THE INTERACTION OF 17 BETA-ESTRADIOL AND GHSR EXPRESSION IN ARCUATE KNDFY NEURONS AND THE IMPACT ON REPRODUCTION AND ENERGY BALANCE IN FEMALE MICE

By

KRISTINE CONDE

A dissertation submitted to the

School of Graduate Studies

Rutgers, The State University of New Jersey

In partial fulfillment of the requirements

For the degree of

Doctor of Philosophy

Graduate Program in Neuroscience

Written under the direction of

Dr. Troy A. Roepke, Ph.D.

And approved by

_____________________________________

_____________________________________

_____________________________________

_____________________________________

New Brunswick, New Jersey

JANUARY 2021
ABSTRACT OF THE DISSERTATION

The interaction of 17 beta-estradiol and GHSR expression in arcuate KNDy neurons and the impact on reproduction and energy balance in female mice

By KRISTINE CONDE

Dissertation Director:
Troy A. Roepke, Ph.D.

Estrogen signaling is a primary central regulator of reproduction and energy balance and ghrelin signaling is a critical modulator in the coordination between the two. In this dissertation, I elucidate the influence of E2 on GHSR signaling in KNDy neurons in the ARC of the hypothalamus and the critical yet understudied role it plays in modulating LH pulsatility, arcuate gene expression, the metabolic response to ovariectomy, diet-induced obesity, and the thermoregulatory response to cold stress. First, I characterized the 17β-estradiol (E2)-enhanced sensitivity to ghrelin in KNDy neurons utilizing Tac2-EGFP female mice. I discovered that KNDy neurons are indeed more sensitive to the inhibitory effect of ghrelin on the M-current, making these neurons more easily excitable.

The next phase of investigation led to the development of a Kiss1-specific GHSR knockout mouse model utilizing Cre-lox technology. With this model, I further investigated the physiological importance of Ghsr expression in KNDy neurons and the regulation by E2. I found that deleting the GHSR in KNDy neurons alone is not sufficient to alter fertility, age of puberty or estrous cyclicity. Furthermore, I determined that KNDy regulation of LH pulsatility is strongly influenced by the interaction of E2 and Ghsr expression and exogenous ghrelin was able to significantly alter KNDy gene expression, revealing that reproduction and LH pulsatility are tightly regulated by an interaction of
both ghrelin and E2. In addition, I found that our experimental (KNDy- GHSR KO) females treated with E2 had reduced metabolic rates. On a HFD, experimental females were resistant to diet-induced obesity exhibiting less adiposity and delayed glucose clearance. Finally, experimentals also respond to short-term cold stress overnight with increased activity and energy expenditure. In summary, my results indicate that there is a central pathway for the control of reproduction and metabolism coordination in female mice and it involves E2 and Ghsr expression ARC KNDy neurons.
ACKNOWLEDGEMENTS

First and foremost, I would like to thank my advisor, Dr. Troy A. Roepke, without whom I would not be here today. Since our first meeting at an SfN meeting in 2014 he encouraged me to apply to Rutgers to work with him. It is now 2020 and he has not stopped encouraging me. Thank you for teaching me how to be a good mentor to others, how to be inclusive, and how to be persistent in this competitive field. I’d also like to thank my committee members, Dr. Zhiping Pang, Dr. Nicholas Bello, and Dr. Sally Radovick for their guidance through my program, through this research, and through my project. Additionally, I would like to thank my program directors and staff Dr. John Pintar and Joan Mordes, for keeping me grounded and focused on this journey. I also need to say thank you to the staff in Bartlett Hall and in the department of Animal Sciences for their continued support.

I certainly would not have made it this far without our lab manager, Ali Yasrebi, who has been the most patient teacher, a great listener, and a caring friend. My fellow graduate students, Dr. Gwyndolin Vail and Thomas Degroat as well as our postdoc, Dr. Kimberly Weirsilis who I have learned so much from and have provided me with a lab family in the absence of my own here in New Jersey. Thank you to all of our undergraduates, especially Allison Vanschaik, Danielle Kulyk, and Sierra Daisey without whom my projects would not have progressed as quickly, I am so proud of you all.

Finally, I need to thank my fellow Neuroscience grad students, especially Andrew Dieterich and Monal Mehta, who have become my closest friends during my time here. I need to thank my family who have been so supportive of my journey and who have forgiven my long absence in their lives while I work toward my dreams. Finally, and most importantly, I’d like to thank my fiancé, Alex McDermott for his unwavering love, patience, and support. Thank you for always being there to celebrate my accomplishments, to wipe
my tears when I failed and for making sure I had everything I needed reach this point. Without all of these amazing people, this journey would have been impossible.
# TABLE OF CONTENTS

ABSTRACT OF THE DISSERTATION ........................................................................... ii

ACKNOWLEDGEMENTS ............................................................................................ iv

TABLE OF CONTENTS ............................................................................................... vi

LIST OF TABLES ......................................................................................................... viii

LIST OF FIGURES ...................................................................................................... ix

ASSOCIATED PUBLICATIONS .................................................................................... xi

CHAPTER 1: LITERATURE REVIEW ........................................................................ 1

1.1 Neural Substrates of Reproduction ................................................................. 2

1.2 Neural Substrates of Energy Balance ............................................................... 7

1.3 Ghrelin: The hunger hormone ........................................................................ 11

1.4 Summary, Hypothesis and Overview ............................................................... 14

References ................................................................................................................. 17

Figures ...................................................................................................................... 26

CHAPTER 2: 17β-ESTRADIOL INCREASES ARCUATE KNDY NEURONAL SENSITIVITY TO GHRELIN INHIBITION OF THE M-CURRENT IN MICE ...................... 28

2.1 Abstract ............................................................................................................ 29

2.2 Introduction ...................................................................................................... 30

2.3 Materials and Methods ................................................................................... 33

2.4 Results ............................................................................................................. 38

2.5 Discussion ....................................................................................................... 43

References ................................................................................................................. 48

Figures ...................................................................................................................... 52

CHAPTER 3: THE ROLE OF GHSR EXPRESSION IN KNDY NEURONS IN THE CONTROL OF REPRODUCTION AND ARCUATE GENE EXPRESSION ..................... 61

vi
# LIST OF TABLES

<table>
<thead>
<tr>
<th>Table</th>
<th>Title</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td><strong>Chapter 2</strong></td>
<td></td>
</tr>
<tr>
<td>1</td>
<td>Resting membrane potentials (RMP) and input resistance (Rin)</td>
<td>55</td>
</tr>
<tr>
<td></td>
<td><strong>Chapter 3</strong></td>
<td></td>
</tr>
<tr>
<td>2</td>
<td>List of primers for ARC qPCR</td>
<td>84</td>
</tr>
<tr>
<td></td>
<td><strong>Chapter 4</strong></td>
<td></td>
</tr>
<tr>
<td>3</td>
<td>List of primers for liver qPCR</td>
<td>131</td>
</tr>
<tr>
<td>Table</td>
<td>Title</td>
<td>Page</td>
</tr>
<tr>
<td>-------</td>
<td>------------------------------------------------------------</td>
<td>------</td>
</tr>
<tr>
<td></td>
<td><strong>Chapter 1</strong></td>
<td></td>
</tr>
<tr>
<td>1</td>
<td>Central hypothesis for E2 regulation of GHSR in KNDy neurons</td>
<td>27</td>
</tr>
<tr>
<td></td>
<td><strong>Chapter 2</strong></td>
<td></td>
</tr>
<tr>
<td>2</td>
<td>M-current Rundown in KNDy neurons</td>
<td>56</td>
</tr>
<tr>
<td>3</td>
<td>XE-991 inhibits the M-current in KNDy neurons</td>
<td>57</td>
</tr>
<tr>
<td>4</td>
<td>Ghrelin inhibits the M-current in KNDy neurons</td>
<td>58</td>
</tr>
<tr>
<td>5</td>
<td>E2 enhances ghrelin sensitivity in KNDy neurons</td>
<td>59</td>
</tr>
<tr>
<td>6</td>
<td>Ghrelin inhibits the M-current via PLC-PKA pathway in KNDy neurons</td>
<td>60</td>
</tr>
<tr>
<td></td>
<td><strong>Chapter 3</strong></td>
<td></td>
</tr>
<tr>
<td>7</td>
<td>Effects on puberty and estrous cyclicity</td>
<td>85</td>
</tr>
<tr>
<td>8</td>
<td>Representative LH pulsatility</td>
<td>86</td>
</tr>
<tr>
<td>9</td>
<td>LH dynamics in intact experimental females in diestrous and proestrous and experimental and control females in diestrous after a 24h fast</td>
<td>87</td>
</tr>
<tr>
<td>10</td>
<td>LH dynamics in OVX experimental females treated with EB or Oil and experimental and control OVX females treated with EB and ghrelin (1mg/kg; IP) 30 minutes prior to collection</td>
<td>88</td>
</tr>
<tr>
<td>11</td>
<td>Arcuate gene expression in OVX control and experimental females with or without E2 replacement</td>
<td>89</td>
</tr>
<tr>
<td>12</td>
<td>Arcuate gene expression in OVX control and experimental females with Ghrelin injection with or without E2 replacement</td>
<td>90</td>
</tr>
<tr>
<td></td>
<td><strong>Chapter 4</strong></td>
<td></td>
</tr>
<tr>
<td>13</td>
<td>Experiment #1: Body weight gain and body composition, and cumulative food intake</td>
<td>132</td>
</tr>
<tr>
<td>14</td>
<td>Experiment #1: Glucose and Insulin tolerance tests.</td>
<td>133</td>
</tr>
<tr>
<td>15</td>
<td>Experiment #1: Average daytime, nighttime and hourly X-activity, Y-activity, Food and Water intake</td>
<td>134</td>
</tr>
<tr>
<td>16</td>
<td>Experiment #1: Average daytime and nighttime V.O2 and V.CO2, RER, and Energy expenditure</td>
<td>135</td>
</tr>
<tr>
<td>17</td>
<td>Experiment #1: Meal pattern analysis</td>
<td>136</td>
</tr>
<tr>
<td>18</td>
<td>Experiment #2: Body weight gain and body composition, and cumulative food intake</td>
<td>137</td>
</tr>
<tr>
<td>19</td>
<td>Experiment #2: Glucose and Insulin tolerance test</td>
<td>138</td>
</tr>
<tr>
<td>20</td>
<td>Experiment #2: Average daytime, nighttime and hourly X-activity, Y-activity, Food and Water intake</td>
<td>139</td>
</tr>
<tr>
<td>21</td>
<td>Experiment #2: Average daytime and nighttime V.O2 and V.CO2, RER, and Energy expenditure</td>
<td>140</td>
</tr>
<tr>
<td>22</td>
<td>Experiment #2: Meal pattern analysis</td>
<td>141</td>
</tr>
<tr>
<td>23</td>
<td>Experiment #3: Fasting Meal pattern analysis</td>
<td>142</td>
</tr>
<tr>
<td>Page</td>
<td>Experiment #3: Ghrelin Meal pattern analysis</td>
<td>143</td>
</tr>
<tr>
<td>------</td>
<td>-------------------------------------------</td>
<td>-----</td>
</tr>
<tr>
<td>25</td>
<td>Experiment #4: Average daytime and nighttime V.O2 and V.CO2, RER, and Energy expenditure</td>
<td>144</td>
</tr>
<tr>
<td>26</td>
<td>Experiment #4: Average daytime, nighttime and hourly X-activity and Wheel Counts</td>
<td>145</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Chapter 6</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
</tr>
<tr>
<td>1</td>
</tr>
</tbody>
</table>
ASSOCIATED PUBLICATIONS

Chapter 2


Additional publications


CHAPTER 1: LITERATURE REVIEW
Chapter 1: Introduction

1.1 Neural Substrates of Reproduction

1.1.1 HPG Axis and basic pathways involved in reproduction

The hypothalamic-pituitary-gonadal (HPG) axis is a highly conserved pathway that regulates the onset of puberty and reproduction. The hypothalamus is the “control center” of the brain wherein signals from the body, like gonadal steroids, are sensed, interpreted, and responded to in order to maintain homeostasis (1, 2). The HPG axis works in a tightly regulated manner to control reproductive behaviors while simultaneously responding to and coordinating the immediate needs of the body, like hunger for example. The basic pathway is initiated when gonadotropin releasing hormone (GnRH) is released from gonadotropin releasing hormone expressing neurons, whose cell bodies reside in the anterior hypothalamus and preoptic region (3). GnRH is released from axon terminals located in the median eminence, a “leaky” portion of the blood brain barrier, which allows for the travel of GnRH to gonadotropin cells within the anterior pituitary (AP), which express GnRH receptors. GnRH will bind to its receptor within the AP, resulting in the release of two gonadotropins: Follicle Stimulating Hormone (FSH) and Luteinizing Hormone (LH). FSH and LH then circulate throughout the blood stream until they reach their receptors located within the gonad. These gonadotropins stimulate both steroidogenesis, and gametogenesis in males and females (2, 4). As a result, serum testosterone and estrogen concentrations rise and act as negative feedback to reduce GnRH release via Kisspeptin/Neurokinin B/Dynorphin expressing neurons, also known as KNDy neurons (5). There are several physiological mechanisms that influence this pathway which may result in dysfunction of the HPG axis and therefore loss of fertility. While the hypothalamus is made up of several distinct nuclei, the arcuate nucleus (ARC) is the primary location for this highly specific coordination (6). Within the ARC, many different neuronal populations involved
in the regulation of reproduction reside, including KNDy neurons. These neurons within this critical brain region are primarily responsible for the onset of puberty, steroidogenesis, and gametogenesis (7-9).

Because this pathway is multifaceted, complex, and adaptable, there is an ever-growing gap in our understanding of how each of these elements (steroids, hormones, and peripheral signaling) are coordinated. The focus of this dissertation is to unravel the neuronal control of reproduction in coordination with steroids (estrogen) and energy status.

1.1.2 Estrogenic influence on the control of reproduction
Steroid hormones most commonly associated with female reproduction are comprised of three isoforms: estrone (E1), estradiol (E2), and estriol (E3) (10). E2, more specifically, 17β-estradiol, is the most biologically potent estrogen and is produced primarily in the ovary. In addition to ovarian production of E2, there is also strong evidence of central neuroestrogen production in several brain regions across multiple species, including songbirds, primates, and the rodent hypothalamus (11-14). The cyclical production of E2, along with other reproductive hormones, like progesterone, are critical to the maintenance of ovarian cyclicity in females, although E2 is also produced by the testes and functions in the HPG axis of male mammals (10, 15).

In addition to reproduction, E2 functions in various roles outside of reproduction and its feedback mechanisms are involved in many other metabolic processes including core body temperature regulation, feeding behavior, and body weight gain (16, 17). E2 is highly conserved among mammals and has critical functions in the central nervous system, endocrine system, immune system, cardiovascular system, renal system, and musculoskeletal system, among others (18). E2 has a plethora of mechanisms of altering cellular physiology. These include classical genomic signaling
initiated via estrogen receptor alpha and beta (ERα and ERβ). Once E2 binds these receptors, they translocate to the nucleus to activate transcription through their interaction with the DNA binding site known as the estrogen response element (ERE) or with other DNA-bound transcription factors (15, 19). Furthermore, E2 can function through rapid membrane-initiated estrogen signaling via nuclear ERs associated with the plasma membrane or E2-responsive G-protein coupled receptors (GPCRs) (20-22). These membrane-mediated effects of E2 signaling initiate intracellular signaling cascades via protein kinase C (PKC), protein kinase A (PKA), phosphatidylinositol-3 kinase (PI3K), and mitogen-activated protein kinase (MAPK) leading to protein phosphorylation, gene transcription, and regulation of ion channels and neuronal excitability (20, 23).

