV kisspeptin (Chassard et al., 2015). While it has been shown that many reproductive organs including the uterus and ovaries have circadian regulators independent of the suprachiasmatic nucleus (SCN), only AVPV kisspeptin neurones have been studied in the mouse (Chassard et al., 2015). The AVPV kisspeptin neurons are responsible for the LH surge during female estrous cycles, which is highly controlled by the SCN to occur shortly after dark on the day of proestrous (Bosch et al., 2013,Brock and Bakker, 2013). Therefore, our study aims to examine the ARC KNDy expression over a 24 h period in ovx female mice. We hypothesise that ARC KNDy expression will exhibit a distinct circadian rhythm throughout the 24 h.

6.2.2 Methods

All animal procedure were performed in compliance with standards, as previously stated. Adult Swiss Webster (SW) animals were used for this experiment. Ovariectomy procedures were conducted as stated in Chapters 2-5.

6.2.2.1 Experiment #1: E2B and time effects on arcuate KNDy-associated gene expression

To determine if there is an effect of time on KNDy-associated gene expression between the morning and night, we collected ARC from females 24 h and 33 h after the last injection. Chapter 4 includes data on females sacrificed 24 h post-injection. We did not include additional data on animals sacrificed 33 h post-injection because it did not fit in with the other experiments conducted in our manuscript submission. The data of both females sacrificed 24 h- and 33 h-post injection is included in the appendix. Note that data for animals sacrificed at 24 h is already presented in Chapter 4. However, to compare between 24 h and 33 h, we analyzed data using a two-way ANOVA (time x steroid) followed by Newman-Keuls post-hoc analysis and thus both data are included in the appendix. At sacrifice, we collected ARC for gene expression analysis and blood for plasma hormone concentration analysis.

6.2.2.2 Experiment #2: Circadian effects on arcuate gene expression

SW females were ovx one week prior to sacrifice, to minimize the role of E2 in regulating circadian rhythmicity and gene expression. Animals were sacrificed on post-ovx day 7 at the following time points: 100, 400, 700, 1000, 1300, 1600, 1900, and 2200 h (n = 8 per time point). Following sacrifice, the ARC was microdissected and used for gene expression analysis as described. Table 3 includes the primer sequences used for qPCR analysis of gene expression. ARC microdissection, RNA extraction, DNAse-I treatment, reverse transcription, and qPCR were conducted as described in Chapter 4.

6.2.3 Results

6.2.3.1 Experiment #1: E2B regulates ARC KNDy gene expression through time-dependent differences

To determine if there is a time-dependent effect on gene expression, we sacrificed animals at two time points: 1100 h and 1900 h. There was an effect of steroid on *Kiss1* gene expression (Figure 3A: $F(1, 21) = 18.67$, $p < 0.001$), which was

decreased in E2B-treated females at 1100 h ($p < 0.01$) and 1900 h ($p < 0.05$). There was a decrease in *Tac2* in E2B-treated females at 1100 h and 1900 h ($p < 0.05$, both; Figure 3A: Steroid: $F(1, 23) = 15.68$, $p < 0.001$).There was no change in *Pdyn* or *Kiss1r* expression by time or steroid. *Tac3r* was increased from 1100 h to 1900 h in oil-treated animals ($p < 0.05$) and decreased by E2B at 1900 h (Figure 3B: Steroid: $F(1, 22) = 6.274$, $p < 0.05$; Time: $F(1, 22) = 5.495$, $p < 0.05$). For *Kor*, the κ-opioid receptor, there was a time (Figure 3B: $F(1, 21) = 25.04$, $p < 0.0001$) and time*steroid effect (Figure 3B: $F(1, 21) = 7.988$, $p < 0.05$). *Kor* was increased in oil-treated females sacrificed at 1900 h compared to females sacrificed at 1100 h ($p < 0.0001$) and was decreased by E2B treatment, compared to oil treatment, at 1900 h ($p < 0.05$).

Expression of the two steroid hormone receptors that mediate negative feedback, *Esr1* and *Pgr*, was also regulated by E2B. *Esr1* ($ER\alpha$) expression was regulated by time (Figure 3C: $F(1, 21) = 14.08$, $p < 0.01$) and increased in oil-treated females at 1900 h compared to 1100 h, similar to *Kor* ($p < 0.05$). There was a steroid effect on *Pgr* expression (Figure 3C: $F(1, 23) = 42.74$, $p < 0.0001$). As expected, E2B increased expression of *Pgr* at both 1100 h ($p < 0.001$) and 1900 h ($p < 0.01$).