In addition, the expression of E2’s receptors in non-reproductive tissues also suggests E2 has roles outside of reproduction (24, 25). In females, E2 levels differ throughout the ovarian cycle, complicating the use of female rodents as research models (26). It is therefore imperative to consider cyclical levels of E2 and the subsequent feedback mechanisms that impact the HPG axis. These feedback mechanisms for E2 are regulated through both negative and positive feedback. E2 regulation is dependent on additional efferent pathways that converge on 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 for reproductive success.

1.1.3 The role of kisspeptin in regulation of reproduction

Kisspeptin was recently discovered to be the endogenous ligand for G-protein coupled receptor 54, GPR54, circa 2001, now more commonly known as the Kiss1 receptor. Kisspeptin has been shown to project to and control GnRH neuronal excitability (5, 27,
ARC kisspeptin neurons, specifically, have been identified as the major influencing neuronal population which drive the tonic/pulsatile release of GnRH. It is established that ARC kisspeptin neurons express steroid hormone receptors as well as metabolic hormone receptors. For example, similar to proopiomelanocortin (POMC) neurons, kisspeptin neurons can be regulated by insulin and leptin (29).

There are two populations of kisspeptin neurons in the central nervous system: ARC kisspeptin neurons, which also coexpress neurokinin B and dynorphin and kisspeptin neurons in the anteroventral periventricular nucleus (AVPV), both play separate but linked critical roles in regulating reproduction. Both populations of kisspeptin neurons respond to circulating steroids, like E2, but do so differently. AVPV kisspeptin neurons respond to high, preovulatory, levels of E2, like during proestrus or ovariectomy (OVX) with E2 replacement in rodents, leading to a surge in GnRH and subsequently an LH surge to drive the positive feedback regulation of ovulation (30). On the other hand, ARC KNDy neurons mediate negative feedback and GnRH pulsatility by responding to lower levels of E2 and progesterone, as during metestrus and diestrus. Neurokinin B and dynorphin act as positive and negative autoregulators of KNDy neuronal excitability via their receptors NK3R and KOR, respectively, resulting in the generation of a GnRH and subsequent LH pulse (31-36). KNDy neurons have also been found to release excitatory glutamate onto AVPV kisspeptin expressing neurons to control activation of GnRH neurons (27).

1.1.4 Influence of Estradiol on KNDy neurons and LH pulsatility

KNDy neurons are highly susceptible to the influence of E2. With KNDy neurons, E2 not only regulates cell excitability, but also cell receptor expression and ion channel expression, altering the sensitivity to certain regulators in synchrony with the rodent estrous cycle (37). Recent studies found that preovulatory levels of E2 increased ARC
growth hormone secretagogue receptor (GHSR), also known as the ghrelin receptor by 6-fold (38). Furthermore, this increase in Ghsr expression was specifically found to be in KNDy neurons (Tac2-GFP), but not NPY neurons (39). Thus, an objective of my dissertation is to characterize GHSR activity in KNDy neurons.

ARC kisspeptin activity has been greatly associated with pulsatile LH release (40). In fact, ARC lesion studies in the late 1970’s and early 80’s discovered that if the ARC was lesioned, LH pulsatility was abolished in rats and monkeys (41, 42). Not only has this association been solidified through further work, but ARC kisspeptin neurons have also been found to directly sense steroid (E2) negative feedback (7). These findings taken together suggest that LH pulse generation and steroid regulation of GnRH pulses may both converge on KNDy neurons. Proof of this interconnection was established when bath application of neurokinin B (NKB) and dynorphin, both expressed in KNDy neurons, to mouse brain slices had an opposing effect on KNDy neurons; NKB is excitatory, and dynorphin, inhibitory (27, 31, 32). Long-term electrophysiological monitoring of KNDy neuron activity revealed that they exhibit spontaneous peaks and nadirs in firing rate (43). This activity is closely associated with peaks and nadirs of LH concentration in whole blood samples (3, 44).

Furthermore, in 2014, Campos and Herbison determined the GnRH neuronal firing rate necessary to generate a pulse-like increment in LH secretion, which was achieved by activating the GnRH neuron cell bodies or distal processes around the median eminence via optogenetic stimulation (45). A follow up study in which ARC KNDy neurons were specifically targeted and stimulated using optogenetic techniques provided more direct evidence that the synchronous activation of ARC KNDy neurons can generate pulsatile LH release (46). Together these observations point to a hypothesis that KNDy neurons form an interconnected network that is modulated by its neuropeptides in addition to negative feedback by E2 to alter LH pulsatility.
Recent studies have found that E2 reduces the firing rate of KNDy neurons (47), furthermore, in an OVX mouse, where E2 can no longer provide negative feedback, LH pulse frequency increases (44). This work suggests that estradiol modulates firing patterns of KNDy neurons, which in turn modulates LH pulse frequency and concentration. Changes in cell excitability and receptor expression by E2 can lead to the potential to alter LH pulse frequency and ultimately the ability to reproduce.

1.2 Neural Substrates of Energy Balance

1.2.1 Hypothalamic pathways involved in energy balance

Hypothalamic control of fertility is the archetypal homeostatic function. However, reproduction, particularly in females, is extremely metabolically demanding, therefore, there must be coordination between energy status and reproductive function. Because GnRH neurons do not express all of the critical metabolic hormone receptors for sensing nutrient levels, Divall et al. 2010, demonstrated that GnRH neuron-specific deletion of the insulin receptor had no effect on fertility, while a global insulin receptor deletion did (48). Therefore, sensing of energy stores must be accomplished by neurons presynaptic to GnRH neurons. These include anorexigenic proopiomelanocortin (POMC) and orexigenic neuropeptide Y (NPY)/agouti-related peptide (AgRP) neurons, both of which are located within the ARC, near to the median eminence and have been shown to share reciprocal projections with KNDy neurons as well as project to axon terminals of GnRH neurons (49-52).

NPY/AgRP and POMC neurons receive direct, real-time information about the availability of food in the external world, suggesting a primary role for these neurons in controlling appetitive behaviors (53). POMC and NPY/AgRP neurons are also inversely regulated by glucose, insulin, and leptin (54-58). However, GnRH neurons are lacking the postsynaptic receptors, likely to mediate the direct physiological response. There is
support for indirect action of leptin and insulin on GnRH neurons, however a lack of evidence for the direct interaction and lack of expression of their respective receptors indicates that these effects must be mediated by other neurons, like KNDy neurons which do express the appropriate receptors (59, 60).

Therefore, ARC KNDy neurons, acting through POMC and NPY/AgRP neurons are the proposed key neurons coordinating energy status with reproduction (61). In fact, both POMC and NPY/AgRP neurons receive direct glutamatergic input from ARC KNDy neurons in mice, which may be a critical pathway for KNDy neurons to coordinate energy homeostasis and reproduction (52). An in-depth investigation revealed that a high-frequency optogenetic stimulation of ARC Kiss1 neurons coupled with whole-cell patch clamp electrophysiology resulted in a slow excitatory post synaptic potential (EPSP) in POMC neurons but a slow inhibitory post synaptic potential (IPSP) in NPY/AgRP neurons. Therefore, ARC Kiss1 neurons can differentially excite POMC neurons and inhibit AgRP neurons via different classes of metabotropic glutamate receptors (mGluR) (52).

One pathway for KNDy neurons to control energy homeostasis is interactions with the melanocortin circuitry by directly depolarizing POMC neurons through kisspeptin and/or glutamate release and directly or indirectly hyperpolarizing NPY/AgRP neurons through glutamate release and/or through an enhancement of inhibitory GABA-ergic tone (52, 62). Furthermore, POMC and NPY/AgRP neurons modulate KNDy activity (51, 52, 63-65), thus producing a neuronal network of at least three cell types (POMC, NPY/AgRP, and KNDy) that respond differently to steroids, nutrients, and peripheral peptide hormones to modulate energy homeostasis and reproduction simultaneously.

1.2.2 Estrogen’s control of energy homeostasis
17β-estradiol (E2) plays a major role in regulating the hypothalamic control of metabolism in females (16, 63, 66). E2 is known to reduce food intake and increase energy expenditure and activity, in part, through actions in the mediobasal hypothalamus (67-70). OVX female rats showed an increase in adiposity, which is prevented with E2 replacement (71, 72). In ERα knockout female mice, an obese phenotype is observed with an increase in visceral adiposity, decreased energy expenditure, and altered glucose homeostasis and insulin resistance (72-74). E2 has also been found to rapidly increase excitatory input on POMC neurons (75), enhancing anorectic feeding behavior. Furthermore, E2 can also rapidly inhibit NPY/AgRP neurons to reduce orexigenic activity (62, 76). ERα is highly expressed in POMC neurons, and in ERα knockout mice there is an observation of reduced Pomc gene expression (77, 78). In NPY neurons, ERα is expressed but most of estradiol actions are membrane-mediated (79).

1.2.3 KNDy-mediated control on Energy Balance

Work in 2016 suggests that KNDy neurons also contribute to the control of energy homeostasis in a steroid-dependent manner in mice (52). When KNDy neurons are ablated, the post-ovariectomy weight gain associated with E2 in rats is abrogated, suggesting that KNDy neurons mediate, in part, the anorectic effects of E2 (80). Yang et al., 2016 found that both negative (24 h fast and 30% caloric restriction) and positive (diet induced obesity) states of energy balance differentially impact the expression of ARC KNDy neuropeptides and their receptors. They demonstrated that E2 can have protective effects by both augmenting and opposing the effects of positive or negative energy states on KNDy neuropeptides and receptors (38).

More recently, Negrón and Radovick, 2020, demonstrate that overnutrition (diet induced obesity) increases LH pulsatility in diestrus females, but not during estrus when
E2 is elevated. This may indicate that long-term (12 week) HFD exposure leads to inhibited sensitivity to estradiol negative feedback, which is potentially mediated by KNDy neurons, although further work is required to make this determination (81). While there is evidence of E2 production in adipose tissue (82, 83), we do not see elevated E2 in the serum of OVX wild type mice fed a HFD compared to a LFD, therefore E2 derived from adipose tissue is likely not involved in the metabolic effects of OVX mice.

1.2.4 KNDy influence on thermoregulation

Kisspeptin neurons have been found to be involved in other homeostatic processes including thermoregulation and bone remodeling (84, 85). In fact, current studies have confirmed that neurokinin B, expressed in ARC KNDy neurons, mediate hot flushes in response to reduced E2. In this study, optogenetic activation of KNDy axon terminals in the preoptic area of the hypothalamus increased heat dissipation and vasodilation in the tail skin of mice, which was abolished by a neurokinin B receptor antagonist (9, 86).

1.2.5 Energy balance regulation by leptin and insulin

Energy balance is not only regulated by E2 and ARC neurons in the hypothalamus, but also influenced by peripheral hormones like leptin, insulin, and ghrelin, to name a few. Leptin is primarily produced by adipocytes, insulin is produced by the pancreas, and ghrelin is primarily produced by the stomach. All three of these hormones bind to their receptors, located within the ARC and highly expressed in POMC, NPY/AgRP and KNDy neurons to communicate the energetic and metabolic needs of the body to the central nervous system.

Leptin is known to decrease body weight and adiposity and improve metabolic control by regulating central and peripheral effector pathways. Leptin has been found to reduce excitatory input to NPY/AgRP neuronal activity (87), decrease Npy mRNA
expression and reduce food intake in non-obese rodent models (88). Furthermore, leptin increases the frequency of action potentials in POMC neurons by depolarization (57). Taken together, leptin concentrations are important for signaling energy deficits to the HPG axis, whereas high leptin concentrations, often seen in obesity are associated with leptin resistance (63, 89).

Insulin signaling in the hypothalamus plays a significant role in regulation of food intake and energy homeostasis. Circulating levels of insulin have been found to be positively correlated with body weight and adiposity (90-92). In addition, insulin relays information on peripheral energy stores to the central nervous system by acting through POMC and NPY/AgRP neurons in the ARC. Insulin has been shown to hyperpolarize and inhibit both AgRP and POMC neurons (78, 93, 94), however, there are reports that insulin can also activate AgRP and POMC neurons (95, 96). Intracerebroventricular (ICV) administration as well as intranasal spray administration of insulin results in reduced food intake and body weight in rodents (97) and human men, but not women (98). Studies of insulin resistance or insulin receptor knock out show the opposite effects, an increase in food intake and body weight (60, 99, 100).

1.3 Ghrelin: The orexigenic peptide

1.3.1 Ghrelin’s implications in energy balance

Ghrelin, known as the hunger hormone, is a brain-gut peptide hormone secreted from the stomach to stimulate food intake by acting on its receptor, GHSR, expressed in the hypothalamus (101-103). Proghrelin is primarily synthesized by exocrine X/A-like cells in the stomach and is acylated by an enzyme ghrelin O-acyltransferase (GOAT) (104). Acylated proghrelin is then cleaved by proprotein convertase 1/3 (PC1/3) to form ghrelin. PC1/3 might also cleave non-acylated proghrelin to produce desacyl ghrelin (105, 106). While ghrelin is primarily responsible for driving food intake and regulating
metabolic functions, desacyl ghrelin has also been found to play a role in feeding as well as anxiety-like behaviors in male mice (107, 108).

Ghrelin-expressing neurons are also found in the periventricular region of the hypothalamus, dorsal to the ARC (109, 110). Ghrelin mRNA has been found primarily in the stomach, but also in the intestine, pancreas, kidneys, and even placental tissue (111, 112). Long-term ghrelin treatment increases body weight by promoting adipogenesis and reducing energy expenditure (113). Additionally, ghrelin is known to regulate glucose homeostasis via uncoupling protein 2 (UCP2)-mediated inhibition of glucose-induced insulin secretion (114) and GHSR is responsible for maintaining glucose levels in calorie-restricted rodents (115). Central and peripheral ghrelin administration induces NPY/AgRP gene expression (116-119), NPY activation (120), and potently depolarizes NPY neurons (102, 121). Furthermore, ghrelin activation of GHSR excites NPY neurons and controls calcium homeostasis (102, 121) by activating calcium channels (122) and inhibiting the M-current, a KCNQ channel potassium current (39, 123). Recently, we reported that E2 increases ARC Ghsr expression, but not in NPY neurons (39, 124). Because ghrelin is orexigenic and E2 is anorexigenic, this E2-induced increase in Ghsr expression in the ARC occurs in other ARC neurons to mediate ghrelin’s effects on a range of homeostatic and physiological functions.

1.3.2 Ghrelin and reproduction

While ghrelin actions on NPY neurons are well characterized, only a few studies have examined the actions of ghrelin on KNDy neurons. In one study, central ghrelin administration had no effect on ARC Kiss1 expression in a fed or fasted state (125) and, in a second study, ghrelin depolarized more KNDy neurons in E2-treated females than in vehicle-treated females (124). Ghrelin also depolarizes more KNDy neurons in proestrus and E2-treated OVX females (124) and evokes a greater suppressive effect
on LH secretion without altering ARC *Kiss1* expression (125-127). These data suggest that direct effects of ghrelin on KNDy neuronal excitability, and not kisspeptin transcription, mediate the suppression of LH pulse frequency. Furthermore, GHSR is not regulated by E2 in the AVPV and is expressed in ~3-6% of AVPV Kiss1 neurons (124). Ghrelin suppresses the frequency of pulsatile LH release in male and female rats, in part, due to activation of β-endorphin (POMC) signaling (125-129). Thus, E2 modulation of ghrelin signaling and ghrelin’s inhibition of LH is due to actions in ARC KNDy neurons and not AVPV Kiss1 neurons. Furthermore, we recently demonstrated that E2 increases *Ghsr* expression by 6-fold in KNDy (*Tac2*-GFP) neurons (38), indicating that these two hormones interact at the level of KNDy neurons to control reproduction and metabolism.

### 1.3.3 Ghrelin and thermoregulation

Products of the preproghrelin gene, including ghrelin and obestatin, are involved in sustaining energy stores in mammals and regulating core body temperature (Tc) in response to harsh environmental conditions (130-132). In most mammals, plasma ghrelin concentrations increase and decrease before and after a meal and increase with fasting or food restriction. In mice, which undergo torpor due to food scarcity and low ambient temperatures, ghrelin administration lowers Tc and enhances torpor.

Interestingly, the ghrelin-induced torpor response is eliminated by ablation of the ARC (131). Fasting, which typically stimulates ghrelin, induces torpor in mice and rats housed in cool environments (133, 135). Ghrelin controls the thermoregulatory response to cold stress through time-dependent changes in the circadian rhythm of hypothalamic neurosecretory neurons (135). Intracerebroventricular (ICV) infusion of ghrelin reduces Tc (135) and suppresses BAT activity (136) and ICV des-acyl ghrelin stimulates medial preoptic area (mPOA) neurons leading to downstream
parasympathetic activation and regulation of Tc (137). However, large doses of ghrelin elicit a hyperthermic response, mainly by activating the hypothalamic-pituitary-adrenal axis and corticotropin-releasing hormone (138, 139). Deletion of GHSR in AgRP neurons or in the brain increases thermogenesis by activating BAT and inguinal fat by induction of thermogenic regulatory genes (Ucp1, Ucp3, Pgc1α, and Adrb3) in BAT and inguinal fat (140-142).

1.4 Summary, Hypothesis and Overview

1.4.1 Preliminary Data, Hypothesis & Approach

A former graduate student in the lab, Dr. Jennifer Yang, previously reported that E2 increases GHSR expression in arcuate KNDy neurons by six-fold (38). When these data are considered with the differential control of E2, ghrelin, and KNDy neurons in controlling reproduction, energy homeostasis, and thermoregulation, my dissertation sought to determine what are the neurophysiological, physiological, and behavioral consequences of these interactions. Therefore, my central hypothesis (Figure 1) is that E2 increases KNDy sensitivity to ghrelin in females by augmenting Ghsr expression to suppress the LH production during states of elevated ghrelin (fasting, torpor, or during periods of food scarcity), and to diminish the orexigenic input of ghrelin to NPY neurons by enhancing the activity of POMC neurons, via glutamate, and to control thermogenesis via activation of sympathetic tone from POMC innervation into the PVH and via the release of neurokinin B in the POA, respectively. This dissertation illustrates the importance of examining female animal models within and without the context of sex differences as most of the previous work in this field has been studied in males. In addition, this dissertation, will inform our understanding of how reproduction and energy homeostasis (including thermoregulation) are controlled in part, through common central KNDy/POMC/NPY mediated pathways with similar hormonal inputs, like E2 and
ghrelin from peripheral organs. This will be achieved through a multidisciplinary approach involving electrophysiology, reproductive and metabolic studies. Finally, this dissertation will provide a potential target within the proposed pathway for the amelioration of amenorrhea and other disturbances to the HPG axis as a consequence of positive and negative changes in energy balance. Understanding these physiological integrative controls will have implications for a wide range of pathophysiological conditions including anorexia, cachexia, obesity, and the effects of bariatric surgery.

This project will take a multidisciplinary approach by evaluating the neurophysiological effect of E2 on GHSR expression in KNDy neurons in female mice (Chapter 2) and in control the estrous cycle, LH pulsatility, and ARC gene expression in a novel Kiss1-specific KO of GHSR (Chapter 3), and in the control of energy and glucose homeostasis, the response to ovariectomy, a high-fat diet challenge, in addition to fasting- and ghrelin-induced feeding behavior (Chapter 4).

1.4.2 Project Overview

**Objective 1:** To determine the electrophysiological mechanisms that underlie KNDy neuronal sensitivity to ghrelin when E2 is high, prior to ovulation. In addition, to identify the second messenger cascade involved in the ghrelin inhibition of the M-current. To study E2 enhanced ghrelin signaling in ARC KNDy neurons, we utilized Tac2-EGFP female mice. Females were ovariectomized and treated with E2 or oil to determine differences in KNDy sensitivity to ghrelin (Chapter 2).

**Objective 2:** To characterize the role of E2-induced GHSR expression in KNDy neurons in the suppression of LH pulsatility in females. To study LH pulsatility in response to different energy states, we developed a Kiss1-specific GHSR knockout female mouse model and performed a LH assay in different phases of the estrous
cycle in conjunction with a fasting state in addition to ovariectomizing and treated with E2 or oil in addition to ghrelin injection prior to the LH assay. We also explored the ARC gene expression in response to these different conditions (Chapter 3).

Objective 3: To examine the importance of E2-induced GHSR expression in KNDy neurons in the maintenance of energy balance and thermoregulatory responses to temperature stress. To study E2-induced Ghsr expression in KNDy neurons and its role energy balance and thermoregulation, we used the Kiss1-specific GHSR knockout female mouse model, ovariectomized and treated them with E2 or oil and measured body weight, glucose and insulin tolerance, metabolic rates (V.O2, V.CO2), substrate utilization (RER), food intake, activity, and meal patterns. In a separate cohort of mice, females remained intact and were fed either a low -diet or high-fat diet and the same measurements were taken. In a third cohort of mice, females remained intact and we surgically placed a core body temperature probe inside the abdomen and measured the response to 6h cold stress in conjunction with metabolic rates (V.O2, V.CO2), substrate utilization (RER), food intake, and activity. Finally, in a fourth cohort of mice, females remained intact, in two separate experiments, mice were fasted for 24h and refed or injected with ghrelin (IP; 1mg/kg) or saline while we measured meal patterns (Chapter 4).
References


Central Infusion of Ghrelin Increases Hypothalamic Neuropeptide Y and Agouti-Related Protein mRNA Levels and Body Weight in Rats. Diabetes. 2001;50(7-12):2438-43.


2010;151:4236-46.


Figures

Figure 1: Central hypothesis for E2 regulation of GHSR in KNDy neurons. 1) When estrogen is high, there is an increase in GHSR expression in KNDy neurons, potentially making them more sensitive to the effects of ghrelin. Ghrelin depolarizes KNDy neurons, leading to more output of kisspeptin as well as glutamate. This output will directly excite POMC neurons which release β-endorphin onto the terminals of 2) GnRH neurons, inhibiting LH pulses. Furthermore, while POMC is excited, 3) NPY is inhibited leading to enhanced α-MSH release to MC4R expressing neurons in the 4) PVH which can alter energy expenditure and activity and finally alter 5) thermoregulation through NKB signaling in the POA.
Figure 1.
CHAPTER 2: 17β-ESTRADIOL INCREASES ARCUATE KNDF NEURONAL SENSITIVITY TO GHRELIN INHIBITION OF THE M-CURRENT IN MICE
2. 17β-estradiol increases arcuate KNDy neuronal sensitivity to ghrelin inhibition of the M-current in female mice

2.1 Abstract

Obesity and anorexia result in dysregulation of the hypothalamic-pituitary-gonadal axis, negatively impacting reproduction. Ghrelin, secreted from the stomach, potentially mediates negative energy states and neuroendocrine control of reproduction by acting through the growth hormone secretagogue receptor (GHSR). GHSR is expressed in hypothalamic arcuate (ARC) Kisspeptin/Neurokinin B (Tac2)/Dynorphin (KNDy) neurons. Ghrelin is known to inhibit the M- current produced by KCNQ channels in other ARC neurons. In addition, we have shown 17β-estradiol (E2) increases Ghsr expression in KNDy neurons 6-fold and increases the M- current in NPY neurons. We hypothesize that E2 increases GHSR expression in KNDy neurons to increase ghrelin sensitivity during negative energy states. Furthermore, we suspect ghrelin targets the M- current in KNDy neurons to control reproduction and energy homeostasis. We utilized ovariectomized (OVX) Tac2-EGFP adult female mice, pre-treated with estradiol benzoate (EB) or oil vehicle and performed whole-cell-patch-clamp recordings to elicit the M- current in KNDy neurons using standard activation protocols in voltage-clamp. Using the selective KCNQ channel blocker XE-991 (40 µM) to target the M-current, oil- and EB-treated mice showed a decrease in the maximum peak current by 75.7 ± 13.8 pA (n=10) and 68.0 ± 14.7 pA (n=11), respectively. To determine the actions of ghrelin on the M-current, ghrelin was perfused (100 nM) in oil- and EB-treated mice resulting in the suppression of the maximum peak current by 58.5 ± 15.8 pA (n=9) and 59.2 ± 11.9 pA (n=9), respectively. KNDy neurons appeared more sensitive to ghrelin when pre-treated with EB, revealing that ARC KNDy neurons are more sensitive to ghrelin during states of high E2.
2.2. Introduction

Periods of undernutrition caused by food scarcity as well as overnutrition caused by an abundance of food are both associated with an imbalance in energy status that leads to a suppressed hypothalamic-pituitary-gonadal (HPG) axis, resulting in cessation of reproduction. While obesity has been linked to coronary heart disease, and type 2 diabetes mellitus (1), it also negatively impacts reproduction. In women, obesity leads to irregular menses, infertility, and miscarriages, among other complications (2, 3). These problems in reproductive health extend to individuals suffering from undernutrition. Amenorrhea is prominent in athletes and anorexia nervosa patients, largely due to their decreased body mass index (4). These perturbations in energy balance can lead to problems in the HPG axis, though the precise mechanisms remain unclear.

Energy balance and reproduction are centrally regulated processes that are controlled, in part, by neurons in the arcuate nucleus of the hypothalamus (ARC). Regulation of reproduction is controlled by negative and positive feedback of 17β-estradiol (E2) on the HPG axis mediated, to some extent though not entirely, by neurons expressing kisspeptin. There are two main groups of kisspeptin neurons located in the rodent brain (5). Kisspeptin is expressed in neurons in the anteroventral periventricular nucleus (AVPV) and ARC. ARC kisspeptin neurons co-express neurokinin B (Tac2), and dynorphin (Pdyn), and are therefore termed KNDy neurons. KNDy neurons mediate the negative feedback of E2 and progesterone on the HPG axis in females (6, 7). Neurokinin B and dynorphin in KNDy neurons act as positive and negative autoregulators of KNDy neuronal excitability, respectively. Their combined actions produce the pulse generator that controls gonadotropin releasing hormone (GnRH) release into the median eminence and subsequent release of Luteinizing
Hormone (LH) and Follicle Stimulating Hormone (FSH) from the anterior pituitary (8-12). Both AVPV and ARC kisspeptin regulate the HPG axis by binding to its receptor *Kiss1r* expressed on the soma and axons of GnRH neurons (7-9, 13). KNDy neurons also contribute to the control of energy homeostasis, especially in females. Ablation of KNDy neurons abrogates the post-ovariectomy weight gain associated with E2 in rats, suggesting that KNDy neurons mediate, in part, the anorectic effects of E2 (14). One pathway for KNDy neurons to control energy balance is by directly depolarizing POMC neurons via kisspeptin and/or glutamate (15, 16).

Although the neuroendocrine mechanisms that link reproduction and energy balance are not well understood, it is known that ghrelin suppresses the frequency of pulsatile LH release in male and female rats as well as humans, in part, due to activation of β-endorphin (POMC) signaling (17-19). Ghrelin is a brain-gut peptide hormone secreted from the stomach to stimulate food intake by acting on its receptor, growth hormone secretagogue receptor (GHSR) expressed in the hypothalamus (20, 21). GHSR is expressed throughout the brain particularly in NPY/AgRP neurons in the ARC, while only 10% of POMC neurons express GHSR (22, 23). Furthermore, ghrelin activation of GHSR excites NPY neurons and controls calcium homeostasis (23, 24) by activating calcium channels (25, 26) and inhibiting the M-current, a hyperpolarizing, inwardly-rectifying potassium channel current (26, 27). Recently we reported that E2 increases *Ghsr* expression in the ARC, but not in NPY neurons (26, 28). While ghrelin actions on NPY neurons are well characterized, only a few studies have examined the actions of ghrelin on KNDy neurons. In one study, central ghrelin administration had no effect on ARC *Kiss1* expression in a fed or fasted state (29) and, in second study, ghrelin depolarized more KNDy neurons in E2-treated ovariectomized (OVX) females than in oil- treated females (29, 30). These data suggest that direct effects of ghrelin
on KNDy neuronal excitability, and not kisspeptin transcription, mediate the suppression of LH pulse frequency.

Furthermore, we recently demonstrated that E2 increases Ghsr expression by 6-fold in KNDy (Tac2-GFP) neurons (30), which can potentially increase KNDy neuronal sensitivity to ghrelin. The M-current is a subthreshold voltage gated potassium current generated by KCNQ channels which modulate firing frequency in neurons. KCNQ channels are expressed in numerous brain regions including: the hippocampus, the cortex, and the hypothalamus (27). Additionally, KCNQ channels are negatively modulated by Gq-coupled G-protein coupled receptors, including GHSR. The binding of ghrelin to GHSR activates the alpha subunit which activates the phospholipase C (PLC) signaling cascade. Once PLC becomes activated, it hydrolyzes the membrane phospholipid, phosphatidylinositol 4, 5-bisphosphate (PIP2) for inositol triphosphate (IP3) and diacylglycerol (DAG). IP3 is released into the cytosol which goes on to increase intracellular calcium. Additionally, PLC activation of the membrane associated DAG can activate protein kinase C (PKC) which then decreases KCNQ channel (or M-current) conductance (27, 31). Our previous work has shown that the M-current can be modulated by fasting and 17β-estradiol in NPY neurons of the hypothalamus (32). In addition, activation of the 5-HT2c receptors inhibits the M-current in POMC neurons of the hypothalamus (33). Because KNDy neurons can directly depolarize POMC neurons via kisspeptin and glutamate, (15, 16) and because fasting (increasing ghrelin) suppresses the M-current in NPY neurons (32), we speculate that KNDy neuronal ghrelin sensitivity and the M-current play a significant role in the control of energy balance and reproduction.