Body weight and uterine wet weight were measured at sacrifice. Animals did not have any differences in body weight in steroid treatment or time. There was a significant effect of steroid on uterine weight (Table 4: $F(1, 28) = 97.33$, $p < 0.0001$) and uterine weight relative to body weight (Table 4: $F(1, 28) = 157.6$, $p < 0.0001$). Both were higher in E2B-treated females at both 1100 h ($p < 0.0001$) and 1900 h ($p < 0.0001$).

Plasma E2 levels were measured with an ELISA specific to E2. There was a significant effect of steroid (Table 4: $F(1, 27) = 18.98$, $p < 0.001$) and time*steroid (Table 4: $F(1, 27) = 8.137$, $p < 0.01$). E2 levels in E2B-treated females increased compared to oil-treated females at 1100 h ($p < 0.001$). There was no significant difference in E2

plasma levels between oil- and E2B-treated females at 1900 h, possibly due to the longer time lapsed from the last injection. In addition, there was a significant difference between E2 levels in E2B-treated animals at the two sacrifice times ($p < 0.05$). LH expression was significantly decreased in oil- ($p < 0.05$) and E2B-treated ($p < 0.01$) at 1900 h compared to 1100 h (Figure 3D: $F(1, 27) = 20.49$, $p < 0.0001$). There was no difference between oil- and E2B-treated females, though E2B-treated animals across both time points showed a trend to increased LH levels.

6.2.3.2 Experiment #2: Circadian rhythm does not regulate arcuate KNDy-associated gene expression in the absence of E2

Due to differential expression of *Kor* and *Esr1* in oil-treated animals at 1100 and 1900 h, we examined ARC KNDy gene expression at 3 h intervals across a 24 h time period in untreated, ovx females, which were sacrificed one week following ovx. Body weight, uterine weight, and uterine weight relative to body weight were not significantly different across sacrifice times (Table 4). Gene expression data for all genes were normalized to 700 h. For KNDy genes (*Kiss1, Tac2, Pdyn),* data were normalized to *Kiss1* at 700 h. For these three genes, there was a gene effect (Figure 4A: $F(2, 167)$: 49.08, $p < 0.0001$). *Tac2* expression was elevated compared to *Kiss1* at 100, 400, 1000, 1300, 1600, and 1900 h and compared to *Pdyn* at 100, 1000, 1600, and 1900 h. There were no differences in expression of *Kiss1, Tac2,* or *Pdyn* across time.

KNDy-associated receptors (*Kiss1r, Tac3r, Kor)* were normalized to *Kiss1r* at 700 h. *Kor* expression was elevated compared to *Kiss1r* and *Tac3r* at all time points (Figure 4B: $F(2, 168) = 156.3$, $p < 0.0001$). There were no differences in expression of *Kiss1r, Tac3r,* or *Kor* across time. For the steroid hormone receptors, there were no differences observed over time for *Esr1* or *Pgr.* However, *Pgr* expression, which was normalized to *Esr1* at 700 h, was expressed at lower levels compared to *Esr1* across all time points, except 1300 h (Figure 4C: $F(1, 112) = 117.7$, $p < 0.0001$).

To compare the KNDy-associated genes to genes regulated by circadian rhythms, the following circadian genes were selected for analysis: *Clock, Bmal, Per1,* and *Per2.* Data were normalized to *Clock* at 700 h. Across all time points, *Clock* expression was elevated compared to the remaining genes (time: Figure 4D: $F(7, 224) = 2.424$, $p < 0.05$; time*gene: Figure 4D: $F(3, 224) = 135.2$, $p < 0.0001$). *Per1* expression was significantly different over time (Figure 4D: $F(7, 56) = 5.097$, $p < 0.001$). Post-hoc Newman-Keuls test showed a difference in *Per1* expression at 1300 h compared to 1600 h ($p < 0.05$). *Per2* expression was also different over time (Figure 4D: $F(7, 56) = 4.983$, $p < 0.001$). There was no significant difference in *Clock* or *Bmal* over time. As we did not observe differences in gene expression across time for KNDy genes and the associated receptors, we used an experimental design in which animals were sacrificed at 1000 h for the remaining experiments.

6.2.4 Discussion

To conduct the circadian study, we ovx animals one week prior to euthanasia to allow for E2 clearance and sacrificed the females at 3 h intervals on post-ovx day 7 (24 h). In accordance to our previous experiment, we did not find a time-dependent effect on gene expression of *Kiss1, Tac2, Pdyn, Kiss1r, Tac3r*, and *Pgr.* Interestingly, when analysed over a 24 h cycle, gene expression of *Kor* or *Esr1* did not change, as it did in the previous experiment. To confirm that circadian effects were present using the ARC, we analyzed known “clock” genes (*Clock, Bmal, Per1,* and *Per2*) (Chassard et al., 2015,Williams et al., 2011)**.** Of these, *Per1* and *Per2* expression were different across the 24 h cycle, indicating that circadian influences on gene expression do occur in the ARC.

We were also interested in differences in relative gene expression between the neuropeptide and receptor genes. For the KNDy neuropeptide and receptor genes, *Tac2*

was significantly higher than *Kiss1* and *Pdyn* and *Kor* was elevated compared to *Kiss1r* and *Tac3r*. For females that lack circulating E2, *Tac2* levels are elevated, potentially increasing the actions of the pulse generator on kisspeptin release. Similarly, higher *Kor* expression may counteract the elevated NKB expression by increasing neuronal sensitivity to dynorphin (*Pdyn*), the negative regulator of the pulse generator in the ARC. Physiologically, these differences in gene expression suggest that, at least in ovariectomised females, disruption to NKB and dynorphin signaling are involved in disturbances in the HPG axis. Interestingly, *Esr1* was significantly higher than *Pgr*, which is expected since E2 suppresses *Esr1* and augments *Pgr* in the ARC (Yang et al., 2016). For the circadian genes, *Clock*, whose posttranslational product when bound to *Bmal* is an essential activator of *Per1* and *Per2* expression (Chassard et al., 2015), was expressed at higher relative levels than *Bmal, Per1,* and *Per2* across all time points. These data provide baseline expression levels to further investigate the impacts of steroids, peripheral hormones, and energy states on the relative neuropeptide and receptor gene expression.

Future studies on circadian cyclicity will include animals that are ovx and given an E2B injection. As previously mentioned, E2 signaling is important in regulation of negative feedback on ARC KNDy neurons. Thus, we expect KNDy-associated genes to be differentially regulated across a 24 h cycle when E2 is present. Interestingly, in the current study, we did not see differences in gene expression for any of the KNDy-associated genes across a 24 h cycle, but did see differences in relative expression within KNDy-neuropeptides, -receptors, and steroid hormone receptors. The significance of differences in relative expression is unknown, though may be important in feedback and regulation of KNDy neurons. Understanding these relative gene expression differences with E2 may be important to understanding this feedback. In conclusion, we hypothesize that with E2, we will see differences in KNDy-associated gene expression

across a 24 h cycle, indicative of the changes associated with ARC kisspeptin's control of reproduction.

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Tables

Table 1. Body and uterine weights for all treatments and genotypes

Genotype	Treatment	Body weight (g)	Uterine weight (mg)
WT	Oil	18.5 ± 0.5	21.5 ± 4.2
	E2B	18.2 ± 0.5	88.3 ± 11.9 ****
KIKO	Oil	22.3 ± 0.7	32.1 ± 3.9
	E2B	21.3 ± 0.5	32.3 ± 2.1
ERKO	Oil	23.2 ± 0.5	15.3 ± 5.6
	E2B	24.4 ± 0.3	11.6 ± 1.6

Body weights of females did not show any significant difference among genotypes or within treatments. Uterine weights of WT females were significantly increased with E2B ($p < 0.0001$), although no significant differences were observed in KIKO and ERKO E2B- vs. oil-treated females. ****$p < 0.0001$, two-way ANOVA with Bonferroni's multiple comparison test.