Because E2, ghrelin, and KNDy neurons all control reproduction and energy homeostasis (14, 34-36), we hypothesize one potential mechanism could be that
Steroids (E2) increase ghrelin sensitivity in KNDy neurons by augmenting KCNQ channel conductance and Ghsr expression to modulate the control of reproduction and other hypothalamic pathways. During states of elevated E2 (proestrus or ovariectomy with E2 replacement), GHSR expression is increased in KNDy neurons, augmenting KNDy sensitivity to ghrelin. These transcriptional effects produce KNDy neurons more sensitive to states of elevated ghrelin (fasting, caloric restriction) increasing KNDy neuronal output, kisspeptin or glutamate release onto ARC POMC and NPY neurons (37). POMC activation would increase the release of β-endorphin on GnRH neurons to suppress LH pulsatility (23). To begin to elucidate this circuit and the interactions of E2 and ghrelin in KNDy neurons, we used whole-cell patch-clamp electrophysiology in a Tac2-GFP mouse model and determined the impact of E2 and ghrelin on M-current activity and excitatory postsynaptic currents.

2.3 Materials and Methods

2.3.1 Animals

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 (6-12 weeks of age) were housed under constant photoperiod conditions (12/12 h light/dark cycle), with lights on/off at 7:00 h and 19:00 h and maintained at a controlled temperature (25°C). Animals were given food (LabDiet 5V75) and water ad libitum. Animals were weaned at postnatal day 21 (PD21). Sexually mature Tac2-EGFP females were used for all electrophysiology experiments. Genotype was determined by using PCR products of extracted DNA from ear clippings. Tac2-EGFP mice were generated by mating either positive EGFP males to positive EGFP females or positive EGFP males to WT EGFP females.
2.3.2 Surgical Procedure

To elucidate the interactions of E2 and the M-current, adult females were bilaterally ovariectomized (OVX) under isoflurane anesthesia 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, females were separated into 2 treatment groups – oil and estradiol benzoate (EB) (n = 8-10 cells, 7-8 mice per group). An EB injection protocol was used that has previously been shown to alter gene expression in the hypothalamus and mimic a proestrus state (13, 38). Animals were injected subcutaneously (s.c.) at 1000 h on post-OVX day 5 with either 0.25 μg of EB or oil-vehicle. On post-OVX day 6, a 1.5 μg dose of EB or oil was injected at 1000 h. On post-OVX day 7, mice were rapidly decapitated, and the brain was prepared for electrophysiology.

2.3.3 Drugs

All drugs were purchased from Tocris unless otherwise specified. Tetrodotoxin (TTX, 1 mM stock), Ghrelin, a GHSR agonist, (100 mM stock) and the GHSR antagonist [D-Lys3]-GHRP-6 (100 mM stock) were all dissolved in ultrapure H2O. The PKA inhibitor H-89 dihydrochloride (10 mM stock), the PKC inhibitor rottlerin (10 mM stock), the PLC inhibitor U73122 (20 mM stock), and 10, 10-bis (4-pyridinylmethyl)-9(10h)-anthracenone dihydrochloride (XE-991), a KCNQ channel blocker (40 mM stock) were all dissolved in DMSO. Aliquots of the stock solutions were stored at -20°C until needed. Estradiol benzoate (EB) was purchased from Steraloids (Newport, RI, USA) and dissolved in ethanol (1 mg/ml) prior to mixing in sesame oil (Sigma-Aldrich).
Ethanol was allowed to evaporate off for 24h prior to storage. EB was stored at 4°C until needed.

### 2.3.4 Tissue Preparation

Animals were rapidly decapitated on day 7 following OVX. The brain was quickly removed from the skull, and a block containing the hypothalamus was immediately dissected and submerged in cold (4°C), oxygenated (95% O₂, 5% CO₂), high-sucrose artificial cerebrospinal fluid (aCSF) consisting of 208 mM sucrose, 2 mM KCl, 26 mM NaHCO₃, 10 mM glucose, 1.25 mM NaH₂PO₄, 2 mM MgSO₄, 1 mM MgCl₂, 2 mM CaCl₂, and 1 mM HEPES (pH 7.3; 300 mOsm). Coronal slices (250 μm) were cut on a vibratome at 4°C. The slices were transferred to an auxiliary chamber where they were kept at room temperature (25°C) in aCSF consisting of 124 mM NaCl, 5 mM KCl, 2.6 mM NaH₂PO₄, 2 mM MgCl₂, 2 mM CaCl₂, 26 mM NaHCO₃, and 10 mM glucose (pH 7.3; 310 mOsm) until recording (recovery for 1–2 h). A single slice was transferred to the recording chamber mounted on an Olympus BX51W1 upright microscope. The slice was continually perfused with warm (35°C), oxygenated aCSF at 1.5 mL/min and allowed to acclimate for 15 minutes prior to patching. Targeted neurons were viewed with an Olympus x40 water-immersion lens. Uteri were removed, and wet weight was recorded to confirm the effect of oil or EB treatment. Mean uterine weights from oil-treated females were 0.041 g ± 0.0013, while EB-treated females were 0.15 g ± 0.0043 (p< .0001).

### 2.3.5 Electrophysiology Recordings

Electrophysiology was performed as described previously (33, 39, 40). In hypothalamic slices, standard whole-cell patch-clamp recording procedures and pharmacological
testing were used. Whole-cell patch-clamp recordings were performed using pipettes made of borosilicate glass and pulled using a vertical PC-10 Narishige Glass Micropipette Puller (Narishige Group, Japan). An Axopatch 700B amplifier, Digidata 1322A Data Acquisition System, and pCLAMP software (version 10.2; Molecular Devices) were used for data acquisition and analysis.

Current-voltage (I-V) plots were constructed by voltage steps from −50 to −140 mV at 10- mV increments applied at 1-second intervals from a holding potential of −60 mV. The input resistance (Rin) was determined from the slope of each I-V plot in the range between −60 and −80 mV. Input resistance, series resistance, and membrane capacitance were monitored throughout the experiments. Only cells with stable series resistance (<30 MΩ; <20% change over the course of the recording) and input resistance (>500 MΩ) were used for analysis. Low- pass filtering of the currents was conducted at a frequency of 2 kHZ. The liquid junction potential was calculated to be 10 mV and corrected for during data analysis using pClamp software. All voltage-clamp recordings were performed from a holding potential of −60 mV.

To record M-currents, pipettes (3- to 5 MΩ resistance) were filled with an internal solution containing 10 mM NaCl, 128 mM K-glucuronate, 1 mM MgCl, 10 mM HEPES, 1 mM ATP, 1.1 mM EGTA, and 0.25 mM GTP (pH 7.3; 300 mOsm). In voltage-clamp, a standard deactivation protocol (33, 39) was used to measure potassium currents elicited during 500-millisecond voltage steps from −30 to −75 mV in 5-mV increments after a 300-millisecond prepulse to −20 mV. The amplitude of the M-current relaxation or deactivation was measured as the difference between initial (<10 ms) and sustained current (>475 ms) of the current trace in the control conditions (1 μM TTX; 5 min). After baseline recording (~5 min), XE-991 (40 μM with 1 μM TTX) or ghrelin (100 nM with 1 μM TTX) was perfused for 10 minutes, and the protocol was repeated. Deactivation protocol was repeated twice for each perfusion condition and averaged for analysis.
The ghrelin dose response was recorded in current- and voltage-clamp, where continuous recording was monitored ~2 min before perfusion to establish a baseline. Ghrelin was perfused at a range of concentrations (0.1 to 1000 nM; 3 concentrations per neuron for 5 minutes each in the presence of 1 μM TTX) to determine if there is a change in sensitivity to ghrelin (cells depolarize at lower concentrations of ghrelin). Miniature Excitatory Post-Synaptic Current (mEPSC) frequency and amplitude were analyzed from voltage-clamp recordings using pCLAMP software (version 10.2; Molecular Devices). Briefly, using event detection, for each voltage-clamp trace a mEPSC template was chosen. Once a template was chosen, event detection was utilized to select mEPSCs and each individual mEPSC was manually accepted or rejected. At the end of the trace, the total number of mEPSCs were used to determine frequency. To determine amplitude, mEPSCs were averaged and the resulting trace was measured for amplitude. This process was repeated for each concentration of ghrelin.

To determine the second messenger pathway, the same M-current protocol was utilized where control conditions were 1 μM TTX + inhibitor for 5 minutes followed by 1 μM TTX + inhibitor + 100 nM ghrelin for 10 minutes. For this experiment, all Tac2-EGFP females were gonadally intact and in diestrus, determined by vaginal cytology the morning of the experiment. All uteri were dissected and weighed for confirmation.

### 2.3.6 Statistical analysis

Comparisons of the I-V plots between control and subsequent drug conditions were performed at each voltage (−30 to −75 mV) using a repeated-measures two-way ANOVA with post hoc Bonferroni’s multiple comparison test. Maximum current at −35 mV was analyzed with paired Student's t test. All statistical analysis was conducted using GraphPad Prism (GraphPad). Data were considered significant when P < .05. All
data values were presented as mean ± SEM. Each n represents the number of cells examined.

2.4 Results

2.4.1 Rundown of the M-current

Because the KCNQ channel current (M-current) in KNDy neurons has not been previously studied, we initially established M-current activity using the selective KCNQ channel blocker XE-991, 40 μM a dose based on previous investigation (32, 40). To establish efficacy, we performed current-clamp recordings from Tac2-EGFP neurons and applied XE-991 to the bath solution. This resulted in an average depolarization of 3.5 mV ± 0.86 (n=4 cells), confirming that XE-991 was able to block KCNQ channels in these neurons (Figure 2A), the average RMP was -66.75 mV ± 1.31. To explore whether ghrelin would do the same, we repeated the current-clamp recordings and applied ghrelin (100 nM) to the bath solution. This resulted in an average depolarization of 10.33 mV ± 4.63 (n=3 cells), confirming that ghrelin does depolarize these neurons potentially through blocking KCNQ channels (Figure 2B), the average RMP was -72.33 mV ± 2.03. To evaluate the M-current in voltage-clamp, we measured the deactivation or relaxation of the whole-cell K⁺ currents elicited by an established protocol (41, 42). M-current was calculated by subtracting the current relaxation (the difference between the instantaneous and steady state; arrows) (Figure 2C) during continuous extracellular perfusion of TTX (1 μM). Because the M-current has been previously reported to show a decrease or “rundown” after cell dialysis in whole-cell recordings in some neuronal cell types, but not all (32, 43), the whole-cell K⁺ currents evoked by the deactivation protocol were monitored over a period of 30 minutes to determine the change in the relaxation currents over time. In Tac2 neurons from oil-
treated, OVX females, the outward $\text{K}^+$ currents evoked decreased by approximately 50% over a period of 20 minutes ($\rho < 0.01$; Figure 2D). Therefore, all subsequent recordings were restricted < 20 minutes to limit the rundown and ensure that any changes observed were due to drug application (32).

2.4.2 Regulation of M-current activity by E2

To determine if estrogen replacement alters M-current activity in KNDy neurons, we examined the effects of XE-991 in Tac2 neurons from oil- and EB-treated OVX Tac2-GFP females. In the presence of TTX (1 µM) to block Na$^+$-spike-dependent synaptic input, XE-991 (40 µM) suppressed evoked currents in both oil- and EB-treated OVX females (Figure 3A-B). XE-991 application resulted in an inhibition of the M-current in the range of -60 to -30 mV in both oil- and EB-treated females ($\rho < 0.05$; Figure 3C-D). However, there was no difference between steroid conditions in the amount of XE-991-sensitive current (Figure 3E). The change in maximum peak current at -35 mV after XE-991 perfusion was $75.7 \pm 13.8$ pA ($\rho < 0.001$, n=10 cells, Figure 3F) in oil-treated females and $68.0 \pm 14.7$ pA ($\rho < 0.001$, n=11 cells, Figure 3F) in EB-treated females. In addition, steroid condition did not alter the mean or change in Resting Membrane Potential (RMP) or Rin due to XE-991 perfusion (Figure 3G-H). The RMP mean for oil-treated females before XE-991 was -56.4 mV ± 6.2, after XE-991 was -40.2 mV ± 7.41. The average change in RMP after XE-991 was 16.2 mV ± 5.9. The RMP mean for EB-treated females before XE-991 was -54.82 mV ± 5.04, after XE-991 was -46.82 mV ± 5.39. The average change in RMP after XE-991 was 8.0 mV ± 3.43. The Rin mean for oil-treated females before XE-991 was 0.76 GΩ ± 0.16, after XE-991 was 0.46 GΩ ± 0.08. The average change in Rin after XE-991 was a decrease of 0.31 GΩ ± 0.13. The Rin mean for EB-treated females before XE-991 was 0.93 GΩ ± 0.25, after XE-991 was
0.62 GΩ ± 0.16. The average change in Rin after XE-991 was a decrease of 0.37 GΩ ± 0.31. RMP and Rin values are summarized in Table 1.

2.4.3 Suppression of the M-current by ghrelin

Our previous data in arcuate NPY neurons indicates that the M-current is a target for the peptide hormone ghrelin. To ascertain if ghrelin also suppresses the M-current in Tac2 (KNDy) neurons, ghrelin (100 nM) was perfused for 10 minutes after an initial (control) deactivation protocol. Ghrelin inhibited the M-current in EB-treated, OVX females with a more robust effect compared to oil-treated, OVX females (Figure 4A-D, EB: (F(1, 16)=9.690, P=0.0067)), although steroid condition did not change the magnitude of M-current inhibition by ghrelin (Figure 4E). The change in maximum peak current at -35 mV after ghrelin perfusion was 58.5±15.8 pA (p<0.05, n=9 cells, Figure 4F) in oil-treated females and 59.2 ± 11.9 pA (p<0.01, n=9 cells, Figure 4F). In addition, steroid condition did not alter the ghrelin-induced change in RMP or Rin (Figure 4G-H). The RMP mean for oil-treated females before ghrelin was -58.22 mV ± 4.7, after ghrelin was -47.67 mV ± 5.69. The average change in RMP after ghrelin was 10.56 mV ± 3.17. The RMP mean for EB-treated females before ghrelin was -52 mV ± 4.11, after ghrelin was -46.33 mV ± 4.72. The average change in RMP after ghrelin was 5.67 mV ± 1.82. The Rin mean for oil-treated females before ghrelin was 1.42 GΩ ± 0.39, after ghrelin was 1.70 GΩ ± 0.73. The average change in Rin after ghrelin was an increase of 0.25 GΩ ± 0.77. The Rin mean for EB-treated females before ghrelin was 1.57 GΩ ± 0.67, after ghrelin was 0.82 GΩ ± 0.15. The average change in Rin after ghrelin was a decrease of 0.75 GΩ ± 0.7. RMP and Rin values are summarized in Table 1.