Table 2. Identification of ERE-binding domains in regulated genes

Gene Abbreviation	Forward strand	Reverse complement strand
Abcc8	7	8
Bcl2	3	1
Cacna1g	1	1
Cacna1h	1	0
Calm1	0	0
Esr1	0	1
Esr2	0	1
Gad1	0	0
Gad2	0	0
Gpr30	0	0
Htr2c	0	0
Kcnj11	1	0
Kcnmb1	1	1
Kcnmb4	0	0
Kcnq5	0	0
Kiss1	4	3
Kiss1r	0	0
Mtor	2	2
Ncoa1	2	0
Npy	0	0
Pik3r3	1	0
Pgr	5	0
Pomc	0	0
Sirt1	0	0
Tac2	0	0
Tac3r	0	0
Th	0	1
Adra1b	0	1
Cart	0	0
Chrm1	1	1
Ghsr	0	0
Pdyn	0	1

All genes were analyzed to determine if there were ERE patterns present. The Dragon Estrogen Response Element Finder was used to determine ERE patterns present on both forward and reverse complement strands (Bajic et al., 2003).

Table 3. Primer sequences for qPCR

Gene Name	Product length	% Eff	Primer sequence	Base pair #	Accession #
Kiss1	154	91	F: TGATCTCAATGGCTTCTTGGCAGC	40-63	NM_178260
			R: CTCTCTGCATACCGCGATTCCTTT	170-193	
Kiss1r	138	100	F: CCTTCACCGCACTCCTCTAC	1993-2012	NM_03244
			R: CATACCAGCGGTCCACACTC	2111-2130	
Pdyn	133	105	F: AGCTTGCCTCCTCGTGATG	335-353	NM_018863
			R: GGCACTCCAGGGAGCAAAT	441-459	
Pgr	191	104	F: TGAAAGAGCGTCATTCTTAC	2980-2999	NM_008829
			R: CAATTCGCGGATATAGCTTG	3151-3170	
Tac2	220	103	F: CGTGACATGCACGACTTC	505-522	NM_00119997
			R: CCAACAGGAGGACCTTAC	707-724	1
Tac3r	124	99	F: TACACCATCGTTGGAATTAC	1026-1045	NM_021382
			R: ATGTCACCACCACAATAATC	1130-1149	
Esr1	107	96	F: GCGCAAGTGTTACGAAGTG	919-937	NM_007956
			R: TTCGGCCTTCCAAGTCATC	1007-1025	
β-actin	63	100	F: GCCCTGAGGCTCTTTTCCA	849–867	NM_007393
			R: TAGTTTCATGGATGCCACAGGA	890-911	
Hprt	85	117	F: GCAGTACAGCCCCAAAATGG	599-618	NM_013556
			R: AACAAAGTCTGGCCTGTATCCA	662-683	
Gapdh	98	93	F:TGACGTGCCGCCTGGAGAAA	778-797	NM_008084.2
			R:AGTGTAGCCCAAGATGCCCTTCAG	852-875	
Bmal	77	93	F:GATAAGGACTTCGCCTCTACC	1304-1324	NM_00128968
			R:TAGCCTGTGCTGTGGATTG	1362-1380	0
Per1	92	113	F: CCAGATTGGTGGAGGTTACTGAGT	3248-3271	NM_011065
			R: GCGAGAGTCTTCTTGGAGCAGTAG	3316-3339	
Per2	126	103	F: GGCATTACCTCCGAGTATATCGT	706-728	NM_011066
			R: GGCGTCCTTCTTACAGTGAAAG	810-831	
Clock	185	120	F: AAGACGGCGAGAACTTGG	1540-1557	NM_007715
			R: AGACTGCGGTGTGAGATG	1707-1724	
Kor	237	110	F: TCCTTGGAGGCACCAAAGTCAGGG	799-822	NM_00120437
			R: TGGTGATGCGGCGGAGATTTCG	1014-1035	1

Forward primer (F) is listed first with the reverse primer (R) second. *Kiss1,* kisspeptin; *Kiss1r,* kisspeptin receptor; *Pdyn,* prodynorphin; *Pgr*, progesterone receptor; *Tac2,* tachykinin 2; *Tac3r,* tachykinin 3 receptor; *Esr1*, estrogen receptor alpha; *β-actin,* beta-actin; *Hprt,* hypoxanthine-guanine phosphoribosyltransferase; *Gapdh,* glyceraldehyde-3-phosphate dehydrogenase; *Bmal,*

aryl hydrocarbon receptor nuclear translocator-like; *Per1,* period circadian clock 1; *Per2,* period circadian clock 2; *Clock,* circadian locomotor output cycles kaput; *Kor,* kappa opioid receptor.

Table 4. Summary of animal body and uterine weights and plasma E2 levels

Exp #	Strain	Treatment	Body weight (g) at sacrifice	Uterine weight (mg)	Uterine weight (mg)/Body weight (g)	E2 (pg/µl)
1	Swiss Webster	1100 h oil	41.0 ± 2.2	55.7 ± 3.9	1.4 ± 0.2	6.6 ± 0.7
		1100 E2B	44.0 ± 2.5	146.2 ± 11.2 **d**	3.3 ± 0.2 **d**	36.4 ± 7.9 **c**
		1900 oil	38.8 ± 2.5	45.6 ± 3.6	1.2 ± 0.1	12.3 ± 3.7
		1900 E2B	40.7 ± 2.8	147.5 ± 15.1 **d**	3.6 ± 0.3 **d**	18.6 ± 1.4 **A**
1a	Swiss Webster	700 h	34.5 ± 1.5	47.6 ± 4.2	1.4 ± 0.1	E2 not measured
		1000	32.4 ± 1.2	55.4 ± 3.1	1.7 ± 0.1	
		1300	34.4 ± 0.9	54.3 ± 2.0	1.6 ± 0.0	
		1600	35.8 ± 1.6	61.8 ± 1.3	1.8 ± 0.2	
		1900	34.7 ± 1.8	52.7 ± 10.8	1.8 ± 0.5	
		2200	32.9 ± 1.6	61.4 ± 2.7	1.9 ± 0.1	
		100	32.6 ± 1.3	51.9 ± 4.4	1.6 ± 0.1	
		400	33.1 ± 0.7	53.2 ± 3.4	1.5 ± 0.2	

Lowercase letters signify differences between oil- vs. E2B-treated females, within the same treatment (different according to experiment: time of sacrifice, energy balance state, diet). Uppercase letters signify differences within steroid, across treatment. (a/A = $p < 0.05$; b/B = $p < 0.01$; c/C = $p < 0.001$; d/D = $p < 0.0001$)

Figures

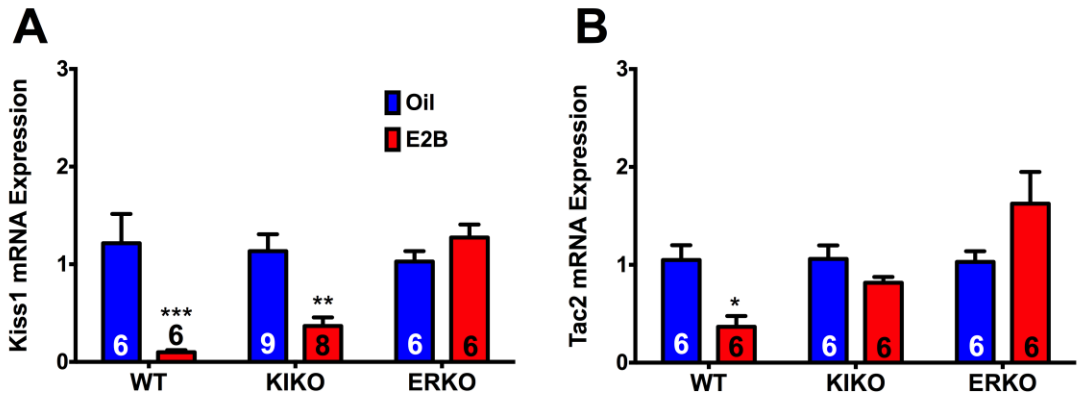
Figure 1. Low dose of E2B regulates neuropeptide gene expression in the ARC. (a) *Kiss1*; (b) *Tac2*. Results of qPCR analyses represent gene expression of oil (black bars) and E2B (gray bars) treated females in WT, KIKO and ERKO genotypes. The number of animals in each treatment group is listed within each bar. Genes are expressed as relative *n*-fold changes, normalized to oil controls, within each genotype (WT, KIKO, ERKO). A two-way ANOVA (genotype x treatment) followed by post-hoc Bonferroni's multiple comparison test was used to determine significant differences between treatments, within genotype. *$p < 0.05$; **$p < 0.01$; ***$p < 0.001$.