2.3.4 Ghrelin dose response and Tac2 neuronal activity
Because of our previous findings demonstrating that E2 increases GHSR expression in KNDy neurons (30), we hypothesize that E2 increases KNDy neuronal sensitivity to ghrelin. As we have established that ghrelin can inhibit the M-current more robustly with EB-treatment, we perfused ghrelin at increasing doses (0.01-1000 nM) and monitored cell excitability and depolarization in current- and voltage-clamp, respectively. During current-clamp recordings, Tac2 neurons from EB-treated, OVX females responded to ghrelin at much lower concentrations eliciting action potential firing at lower doses compared to Tac2 neurons from oil-treated females (Figure 5 A-B). The average RMP for all cells before drug application was -65mV ± 6.2mV. However, not all neurons responded to ghrelin in the same way. In voltage-clamp, cells exhibited an inward current, an outward current, or did not respond at all (Figure 5C). Cells were considered responsive if they showed more than an 8% change in current from the maximum current observed. On average the inward currents for 100 nM ghrelin, the only dose where a difference was observed, in oil-treated females was -4.5 pA ± 0.88, while EB-treated females were -11.43 pA ± 2.8 (p=0.0503). Because there was not an outward current for every dose of ghrelin, the average outward currents across all ghrelin doses from oil-treated females were 4.2 pA ± 1.62, while EB-treated females were 7.86 pA ± 2.19. In oil-treated females, 54 cells responded to ghrelin with an inward current, 5 responded with an outward current, and 14 had no response to ghrelin. In EB-treated females, 66 cells responded to ghrelin with an inward current, 7 responded with an outward current, and 10 had no response to ghrelin. Amongst the cells that responded with an inward current, ghrelin perfusion (100 nM) elicited a more robust current in EB-treated females compared to oil-treated females (p<0.05; Figure 5D). Interestingly, perfusion of 1000 nM ghrelin in EB-treated females did not elicit a stronger current, potentially due to desensitization of GHSR (44). During our voltage-clamp recordings, (Figure 5E-F) we observed a potential effect of steroid on miniature excitatory
postsynaptic currents (mEPSC). Analysis of these traces revealed a reduction of mEPSC frequency, but not amplitude, in EB-treated females compared to oil-treated (p<0.05; Figure 4 G-H), indicating an interaction of E2 on presynaptic glutamate release.

2.4.5 Ghrelin signals through a PLC-mediated pathway

Previous publications have established KCNQ channels are negatively modulated by Gq-coupled G-protein coupled receptors (GPCR), including GHSR (27). The binding of ghrelin to GHSR activates a phospholipase C (PLC) signaling cascade that hydrolyzes phosphatidylinositol 4,5-bisphosphate (PIP2) for inositol triphosphate (IP3) and diacylglycerol (DAG). IP3 is released into the cytosol and increases release of intracellular calcium from internal stores. DAG activates protein kinase C (PKC) which then either directly decreases KCNQ channel (M-current) conductance (27, 31) or activates PKA (40). To confirm the role of this pathway (PLC-PKC) in attenuating the M-current in Tac2 neurons, we applied a series of specific pharmacological interventions. First, to confirm the role of GHSR, we co-perfused a specific GHSR antagonist [D- Lys3]-GHRP-6 (50 µM) with ghrelin (100 nM) and TTX (1 µM) (Figure 6A). The GHSR antagonist eliminated the effect of ghrelin on the M-current, suggesting the M-current inhibition by ghrelin is indeed mediated by GHSR. Next, we inhibited PLC with U73122 (10 µM) and the PKC inhibitor, rottlerin (5 µM) in separate experiments (Figure 6 B-C). Both U73122 and rottlerin blocked the actions of ghrelin, confirming previous reports. GHSR signaling also may activate PKA as a second messenger (45), which blocks Gq-GPCR inhibition of the M-current in hypothalamic neurons (40). Using H89, the selective PKA inhibitor (10 µM), we observed a reduction of the ghrelin-
induced suppression of the M-current (Figure 6D), indicating that PKA may play a role in Tac2 neurons.

2.5 Discussion

The results of the present study confirm previous findings that 17β-estradiol upregulates Ghsr expression (30), increases KNDy neuronal sensitivity to ghrelin, and subsequently enhances the ghrelin-induced inhibition of the M-current via a PLC-PKA pathway in ARC KNDy neurons. These conclusions are based on the following observations: 1) 100 nM Ghrelin was effective in inhibiting the M-current similar to XE-991, the selective KCNQ channel inhibitor; 2) ghrelin was able to elicit action potentials at a lower dose in EB-treated females; 3) there was a more robust inward current in response to ghrelin in EB-treated females compared to oil-treated controls; and 4) blocking the PLC-PKA pathway blocked the M-current inhibition by ghrelin.

The 17β-estradiol-induced increase in KNDy sensitivity to ghrelin is consistent with previous findings (30) and confirmed with our electrophysiological assessment. Additionally, we have confirmed and corroborated that ghrelin binds to GHSR, a Gq-coupled GPCR, activating PLC to hydrolyze PIP2 for IP3 and DAG. DAG in turn activates PKC which decreases KCNQ channel conductance (M-current inhibition). This results in a more depolarized and excitable cell (27, 31). Ghrelin inhibition of KCNQ channels in KNDy neurons is similar to our previous research on the M-current in other ARC neurons (32, 33, 46). This is further supported by the identification of the second messenger pathway (PLC-PKC) in which ghrelin has previously been described to inhibit the M-current (27).

The current study is also an initial characterization of the M-current in KNDy neurons as XE-991 substantially blocked the current and depolarized all KNDy neurons examined. Interestingly, we found that the M-current in ARC KNDy neurons rundown
in less than 20 min, which has not been observed in other ARC neurons, like NPY or POMC (32). This could be due to cell dialysis during whole-cell patch clamp recordings over time (43) or differences in intracellular signaling that impinges on KCNQ activity (43, 47, 48). Because the M-current is a common target for Gq-coupled GPCR such as 5HT2c serotonergic and M1/5 muscarinic receptors in many types of hypothalamic neurons (33), robust M-current activity in KNDy neurons may be a key, although not the only, mechanism for modulation of KNDy activity.

We have also observed that with EB treatment, lower doses of ghrelin induced action potentials (AP) and at higher doses of ghrelin exhibited reduced APs, possibly due to over- excitation or internalization of the receptor, a common mechanism for Gq-coupled GPCRs (44). The EB-induced effects on ghrelin sensitivity is clearly indicated at the 100 nM dose of ghrelin where an increase in the percent maximum inward current is augmented in EB-treated females. Novaria et al., (2014) found that not all Tac2 neurons are active and some are quiescent (49). Furthermore, not all Tac2 neurons express detectable levels of GHSR (30). Thus, a significant subpopulation of KNDy neurons respond to ghrelin and in sufficient numbers to elicit known physiological control of the HPG axis (36, 50).

Lastly, we observed that EB-treatment reduces mEPSC frequency, but not the amplitude, indicating that there is a decrease in the probability of release from presynaptic neurons. This could be due to a reduction in glutamate signaling from neurons that project to KNDy neurons, including other KNDy neurons. Previous studies have demonstrated that ARC KNDy neurons exhibited increased glutamatergic transmission when ERα was knocked down in these neurons (51), which supports our study with proestrous levels of E2. Furthermore, it is critical to consider that high E2 inhibits neuropeptide expression in these neurons (30), and EB treatment can increase negative feedback via dynorphin in these neurons, ultimately reducing excitability. It is
important to note that we only saw a reduction in mEPSC with the 100 nM dose. As mEPSC amplitude did not change, we expect that there is no change in the expression of NMDA or AMPA receptors in KNDy neurons due to E2 (52).

While the effects of E2 and ghrelin in ARC KNDy neurons have become more defined, it is critical to keep in mind that KNDy neurons are one node of a large circuit within the ARC. KNDy neurons not only project locally to POMC and NPY neurons, but also to preoptic GnRH and kisspeptin neurons of the AVPV, playing an important role in puberty and ovarian functions (37, 49). KNDy neurons activate POMC neurons via kisspeptin and glutamate release and inhibit NPY neurons directly via glutamate and indirectly through kisspeptin (16, 37). As all three neurons modulate the excitability of the other two neuronal populations and respond differently to peripheral peptide hormones, any modulation of one neuronal population by a hormone will have disparate effects on the other two populations. Therefore, we hypothesize that ghrelin’s excitation of KNDy neurons and its potentiation by E2 would reduce ghrelin-induced food intake via NPY neurons in female mice due to the excitation of POMC and the inhibition of NPY. Our hypothesis is supported by previous data demonstrating that E2 suppresses ghrelin-induced food intake in female rats (53). This pathway may be sensitive to energy states, both positive (obesity) and negative (fasting/caloric restriction), as it is known that obesity can cause ghrelin resistance in NPY/AgRP neurons (54) and a high fat diet can reduce the amount of plasma ghrelin in females (26). We are currently exploring the effects of diet-induced obesity on ghrelin’s actions in KNDy neurons.

Furthermore, as ghrelin administration suppresses LH pulsatility through β-endorphin produced by POMC neurons in fed female rats (29, 55), ghrelin activation of KNDy neurons may be involved, as GHSR is not highly expressed in POMC neurons (23). Indeed, KNDy neurons can excite POMC neurons via glutamate during states of elevated E2 (37), which may lead to greater β-endorphin release onto GnRH terminals,
inhibiting LH pulsatility. Hence, during states of elevated ghrelin (fasting, food scarcity, etc.), the HPG axis would be suppressed diverting motivation towards food intake and away from reproduction. Because of this, the role of ghrelin signaling in KNDy neurons may prove to be relevant in the control of reproduction, energy homeostasis, and other physiological processes controlled by KNDy neurons. Future experiments will explore if androgens or E2 in males also regulate Ghsr expression in KNDy neurons and if ghrelin activation of KNDy neurons alters the downstream modulation of POMC or NPY neurons utilizing additional transgenic mouse models.

In conclusion, during the normal reproductive cycle, E2 fluctuates to control the growth of ovarian follicles via negative feedback regulated by ARC KNDy neurons. We have shown that when E2 is high (proestrus or OVX + E2), Ghsr expression is increased in KNDy neurons (30) to augment their sensitivity to states of elevated ghrelin (starvation/caloric restriction) to suppress the HPG axis. Ghrelin, in turn, suppressed the M-current with greater potency leading to depolarization and neurotransmitter (glutamate) release to potentially suppress the HPG axis during periods of food scarcity in females.

2.6 Acknowledgement
The authors have no acknowledgements.

2.7 Statement of Ethics
The research presented in the manuscript was ethically conducted in accordance with institutional guidelines based on National Institutes of Health standards and all animal experiments were performed with Institutional Animal Care and Use Committee approval at Rutgers University.
2.8 Funding Sources

This work was supported by the US Department of Agriculture-National Institute of Food and Agriculture (NJ06195) and the National Institutes of Health (R21ES027119; P30ES005022), and the Rutgers University ONE Nutrition Initiative.

2.9 Author Contributions

KC & TAR conceived and designed the project; KC performed the experiments; KC & TAR analyzed and interpreted data and drafted the manuscript.
References

17. Fernandez-Fernandez R, Tena-Sempere M, Navarro VM, Barreiro ML, Castellano JM, Aguilar E, et al. Effects of ghrelin upon gonadotropin-releasing hormone and


Figures

Table 1. Resting membrane potentials (RMP) and input resistance (Rin) from Figure 3 and 4 in both Control (before) and after either XE-991 (40 µM) or Ghrelin (100nM) perfusion conditions.

Figure 2. M-current Rundown in KNDy neurons. (A) Representative current-clamp trace of the modest depolarization caused by 40 µM XE-991 in an Oil-treated OVX female. (B) Representative trace of the depolarization caused by 100 nM Ghrelin in an intact, diestrus female. (C) M-current deactivation protocol. (D) I-V plot from -75 to -30 mV after 1 µM TTX for 0, 10, 20 and 30 minutes. After a time period of 20-30 minutes, the M-current ran down by ~50%. All subsequent data was collected in under 20 minutes. Data were analyzed by two-way ANOVA and post hoc Bonferroni’s multiple comparison tests. (b=P<.01; c=P<.001; d=P<.0001). Dark blue letters indicate comparison between 10 and 30 minutes, light blue letters indicate a comparison between 10 and 20 minutes. n=7 cells.

Figure 3. XE-991 inhibits the M-current in KNDy neurons Representative current traces of the M-current inhibition caused by 40 µM XE-991 in (A) Oil- and (B) EB-treated OVX female mice. I-V plot from -75 to -30 mV after XE-991 perfusion in (C) Oil- and (D) EB-treated OVX female mice. (E) I-V plot from -75 to -30 mV of the XE-991- sensitive M-current in Oil- and EB-treated OVX female mice. (F) XE-991 reduced the maximum peak current in Oil- and EB-treated mice. (G-H) The mean Resting Membrane Potential (RMP) and Input Resistance (Rin). (C-F) Data were analyzed by two-way ANOVA with Bonferroni’s multiple comparison tests. (G-H)
Data were analyzed by unpaired t-test (a=P<.05; b=P<.01; c=P<.001; d=P<.0001).
Sample sizes for C-H were Oil n=10 cells and EB n=11 cells.

**Figure 4. Ghrelin inhibits the M-current in KNDy neurons**
Representative current traces of the M-current inhibition caused by 100 nM ghrelin in (A) Oil- and (B) EB-treated OVX female mice. I-V plot from -75 to -30 mV after ghrelin perfusion in (C) Oil- and (D) EB-treated OVX female mice. (E) I-V plot from -75 to -30 mV of the ghrelin-sensitive M-current in Oil- and EB-treated OVX female mice. (F) Ghrelin reduced the maximum peak current in Oil- and EB-treated mice. (G-H) The mean Resting Membrane Potential (RMP) and Input Resistance (Rin). (C-F) Data were analyzed by two-way ANOVA with Bonferroni’s multiple comparison tests. (G-H) Data were analyzed by unpaired t-test (a=P<.05; b=P<.01; c=P<.001; d=P<.0001). Sample sizes for (C-H) were Oil n=9 cells and EB n=9 cells.

**Figure 5. E2 enhances ghrelin sensitivity in KNDy neurons**
Representative current-clamp traces of an estrogen-dependent increase in neuronal sensitivity to increasing doses of ghrelin (0.01 nM to 100 nM) in (A) Oil- and (B) EB-treated OVX female mice. Average RMP for all cells before ghrelin application was -65mV ± 6.2mV (C) Table indicating the number of cells from voltage-clamp experiments that responded with either an inward current, outward current or no response. (D) Ghrelin dose response curve. Representative voltage-clamp traces of an estrogen-dependent decrease in mEPSC frequency in (E) Oil- and (F) EB-treated OVX female mice. mEPSC frequency (G) and amplitude (H) before (baseline) and after 100 nM ghrelin. Data were analyzed by two-way ANOVA with Holm-Sidak’s multiple comparison tests. (G-H) were analyzed by unpaired t-test (a=P<.05). Sample sizes are indicated in table (C).
Figure 6. Ghrelin inhibits the M-current via PLC-PKA pathway in KNDy neurons. I-V plots from -75 to -30 mV before (black) and after (green) ghrelin [100 nM] perfusion in diestrus Tac2 female mice with GHSR antagonist, [D-Lys³]-GHRP-6 [50 µM] n=5 cells (A), PLC inhibitor, U73122 [10 µM] n=6 cells (B), PKC inhibitor, Rottlerin [5 µM] n=6 cells (C), and PKA inhibitor H89 [10 µM] n=7 cells (D). Data were analyzed by two-way ANOVA with Bonferroni’s multiple comparison tests.
Table 1. Resting membrane potentials (RMP) and input resistance (Rin) from Figure 3 and 4.