Figure 2. Low dose of E2B regulates receptor gene expression in the ARC. (a) *Ghsr;* (b) *Pgr;* (c) *Tac3r.* Results of qPCR analyses represent gene expression of oil (black bars) and E2B (gray bars) treated females in WT, KIKO and ERKO genotypes. The number of animals in each treatment group is listed within each bar. Genes are expressed as relative *n*-fold changes, normalized to oil controls, within each genotype (WT, KIKO, ERKO). A two-way ANOVA (genotype x treatment) followed by post-hoc Bonferroni's multiple comparison test was used to determine significant differences between treatments, within genotype. *$p < 0.05$; **$p < 0.01$; ***$p < 0.001$.

Figure 3. Estradiol benzoate (E2B) regulates KNDy-associated ARC gene expression. (A) KNDy genes: *Kiss1, Tac2,* and *Pdyn*; (B) KNDy-associated receptors: *Kiss1r, Tac3r,* and *Kor*; (C) steroid hormone receptors: *Esr1* and *Pgr.* For all genes, n = 5 - 8. Gene expression data were normalized to 1100 h oil controls for each gene and analysed using a two-way ANOVA (time x steroid) followed by Newman-Keuls multiple comparisons tests (* $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$; **** $p < 0.0001$).

Figure 4. Expression of KNDy and Clock genes are differentially regulated by circadian rhythm. Data shown are relative mRNA expression levels normalized to 700 h gene expression of (A) *Kiss1*, (B) *Kiss1r*, (C) *Esr1*, and (D) *Clock*. (A) Gene expression of arcuate KNDy genes. (B) Gene expression of KNDy receptors. (C) Gene expression of hormone receptors. (D) Gene expression of selected circadian genes. Data were analyzed using a two-way ANOVA for time x gene, followed by Newman-Keuls tests. Lowercase letters denote significance between (A) *Kiss1* and *Tac2* or *Pdyn* and (B) *Kiss1* and *Tac3r* or *Kor*. Uppercase letters denote significance between (A) *Tac2* and *Pdyn* and (B) *Tac3r* and *Kor*. For all genes, n = 8. For (D), a = *Clock* vs. *Bmal; a* = *Clock* vs. *Per2;* a = *Clock* vs. *Per1* (a/A = $p < 0.05$; b/B = $p < 0.01$; c/C = $p < 0.001$; d/D = $p < 0.0001$).

Figure 1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Kiss1 mRNA Expression
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E2B
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Figure 2

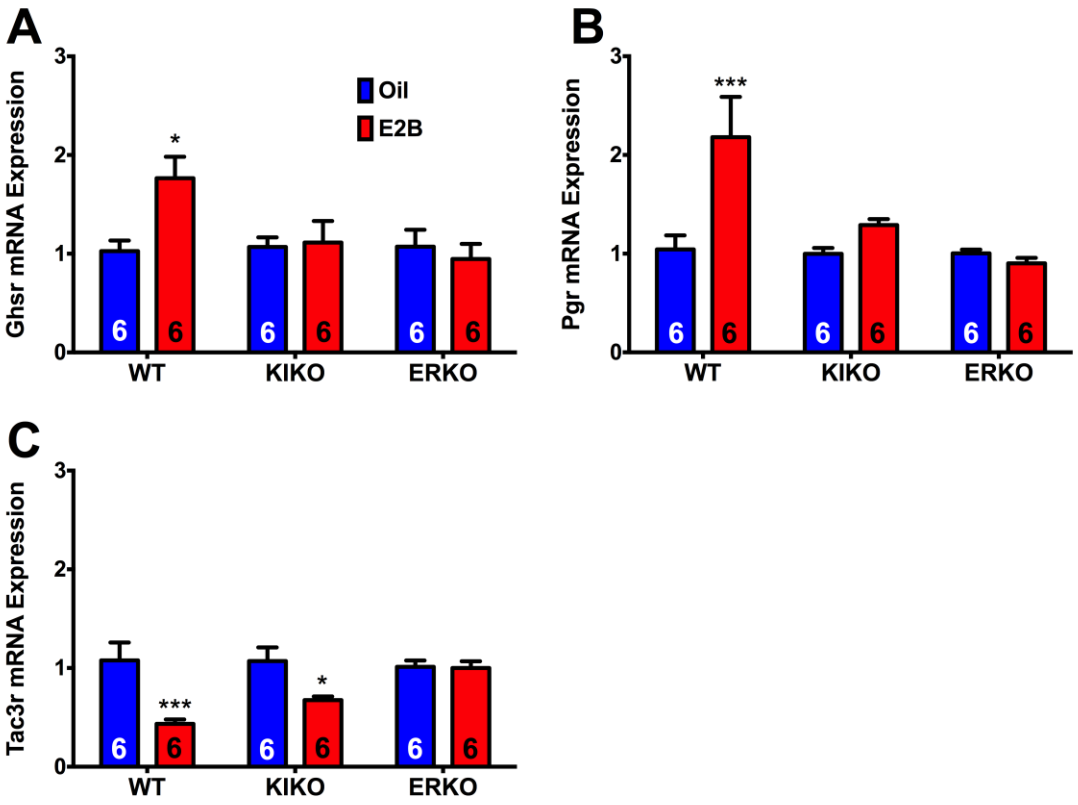
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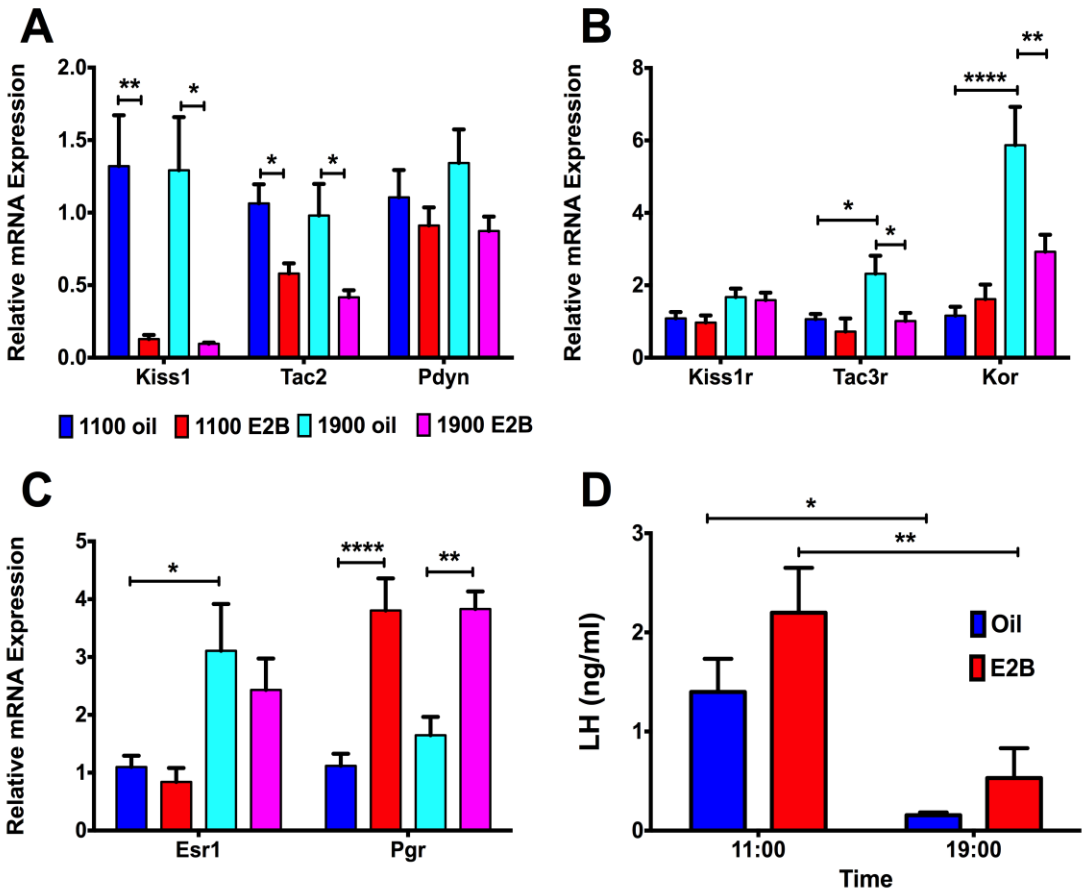
Figure 3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Relative mRNA Expression
2.0
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Kiss1
Tac2
Pdyn
1100 oil
1100 E2B
1900 oil
1900 E2B
B
Relative mRNA Expression
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6
4
2
0
****
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*