<table>
<thead>
<tr>
<th></th>
<th>Figure 2: Control</th>
<th>XE-991(40µM)</th>
<th>Figure 3: Control</th>
<th>Ghrelin (100nM)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Oil</td>
<td>RMP (mV)</td>
<td>-56.4 ± 6.2</td>
<td>-40.2 ± 7.41</td>
<td>-58.22 ± 4.7</td>
</tr>
<tr>
<td></td>
<td>Rin (GΩ)</td>
<td>0.76 ± 0.16</td>
<td>0.46 ± 0.08</td>
<td>1.42 ± 0.39</td>
</tr>
<tr>
<td>E2</td>
<td>RMP (mV)</td>
<td>-54.82 ± 5.04</td>
<td>-46.82 ± 5.39</td>
<td>-52 ± 4.11</td>
</tr>
<tr>
<td></td>
<td>Rin (GΩ)</td>
<td>0.93 ± 0.25</td>
<td>0.37 ± 0.31</td>
<td>1.57 ± 0.67</td>
</tr>
</tbody>
</table>
Figure 2.

A

XE 991 [40 mM]

-66.8 mV

B

Ghrelin [100 nM]

-72.3 mV

C

-20 mV

300 ms

-60 mV

D

Rundown

-150

Current (pA)

0 min

10 min

20 min

30 min

Voltage (mV)
Figure 3.
Figure 4.

A
Control
Oil
Ghrelin

B
Control
EB
Ghrelin

C

D

E

G

H

Max Peak Current (pA)

RMP (mV)

Rin (GΩ)
Figure 5.

A. Oil-treated Current Clamp

B. EB-treated Current Clamp

C. Table:

<table>
<thead>
<tr>
<th></th>
<th>0.01nM</th>
<th>0.1nM</th>
<th>1.0nM</th>
<th>10nM</th>
<th>100nM</th>
<th>1000nM</th>
<th>Total # Cells</th>
</tr>
</thead>
<tbody>
<tr>
<td>Inward</td>
<td>8</td>
<td>8</td>
<td>12</td>
<td>14</td>
<td>6</td>
<td>6</td>
<td>54</td>
</tr>
<tr>
<td>Outward</td>
<td>1</td>
<td>1</td>
<td>2</td>
<td>1</td>
<td>0</td>
<td>0</td>
<td>5</td>
</tr>
<tr>
<td>No Response</td>
<td>1</td>
<td>3</td>
<td>3</td>
<td>4</td>
<td>2</td>
<td>1</td>
<td>14</td>
</tr>
<tr>
<td>Total # Cells</td>
<td>10</td>
<td>12</td>
<td>17</td>
<td>19</td>
<td>8</td>
<td>7</td>
<td>73</td>
</tr>
</tbody>
</table>

D. Ghrelin Dose Response

E. Oil-treated Voltage Clamp

F. EB-treated Voltage Clamp

G. Graph:

H. Graph:

mEPSC Amplitude (pA)

100nM
Figure 6.

A

- TTX+GHSR Antagonist
- TTX+GHSR Antagonist+Ghrelin

B

- TTX+U73122
- TTX+U73122+Ghrelin

C

- TTX+Rottlerin
- TTX+Rottlerin+Ghrelin

D

- TTX+H89
- TTX+H89+Ghrelin
CHAPTER 3: THE ROLE OF GHSR EXPRESSION IN KNDY NEURONS IN THE CONTROL OF REPRODUCTION AND ARCULATE GENE EXPRESSION
3. The role of GHSR expression in KNDy neurons in the control of reproduction and arcuate gene expression

3.1. Abstract

The gut peptide, ghrelin, mediates negative energy homeostasis and the neuroendocrine control of reproduction by acting through its receptor, growth hormone secretagogue receptor (GHSR). GHSR, expressed in hypothalamic Kisspeptin/Neurokinin B/Dynorphin (KNDy) neurons in the arcuate (ARC), is known to regulate reproduction and energy balance. We have previously shown 17-beta-estradiol (E2) robustly increases Ghsr expression in KNDy neurons, enhancing their sensitivity to ghrelin. We hypothesize that E2-induced GHSR expression augments KNDy sensitivity in a fasting state by elevating ghrelin to disrupt reproduction and reduce energy expenditure in females. We developed a Kiss1-specific GHSR knockout to determine the role of GHSR in ARC KNDy neurons and found no differences in age of vaginal opening or estrous cyclicity. Luteinizing Hormone (LH) pulsatility was measured in fasted, ghrelin-injected, and calorically restricted knockouts and controls. We observed that fasting reduced Luteinizing Hormone (LH) pulses in controls but not knockout females; ghrelin reduced LH in OVX+E2 control females about 90 min post-injection. In addition, gene expression was determined using quantitative real-time PCR from the ARC of OVX females with and without GHSR KO, treated with Oil or EB with or without ghrelin injection. We found that changes in Pdyn, Tac3R, Esr1, and Ghsr expression with EB treatment in controls were abrogated by the deletion of GHSR from KNDy neurons. Adding a ghrelin injection appeared to abrogate genotype differences seen with only EB with Kiss1, Tac2, Pdyn and Esr1. GHSR expression in KNDy neurons potentially mediates the changes in ARC gene expression and is clearly regulated by the interaction between EB and GHSR expression, altering downstream effects on reproduction. Collectively, these data suggest that GHSR
activation in KNDy neurons modulates LH pulsatility as well as ARC gene expression, illustrating a novel mechanism for E2 and ghrelin in control of KNDy neurons and their physiological functions.

3.2 Introduction

Periods of food scarcity or torpor are associated with a negative balance in energy status that leads to a suppressed hypothalamic-pituitary-gonadal (HPG) axis, resulting in temporary cessation of reproduction (1). During these sensitive periods of food scarcity, energy conservation becomes prioritized over reproduction. The neuroendocrine mechanisms that link reproduction with metabolism and thermoregulation are not well understood in most mammals. One peripheral hormone that connects the control of reproduction to thermoregulation and negative energy states, is the gut peptide ghrelin (2-4). Ghrelin is produced primarily by the stomach to drive hunger via the ghrelin receptor, growth hormone secretagogue receptor (GHSR), expressed in hypothalamic arcuate (ARC) neuropeptide Y (NPY) and KNDy (Kisspeptin-Neurokinin B-Dynorphin) neurons (5, 6). Ghrelin is known to suppress luteinizing hormone (LH) pulse frequency in rodents, through β-endorphin signaling from proopiomelanocortin (POMC) neurons that typically do not express GHSR (7-11). KNDy neurons have been confirmed to project to both NPY and POMC neurons within the ARC as well as cross-talk with other KNDy neurons (12).

Reproduction is a centrally regulated process that is controlled by cooperation between several hypothalamic and extra hypothalamic nuclei, including the ARC. In fact, these neuronal populations interact with each other through a wide variety of neuropeptides and neurotransmitters. In rodent models, reproduction is controlled by the negative and positive feedback of E2 on the Hypothalamic-Pituitary-Gonadal (HPG) axis, mediated by neurons expressing kisspeptin in the ARC and the anteroventral periventricular (AVPV) nucleus, respectively (13). AVPV kisspeptin neurons respond to
high concentrations of E2 to stimulate the GnRH surge into the portal vein system of the median eminence to control gonadotropin (luteinizing hormone (LH) and follicle-stimulating hormone (FSH)) production, essential for both gametogenesis and steroidogenesis. ARC kisspeptin neurons are called KNDy neurons as they co-express neurokinin B (NKB, Tac2), and dynorphin (Pdyn). KNDy neurons mediate the negative feedback of E2 and progesterone on the HPG axis in females and testosterone and E2 in males (13, 14). Neurokinin B and dynorphin in KNDy neurons act as positive and negative autoregulators of KNDy neuronal excitability, respectively, to produce the pulse generator that controls GnRH release into the median eminence (15-19). Both AVPV and ARC kisspeptin neuropeptide regulate the HPG axis by binding to its receptor KISS1r expressed on the soma and axons of GnRH neurons (14, 20).

$^{17\beta}$-estradiol (E2), the primary sex steroid hormone in females, also plays a major role in regulating the hypothalamic control of reproduction, metabolism and thermoregulation in females (21-23). One of many potential mechanisms for E2 to link these processes is to modulate hormone receptor expression in hypothalamic neurosecretory neurons, like KNDy neurons, which regulate LH pulsatility and respond to negative feedback of E2 (12, 24). Indeed, we have observed that E2 increases GHSR expression in arcuate KNDy neurons by six-fold (25). After further exploration, using whole-cell patch-clamp electrophysiology we have confirmed that when E2 is high (proestrus), the increase in Ghsr expression enhances KNDy sensitivity to ghrelin via inhibition of the M-current (26).

While ghrelin actions on NPY neurons are well characterized, only a few studies have examined the actions of ghrelin on KNDy neurons. In one study, ghrelin depolarized more KNDy neurons in E2-treated females than in oil-treated females (27). Ghrelin also depolarizes more KNDy neurons in proestrus and E2-treated OVX females (27) and evokes a greater suppressive effect on LH secretion without altering ARC Kiss1
expression (8, 10, 28). These data suggest that direct effects of ghrelin on KNDy neuronal excitability, and not kisspeptin transcription, mediate the suppression of LH pulse frequency. Ghrelin suppresses the frequency of pulsatile LH release in male and female rats, in part, due to activation of β-endorphin (POMC) signaling (7-11). Taken together, E2 and ghrelin interact at the level of KNDy neurons to control reproduction and metabolism.

In conclusion, the arcuate neuronal circuit of POMC, NPY/AgRP, and KNDy neurons regulate reproduction, energy balance, and thermoregulation by responding to circulating nutrients (glucose, free fatty acids, etc.), gonadal and adrenal steroids, and appetite-regulating hormones such as leptin, insulin, and ghrelin (29). In particular, KNDy neurons simultaneously excite and inhibit POMC and NPY/AgRP neurons, respectively, through glutamatergic signaling in both male and female mice (12, 30). Therefore, we hypothesize that ghrelin-induced KNDy activity may reinforce the actions of E2 by exciting POMC tone and by reducing the activation of NPY/AgRP neurons by ghrelin to regulate sympathetic output that controls metabolism and thermogenesis while also suppressing LH pulsatility through β-endorphin release during states of elevated ghrelin in females (30, 31). To address this hypothesis, we developed a novel transgenic mouse model that selectively deleted GHSR in Kiss1-expressing cells and measure reproductive parameters, LH pulsatility, and arcuate gene expression after hormonal administration and caloric restriction.

### 3.3. Materials and Methods

#### 3.3.1 Animals

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 (8-10 weeks of age) were housed under constant photoperiod conditions (12/12 h light/dark cycle) and
maintained at a controlled temperature (25°C). Animals were given food and water ad libitum, unless noted otherwise. Animals were weaned at postnatal day 21 (PD21).

### 3.3.2 Production of Kiss1^{Cre/EGFP};Ghsr^{fl/fl} mice

To produce a selective GHSR knockout in KNDy neurons, we mated C57BL/6J-Kiss1^{tm1.1(cre/EGFP)Steij} mice from Jackson Laboratory (#017701) to a floxed GHSR (GHSR^{fl/fl}) mouse strain provided by Dr. Yuxiang Sun (Texas A&M). The Kiss1^{Cre/EGFP} allele expresses a Cre-EGFP fusion protein from the Kiss1 promoter and enhancer elements. Cre-mediated recombination will result in deletion of the Ghsr in the Kiss-expressing neurons in the offspring (Kiss1^{Cre/+}/Ghsr^{fl/fl}). Littermates that did not express the Kiss1^{Cre/EGFP} allele (Kiss1^{WT}/Ghsr^{fl/fl}) were used as controls. Genotype was determined by using PCR products of extracted DNA from ear clippings.

### 3.3.3 Surgical Procedures

Adult females were bilaterally ovariectomized (OVX) under isoflurane anesthesia 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, which was regained by post-surgery day three.

### 3.3.4 Chemicals

An estradiol benzoate (E2B) injection protocol was used that has previously been shown to alter gene expression in the hypothalamus and mimic a proestrous-like state in mice (20). Animals were injected subcutaneously (s.c.) at 1000 h on post-OVX day 5 with either 0.25 μg of E2B or sesame oil-vehicle. On post-OVX day 6, a 1.5 μg dose of E2B or oil was injected at 1000 h. Ghrelin was purchased from AnaSpec Peptides (Fremont, CA,
USA) and was dissolved in saline prior to storage at -20°C until needed.

### 3.3.5 Vaginal Opening and Cytology

To determine if the loss of Ghsr in KNDy neurons altered the onset of puberty, weaned female pups (PND 21) were visually checked daily for vaginal opening as an indication of pubertal age. To determine if the loss of Ghsr in KNDy neurons altered the estrous cycle, after vaginal opening, vaginal lavage was performed to determine the estrous cycle daily for 2 weeks.

### 3.3.6 Fertility

To determine if the loss of GHSR in KNDy neurons alters fertility, control females were bred with experimental males and control males with experimental females. All female breeders were paired on the morning of proestrus. Females were checked for seminal plugs to determine day one of pregnancy. All females remained paired to have three subsequent litters, time to parturition, litter size, and sex ratio were recorded.

### 3.3.7 LH Blood Collection and Assay

All mice were handled daily between 0930-1030hrs for three weeks for 5-10 minutes each prior to tail bleed (32, 33). Cages were not changed during the last week of handling. Special care was taken to reduce changes in scent from the investigator. The person performing the bleeds also performed the daily handling. On the day of the bleed, the tail was numbed with lidocaine 30 minutes prior to the tail cut. All LH bleeds took place from 1000 to 1300 hours. Briefly, a small vertical cut was made to the tip of a cleaned and numbed tail. A small drop of blood was gently massaged from the tip of the tail and 6 mL was collected using a p20 pipette then immediately placed in 54 mL of prepared buffer, mixed and placed on dry ice. Samples were stored at -80°C until shipping for analysis.
Buffer was prepared with 0.2% BSA and 0.05% Tween 20, in PBS. The buffer was prepared on the morning of the experiment and was filtered before adding Tween 20, then placed on ice.

The Ultra-Sensitive Mouse & Rat LH ELISA is an in-house method performed by the Center for Research in Reproduction Ligand Assay and Analysis Core based on a manuscript published by Steyn et al (34). This assay allows for a LH measurement in 2.5 – 5 ml of serum, plasma or whole blood. The capture monoclonal antibody (anti- bovine LH beta subunit, 518B7) is provided by Janet Roser, University of California. The detection polyclonal antibody (rabbit LH antiserum, AFP240580Rb) is provided by the National Hormone and Peptide Program (NHPP). HRP-conjugated polyclonal antibody (goat anti-rabbit) is purchased from DakoCytomation (Glostrup, Denmark; D048701-2). Mouse LH reference prep (AFP5306A; NHPP) is used as the assay standard. The Limit of Quantitation (Functional Sensitivity) is defined as the lowest concentration that demonstrates accuracy within 20% of expected values and intra-assay coefficient of variation (%CV) <20% and was determined by serial dilutions of a defined sample pool. Intra-assay %CV is 2.2%. Inter-assay %CVs are 7.3% (Low QC, 0.13 ng/ml), 5.0% (Medium QC, 0.8 ng/ml) and 6.5% (High QC, 2.3 ng/ml). Functional sensitivity is 0.016 ng/ml. LH pulse peaks were identified by using previously established criteria (32, 35). A LH pulse peak was defined by the following 3 criteria: 1) the LH peak value must have a >20% increase compared with the previous (1 or 2) LH values; 2) the LH peak value must be followed by a decrease of >10% in the subsequent (1 or 2) LH values, and c) the change in pulse amplitude was ≥ 0.32 ng/mL. After identifying pulse peaks for each female, we identified the total number of LH peaks during the three-hour blood collection, the average LH concentration, the peak amplitude, defined as the difference in LH concentration between a peak its preceding nadir, and the interpulse interval, defined as the average time between pulses.
3.3.8 Arcuate RNA Extraction and quantitative real-time PCR

To determine if ghrelin alters ARC gene expression, experimental and control mice were separated into 4 treatment groups: 1) OVX/Oil + ghrelin (12h prior), 2) OVX/EB + ghrelin, 3) OVX/Oil and 4) OVX/EB. For collection of hypothalamic nuclei, mice were decapitated after an injection of ketamine at 1000h (100 µL of 100 mg/mL, IP) and the brain was extracted from the skull and rinsed in ice-cold Sorensen’s buffer for 30 sec. Brain slices were done using a brain matrix (Ted Pella, Redding, CA, USA) that cut the brain 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 (36). Blocks of the basal hypothalamus (BH) were transferred to RNALater (Life Technologies) and stored overnight at 4 °C. The rostral and caudal parts of the arcuate nucleus were dissected from slices using a dissecting microscope and stored at −80 °C until needed for extraction. To determine if the deletion of GHSR in Kiss-1 neurons altered the expression of GHSR or Kiss1 in other brain regions where both are expressed, we extracted RNA from the bed nucleus of the stria terminalis (BNST) and the central amygdala from 6 controls and 6 experimental females using the same protocols.

Pure RNA was extracted from the arcuate nucleus (ARC), BNST, and central amygdala 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). Quantity of RNA was assessed by the NanoDrop™ ND-2000 spectrophotometer (ThermoFisher, Inc., Waltham, MA, USA), followed by the RNA 6000 Nano Kit (Agilent Technologies, Inc., Santa Clara, CA, USA) to assess quality. Samples with an RNA integrity number (RIN) greater than 8 were used for quantitative real-time PCR (qPCR). Complementary DNA (cDNA) was synthesized from 250 ng of total ARC RNA using Superscript III reverse transcription (Life Technologies), 4 µL
5× buffer, 25 mM MgCl₂, 10 mM dNTP (Clontech Laboratories, Mountain View, CA), 100 ng random hexamer primers (Promega, Madison, WI), 40 U/μL Rnasin (Promega), and 100 mM dithiothreitol in diethylpyrocarbonate-treated water (Bioexpress, Kaysville, UT) in a total volume of 20 μL. Reverse transcription was conducted using the following protocol: 5 minutes at 25°C, 60 minutes at 50°C, and 15 minutes at 70°C. cDNA was diluted to 1:20 with nuclease-free water (Gene Mate/Bioexpress) for a final cDNA concentration of 0.5 ng/μL and stored at -20°C. Untreated ARC tissue RNA was used for the calibrator and negative control (no reverse transcription) and processed simultaneously with the experimental samples. All values were normalized and are expressed as relative mRNA expression.

Efficiencies were calculated as a percent efficiency and are approximately equal (90%–110% or one doubling per cycle, See Table 2 for a list of primer sequences). Amplification protocol for genes was as follows initial denaturing at 95 °C for 3 (SsoAdvanced) or 10 minutes (PowerSYBR) followed by 40 cycles of amplification at 94°C for 10 seconds (denaturing), 60°C for 45 seconds (annealing), and completed with a dissociation step for melting point analysis with 60 cycles of 95 °C for 10 seconds, 65°C to 95 °C (in increments of 0.5 °C) for 5 seconds and 95°C for 5 seconds. The reference genes used were Gapdh and Hprt. Positive, negative and water blank controls were included in the qPCR plate design. The geomean of the Cq values from each reference gene was used to calculate relative gene expression (37).

### 3.3.9 Statistical Analysis

All data were analyzed by a 2-way ANOVA (genotype and steroid) followed by a post-hoc Holm-Sidak’s except for q-PCR data which were analyzed by multifactorial ANOVA (genotype and steroid) using Statistica (Dell, Round Rock, TX, USA). All gene expression data were normalized to control females for comparison across genotypes. Values that
exceeded 2 SDs above or below the group mean were considered outliers and dropped. LH values were analyzed by two-tailed unpaired Student *t*-test was to compare LH pulse parameters between experimental females in proestrus and diestrus, experimental and control females in diestrus after a 24h fast, experimental females in diestrous with and without ghrelin, OVX experimental females treated with EB or oil, OVX experimental females treated with EB and treated with EB/ghrelin, and OVX/EB treated control and experimental females with ghrelin. Results were considered statistically significant at *p* < 0.05.

3.4 Results

3.4.1 Experiment #1: Age of Puberty and Fertility

Both experimental females and males were bread to wild type partners. Females were paired with males during proestrus. All mice were determined to be fertile. Average number of days from pairing to birth was 23 ± 2, average litter size 6 ± 1, male to female pup ratio was equal. No differences were observed in onset of puberty in females (day of vaginal opening) or estrous cyclicity (Figure 7a and b).

3.4.2 Experiment #2: Fasting and ghrelin suppression of LH in females.

Mice were serially sampled for whole-blood LH every 10 minutes for 3 hours for intact mice and every 6 minutes for 3 hours for OVX mice. Mice were sampled twice, once intact and again after OVX with four weeks of recovery and handling in between. First, we wanted to determine if our experimental females had similar LH profiles during as previously published work (32, 35, 38). Subsequently, we measured the response to OVX with and without E2 replacement. In a separate cohort of mice, we determined if there were any differences in our experimental and control females in response to a 24h fast, while in diestrus when E2 is low and ghrelin is high. Finally, we explored the importance of *Ghsr*
expression in kisspeptin neurons with elevated E2 on LH pulsatility. Both experimental and control females were OVX, with E2 replacement and ghrelin injection (1mg/kg; IP) 30 minutes prior to the collection. Figure 8 shows representative LH pulse profiles for experimental and control females in diestrous and proestrous (Figure 8a and b), after OVX with EB or Oil replacement (Figure 8c and d), experimental and control females in diestrous after a 24h fast (Figure 8e and f), and experimental and controls after OVX with EB replacement and injected with ghrelin (1mg/kg; IP) 30 minutes prior to the collection (Figure 8g and h). For intact females, there were no differences in LH peaks (Figure 9a), LH concentration (Figure 9b), peak amplitude (Figure 9c), or interpulse interval (Figure 9d).

After OVX, experimental females treated with oil had a significantly higher number of LH peaks compared to EB-treated females (Figure 10a; p = 0.0319). Experimental females treated with oil also had a higher LH concentration (Figure 10b; p = 0.0340) compared to EB-treated experimental females, a common effect of EB treatment. There were no differences in the peak amplitude (Figure 10c) or interpulse interval (Figure 10d).

### 3.4.3 Experiment #3: Regulation of ARC gene expression

It is well known that E2 regulates gene expression in ARC neurons, we selected E2-responsive genes that are involved in energy homeostasis and reproduction to determine the effect of GHSR deletion in KNDy neurons as well as the interaction of ghrelin. In figure 11, expression of neuropeptide KNDy genes *Kiss1* (Figure 11a; steroid: F(1, 20)=34.34, p<0.0001; genotype: F(1, 20)=6.385, p=0.0201; interaction of steroid*genotype: F(1, 20)=5.532, p=0.0290), *Tac2* (Figure 11b; steroid: F(1, 20)=39.06, p<0.0001; genotype: F(1, 20)=6.661, p=0.0178; interaction of steroid*genotype: F(1, 20)=7.573, p=0.0123), *Pdyn* (Figure 11c; steroid: F(1, 20)=32.32, p<0.0001; genotype: F(1, 20)=8.140, p=0.0098; interaction of steroid*genotype: F(1, 20)=7.674, p=0.0118), and *Tac3r* (Figure 11d; steroid: F(1, 20)=16.43, p=0.0006; genotype: F(1, 20)=7.042, p=0.0152) were reduced by EB in
control females. Furthermore, EB reduced $Esr1$ gene expression (Figure 11e; steroid: $F(1, 20)=11.6$, $p=0.0028$; interaction of steroid*genotype: $F(1, 20)=5.296$, $p=0.0323$) and elevated $Ghsr$ expression (Figure 11f; genotype: $F(1, 15)=5.832$, $p=0.0290$) in controls. In contrast, in experimental females, gene expression was only reduced for $Kiss1$ (Figure 11a; $p<0.05$) and $Tac2$ (Figure 11b; $p<0.05$) while $Pdyn$, $Tac3R$, $Esr1$, and $Ghsr$ remained unchanged by EB treatment. In addition, oil- treated experimental females revealed reduced gene expression for $Kiss1$ (Figure 11a; $p<0.01$), $Tac2$ (Figure 11b; $p<0.01$), $Pdyn$ (Figure 11c; $p<0.01$), $Tac3R$ (Figure 11d; $p<0.01$), and $Esr1$ (Figure 11e; $p<0.05$), compared to controls, while $Ghsr$ expression was increased by EB treatment only in controls (Figure 11f; $p<0.05$) and EB-treated experimental females showed reduced $Ghsr$ expression compared to oil- treated (Figure 11f; $p<0.05$). These data confirm that the E2-induced increase in ARC $Ghsr$ expression is solely due to up- regulation in KNDy neurons (25).

To determine the interaction of E2 and ghrelin on ARC gene expression, we repeated the same experiment and added a ghrelin injection (1mg/kg; IP) 12h before sacrifice (39). In Figure 12, expression of neuropeptide KNDy genes $Kiss1$ (Figure 12a; steroid: $F(1, 19)=27.39$, $p<0.0001$), $Tac2$ (Figure 12b; steroid: $F(1, 19)=20.43$, $p=0.0002$), $Pdyn$ (Figure 12c; steroid: $F(1, 19)=16.72$, $p=0.0006$), and $Tac3r$ (Figure 12d; steroid: $F(1, 19)=50.60$, $p<0.0001$; genotype: $F(1, 19)=7.924$, $p=0.0111$; interaction of steroid*genotype: $F(1, 19)=4.457$, $p=0.0482$) were reduced by EB and ghrelin in control females. Furthermore, EB reduced $Esr1$ expression (Figure 12e; steroid: $F(1, 19)=16.83$, $p=0.0006$) and abrogated the change in $Ghsr$ expression (Figure 12f; genotype: $F(1, 17)=10.53$, $p=0.0048$) in controls. In contrast to gene expression without ghrelin (Figure 11), in experimental females treated with EB and ghrelin, gene expression reduction was expanded beyond $Kiss1$ (Figure 12a; $p<0.05$), and $Tac2$ (Figure 12b; $p<0.01$) to include $Pdyn$ (Figure 12c; $p<0.05$), $Tac3r$ (Figure 12d; $p<0.01$), and $Esr1$ (Figure 12e; $p<0.05$), while $Ghsr$ remained unchanged. In addition, there were only genotype differences in oil and ghrelin-treated experimental females for
Tac3r (Figure 12d; p<0.01), while ghrelin injection appeared to abrogate genotype differences seen previously with Kiss1, Tac2, Pdyn and Esr1. These data indicate the importance of the interaction between EB and GHSR expression when it comes to gene regulation in the ARC and specifically in KNDy neurons and the downstream effects on reproduction.

3.4.4 BNST and Central Amygdala gene expression

To determine if the deletion of GHSR in Kiss1 neurons altered the expression of Ghsr or Kiss1 in other brain regions where both are expressed, we determined Kiss1 and Ghsr gene expression in the BNST as well as the central amygdala. Kiss1 was detected in both control and experimental females in both regions without any genotype differences. However, Ghsr expression was only consistently detected in control females, with only 1 out of 6 and 3 out of 6 experimental females had detectable Ghsr expression in the BNST and amygdala, respectively (data not shown). These findings indicate the potential for Kiss1 and Ghsr co-expression in regions outside of the hypothalamus with potential influences on mood, motivation, and reward. Additional experiments would be necessary to make that determination.

3.5 Discussion

The impact of E2 and ghrelin signaling in KNDy neurons on reproduction is an area of research where little is known. It is important to understand these discrete mechanisms of reproductive control, especially as reproductive complications continue to rise as a result of diet or lack thereof. In females, ghrelin, E2 and KNDy neurons work in synchrony to regulate the energetic demands of reproduction. Recent findings from our lab have determined that preovulatory levels of E2 increases ARC GHSR, also known as the ghrelin receptor, expression by 6-fold (25). Furthermore, this increase in Ghsr expression is specific to KNDy
neurons (Tac2-GFP), but not NPY neurons (37). ARC KNDy neurons, specifically targeted and stimulated using optogenetic techniques, provide direct evidence that the synchronous activation of ARC KNDy neurons can generate pulsatile LH release (40). At the same time, ghrelin is known to suppress the frequency of pulsatile LH release in male and female rats, in part, due to activation of β-endorphin release from POMC neurons, which do not express Ghsr at significant levels (7-11, 41). Thus, ghrelin’s inhibition of LH pulsatility may be due to its activity in ARC KNDy neurons and the subsequent downstream activation of POMC neurons and β-endorphin release.

We have found that without challenging energy homeostasis (fasting, diet-induced obesity) deletion of Ghsr from kisspeptin-expressing neurons is not sufficient to dysregulate reproduction or fertility in males or females. We found no differences in the age of pubertal onset, detected by vaginal opening, or estrous cyclicity in control or experimental females. Additionally, we found that quantitively, there were no differences in LH pulsatility parameters between control or experimental females. Experimental, oil-treated females did show an increase in LH pulses and concentration, which is expected in the absence of E2. Although not significant, experimental and control females experienced a longer interpulse intervals when injected with ghrelin. The lack of LH inhibition in control females after ghrelin injection could be due to an insufficient quantity of ghrelin injected or inefficient route of administration to elicit a response, previous publications used 3nM ghrelin injected intracerebroventricularly (i.c.v.) in female (7) and male rats (8), 0.1nM ghrelin injected i.c.v. in female rats (9), and 3nM ghrelin injected intravenously in female rats (10). Additional experiments using higher doses (3 – 10 mg/kg) or different routes of administration (i.c.v or intravenous) would be required to better determine the role of KNDy Ghsr expression in ghrelin-induced LH pulsatility inhibition in female mice. However, qualitatively, control females did show a clear inhibition in LH pulsatility when fasted or injected with ghrelin, compared to experimental females.
Furthermore, results from ARC qPCR reveal that our experimental females have reduced Kiss1, Tac2, Pdyn, Tac3r, and Esr1 when OVX and oil-treated compared to controls. Furthermore, EB treatment reduced Kiss1 and Tac2 expression in both controls and experimentals. However, we only observed a reduction in Pdyn, Tac3r, and Esr1 and an increase in Ghsr expression in control females with EB treatment. Thus, the E2-induced increase in Ghsr expression in the ARC is indeed due to the increase in KNDy neurons. Taken together, these results indicate the importance of Ghsr expression in KNDy neurons at the level of mRNA expression and potentially neuropeptide expression. Further experiments detecting the protein levels of kisspeptin, neurokinin B, dynorphin, neurokinin B receptor and estrogen receptor α would be necessary to confirm this. Ghrelin injection was sufficient to alter ARC gene expression. In ghrelin injected OVX females with and without EB replacement, the differences we observed between control and experimental females in expression of Kiss1, Tac2, Pdyn, and Esr1 was eliminated. This could indicate that the neuropeptides essential for reproduction and LH pulsatility are regulated by an interaction of ghrelin and E2.