*
Kiss1r
Tac3r
Kor
C
Relative mRNA Expression
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1
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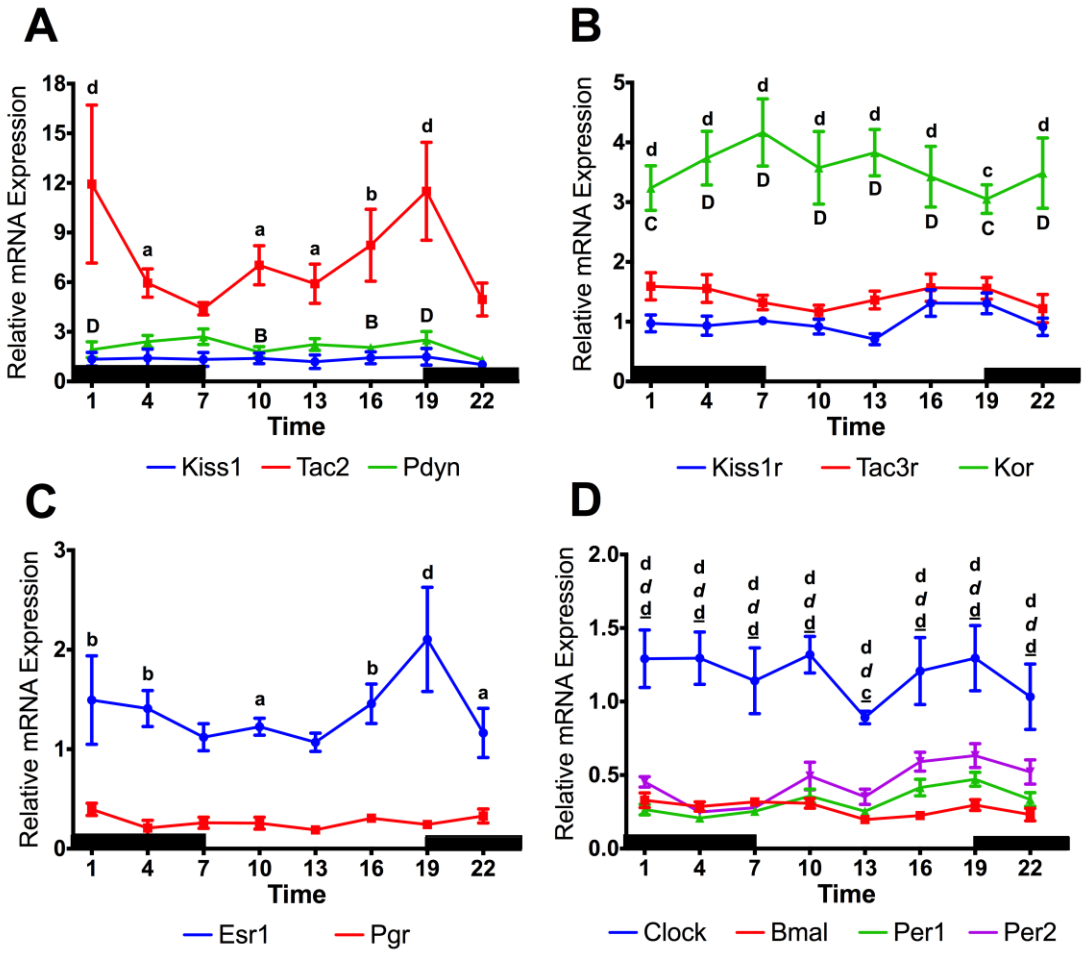
****
**
Esr1
Pgr
D
LH (ng/ml)
3
2
1
0
*
**
Oil
E2B
11:00
19:00
Time

Figure 4


A
Relative mRNA Expression
Time
Kiss1
Tac2
Pdyn
B
Relative mRNA Expression
Time
Kiss1r
Tac3r
Kor
C
Relative mRNA Expression
Time
Esr1
Pgr
D
Relative mRNA Expression
Time
Clock
Bmal
Per1
Per2