It is important to note, that out of 12 ARC samples from oil-treated experimental females, we were only successful in quantifying Ghsr expression in two of them. As mRNA concentration as well as RNA integrity values were in acceptable range and both positive and negative qPCR controls were successfully detected, this lack of Ghsr expression in the absence of E2 and ghrelin could be due to the genetic deletion of Ghsr. It has been established that Pomc-expressing precursor neurons not only differentiate into POMC and NPY/AgRP neurons, but also into KNDy neurons (42). Thus, there is the potential confounding factor that Ghsr was deleted from other ARC neurons, namely NPY/AgRP. Further qPCR analysis of ARC tissue examining Pomc, Cart, Npy, and Agrp expression would be necessary to determine if this is the case. An alternative strategy for future investigation would be viral injection into the ARC of adult Kiss1Cre female mice to selectively
delete Ghsr from KNDy neurons and repeat these studies.

In conclusion, these findings reveal that during time periods when food is scarce and ghrelin becomes elevated and when a female is in proestrus or estrus (E2 levels are high), KNDy neurons are at a heightened state of ghrelin sensitivity. These ghrelin-sensitive neurons will be more easily excited by ghrelin and will therefore provide more excitable input to POMC neurons via glutamate or kisspeptin. This increase in input to POMC neurons will result in an elevated release of β-endorphin, a product of POMC. β-endorphin released near GnRH axon terminals in the median eminence will inhibit GnRH release and therefore LH release from the anterior pituitary, rendering females temporarily infertile, until food is available again and ghrelin levels decrease. Future studies will investigate the reproductive parameters during long-term negative energy balance i.e., chronic caloric restriction and during times of temperature stress (cold-induced torpor).

3.6 Acknowledgement

The authors have no acknowledgements.

3.7 Statement of Ethics

The research presented in the manuscript was ethically conducted in accordance with institutional guidelines based on National Institutes of Health standards and all animal experiments were performed with Institutional Animal Care and Use Committee approval at Rutgers University.

3.8 Funding Sources

This work was supported by the US Department of Agriculture-National Institute of Food and Agriculture (NJ06195) and the National Institutes of Health (R21ES027119; P30ES005022, R01MH123544), and the Rutgers University ONE Nutrition Initiative. The
University of Virginia Center for Research in Reproduction Ligand Assay and Analysis Core
is supported by the Eunice Kennedy Shriver NICHD/NIH Grant R24HD102061.

3.9 Author Contributions
KC & TAR conceived and designed the project; YS provided the mice; KC, AV, and CR
performed the experiments; KC & TAR analyzed and interpreted data and drafted the
manuscript.
References
34. Negrón AL, Radovick S. High-Fat Diet Alters LH Secretion and Pulse Frequency in Female Mice in an Estrous Cycle-Dependent Manner. Endocrinology. 2020;161(10).


Figures

Table 2. List of primers for ARC qPCR

Figure 7. Effects on puberty and estrous cyclicity (A) Vaginal opening in control (Kiss<sup>WT</sup>/GHSR<sup>fl/fl</sup>) and experimental (Kiss<sup>CRE+/+</sup>/GHSR<sup>fl/fl</sup>) females. (B) Average number of days in each stage of the estrous cycle in control and experimental females. Data are represented as mean ± SEM and n=12 mice per group. For A: Data were analyzed by unpaired t-test. For B: Data were analyzed with 2way ANOVA with post hoc Holm-Sidak’s multiple comparison test.

Figure 8. Representative LH pulsatility of: (A) Intact experimental diestrus; (B) Intact experimental proestrus; (C) Experimental OVX/OIL; (D) Experimental OVX/EB; (E) Intact experimental diestrus 24hr fasted; (F) Intact control diestrus 24hr fasted; (G) Experimental OVX/EB/ghrelin (1mg/kg; IP) 30 min prior to collection; (H) Control OVX/EB/ghrelin (1mg/kg; IP) 30 min prior to collection. [LH] analyzed at the University of Virginia Ligand Assay and Analysis Core.

Figure 9. LH dynamics in intact experimental females in diestrous and proestrous and experimental and control females in diestrous after a 24h fast. Parameters analyzed were (A) Total LH peaks; (B) Average LH concentration; (C) Average peak amplitude and (D) Average interpulse interval. [LH] was analyzed at the University of Virginia Ligand Assay and Analysis Core. n=4 mice per group. Data were analyzed by students t-test.

Figure 10. LH dynamics in OVX experimental females treated with EB or Oil and experimental and control OVX females treated with EB and ghrelin (1mg/kg; IP) 30
minutes prior to collection. Parameters analyzed were (A) Total LH peaks; (B) Average LH concentration; (C) Average peak amplitude and (D) Average interpulse interval. [LH] was analyzed at the University of Virginia Ligand Assay and Analysis Core. n=4 mice per group. Data were analyzed by students t-test. * = p<.05.

Figure 11. Arcuate gene expression in OVX control and experimental females with or without E2 replacement: A) Kiss1; B) Tac2; C) Pdyn; and D) Tac3R; E) Esr1; and F) Ghsr. For all graphs, data were analyzed by a multi-factorial ANOVA (steroid, genotype) with a Holm-Sidak comparison test. Lower case letters denote steroid differences within genotype, capped uppercase letters denote genotype effects (A/a = p<.05; B/b = p<.01; C/c = p<.001; D/d = p <.0001). Data is represented as mean ± SEM.

Figure 12. Arcuate gene expression in OVX control and experimental females with Ghrelin injection with or without E2 replacement: A) Kiss1; B) Tac2; C) Pdyn; and D) Tac3R; E) Esr1; and F) Ghsr. For all graphs, data were analyzed by a multi-factorial ANOVA (steroid, genotype) with a Holm-Sidak comparison test. Lower case letters denote steroid differences within genotype, capped uppercase letters denote genotype effects (A/a = p<.05; B/b = p<.01; C/c = p<.001; D/d = p <.0001). Data is represented as mean ± SEM.
<table>
<thead>
<tr>
<th>Gene</th>
<th>Accession #</th>
<th>Forward Primer</th>
<th>Reverse Primer</th>
</tr>
</thead>
<tbody>
<tr>
<td>Esr1</td>
<td>NM_007956</td>
<td>GCGCAAGTGTTACGAAGTG</td>
<td>TTCGGCCTTCCAAAGTCATC</td>
</tr>
<tr>
<td>Gapdh</td>
<td>NM_008084.2</td>
<td>TGACGTGCCGCCTGGAGAAA</td>
<td>AGTGTAGCCCAAGATGCCCTTCAAG</td>
</tr>
<tr>
<td>Ghsr</td>
<td>NM_177330</td>
<td>CAGGGACCAGAACAAACAAC</td>
<td>AGCCAGGCTCGAAAGACT</td>
</tr>
<tr>
<td>Hprt</td>
<td>NM_013556</td>
<td>GCTTGCCTGGTAAAGGACCTCTCGAAG</td>
<td>CCCTGAAGTACTCATTATAGTCAAGGCAAT</td>
</tr>
<tr>
<td>Kiss1</td>
<td>NM_178260</td>
<td>TGATCTCAATGGCTTTCTGGCAACG</td>
<td>CTCTCTGCACTACCCGAGTTCCTTT</td>
</tr>
<tr>
<td>Pdyn</td>
<td>NM_018863</td>
<td>AGCTTGCTCCCTCGTGATG</td>
<td>GCCACTCCAGGGGAGCAGAT</td>
</tr>
<tr>
<td>Tac2</td>
<td>NM_001199971</td>
<td>CGTGACATGCACGACTTC</td>
<td>CCAACAGGAGGACCTTAC</td>
</tr>
<tr>
<td>Tac3R</td>
<td>NM_021382</td>
<td>TACACCACGTTGGAATTAC</td>
<td>ATGTCACCACCAATAATC</td>
</tr>
</tbody>
</table>
Figure 7.

A

Age of vaginal opening (days)

Control
Experimental

12
12

B

Days per cycle

D/M  P  E
12 12 12 12 12

Control  Experimental
Figure 8.
Figure 9.

A. Total Peaks

Number of Peaks

B. LH Concentration

ng/ml

C. Peak Amplitude

ng/ml

D. Interpulse Interval

Minutes
Figure 10.

A. Total Peaks

B. LH Concentration

C. Peak Amplitude

D. Interpulse Interval

- **EB**
- **Oil**
- **Exp**
- **Con**
- **Ghrelin**
Figure 11.

A. Kiss1

B. Tac2

C. Pdyn

D. Tac3r

E. Esr1

F. Ghsr

Fold Change

Control
Experimental

Oil
EB

Fold Change

Oil
EB

Fold Change

Oil
EB

Fold Change

Oil
EB

Fold Change

Oil
EB

Fold Change

Oil
EB

Fold Change

Oil
EB

Fold Change

Oil
EB

Fold Change
CHAPTER 4: THE INFLUENCE OF GHSR ACTIVITY IN KNDY NEURONS ON ENERGY HOMEOSTASIS IN INTACT AND OVARIECTOMIZED FEMALE MICE
4. The influence of GHSR activity in KNDy neurons on energy homeostasis in intact and ovariectomized female mice

4.1 Abstract

The gut peptide, ghrelin, responds to negative energy balance and in part, mediates neuroendocrine control of energy homeostasis by acting through its receptor, growth hormone secretagogue receptor (GHSR). GHSR, expressed in hypothalamic Kisspeptin/Neurokinin B/Dynorphin (KNDy) neurons in the arcuate (ARC), is well known to regulate energy balance. We have previously shown 17-beta-estradiol (E2) robustly increases Ghsr expression in KNDy neurons, enhancing their sensitivity to ghrelin. We hypothesize that E2-induced increase in GHSR expression augments KNDy sensitivity in a fasting state by elevating ghrelin to reduce energy expenditure in females. We developed a Kiss1-specific GHSR knockout to determine the role of GHSR in ARC KNDy neurons and found in ovariectomized females with or without E2 replacement, locomotor activity was elevated as well as food and water intake. Furthermore, metabolic rates (V.O2, V.CO2) were decreased compared to EB-treated controls. Fasting glucose levels were elevated in the knockout mice, regardless of steroid. In addition, we found that on a high fat diet, knockout females were resistant to body weight gain. In another experiment, knockout females did not refeed to the same extent as controls in response to a 24h fast (increase in ghrelin). Finally, in response to an overnight cold stress, experimental females had more elevated metabolic parameters (V.O2, V.CO2, and heat) than the controls did. Collectively, these data suggest that GHSR activation in KNDy neurons modulates metabolism and glucose homeostasis, and thermoregulation illustrating a novel mechanism for E2 and ghrelin in the control of KNDy neurons and their physiological functions.
4.2 Introduction

Rodents have been known to exhibit torpor during periods of food scarcity to compensate for a negative energy balance and the suppression of the hypothalamic-pituitary-gonadal axis (1). These periods induce a state of hypothermia and a reduction in metabolism that allow for survival. Many mammals, including rodents, exhibit a torpor response to periods of food scarcity or unpredictable food availability. During these sensitive periods, reproduction becomes less of a priority than conserving energy. There are numerous neuroendocrine mechanisms that link reproduction with metabolism and thermoregulation, however they are not well understood in most mammals. Ghrelin is one peripheral hormone that could potentially link the neuromodulation of reproduction and metabolism during times of food scarcity (2-4). Ghrelin acts via its receptor, growth hormone secretagogue receptor (GHSR) and is primarily produced by the stomach. GHSR is commonly expressed in arcuate (ARC) neuropeptide Y (NPY) neurons as well as KNDy (Kisspeptin-Neurokinin B-Dynorphin) neurons. Studies determining the role of GHSR in NPY neurons have been well documented, showing ghrelin increases the activity of NPY neurons leading to orexigenic behavior in mammals (5, 6). However, the same cannot be said for the role of GHSR in KNDy neurons, which regulate negative feedback by 17β-estradiol (E2). In fact, ghrelin has been found to suppress luteinizing hormone (LH) pulse frequency in rodents through β-endorphin signaling from proopiomelanocortin (POMC) neurons, which less than 10% express GHSR (7-11).

While KNDy neurons have been primarily implicated reproduction, they also contribute to the control of energy homeostasis, especially in females. Ablation of KNDy neurons abrogates the post-ovariectomy weight gain associated with E2 in rats, suggesting that KNDy neurons mediate, in part, the anorectic effects of E2 (12). One pathway for KNDy neurons to control energy balance is by directly depolarizing POMC...
neurons through kisspeptin and glutamate release (13, 14). Recently, kisspeptin neurons were found to be involved in other homeostatic processes including thermoregulation and bone remodeling (15, 16). In fact, recent studies have confirmed that neurokinin B, expressed in ARC KNDy neurons, mediate hot flushes in response to reduced E2. In this study, optogenetic activation of KNDy axon terminals in the preoptic area of the hypothalamus increased heat dissipation and vasodilation in the tail skin of mice, which was abolished by a neurokinin B receptor antagonist (17, 18). Collectively, these arcuate neuronal populations regulate energy balance, thermoregulation, and reproduction, and respond to peripheral endocrine and nutrient signals such gonadal and adrenal steroids, appetite-regulating hormones such as leptin, insulin, and ghrelin, and circulating nutrients (glucose, free fatty acids, etc.) (19).

E2 also plays a major role in regulating the hypothalamic control of metabolism and thermoregulation in females (20-22). E2 reduces food intake and increases energy expenditure and activity, in part, through actions in the mediobasal hypothalamus (23-26). One of many potential mechanisms for E2 to link these processes is to modulate hormone receptor expression in hypothalamic neurosecretory neurons involved in reproduction and energy homeostasis, like arcuate KNDy neurons. While ghrelin actions in NPY neurons are well characterized, few studies have examined the actions of ghrelin in KNDy neurons. In one study, central ghrelin administration had no effect on ARC Kiss1 expression in a fed or fasted state in female rats (10). However, we have reported that E2 increases GHSR expression in arcuate KNDy neurons by six-fold (27). Using whole-cell patch-clamp electrophysiology, we confirmed that when E2 is high, the increase in Ghsr expression enhances KNDy sensitivity to ghrelin through the inhibition of the M-current (28).

The arcuate melanocortin circuit (POMC, NPY/AgRP) are also modulated by E2 and peripheral peptide hormones (23, 24, 26, 29, 30). KNDy neurons modulate this circuit
by simultaneously exciting and inhibiting POMC and NPY neurons, respectively, through glutamatergic signaling in both male and female mice (13, 31). Therefore, while high E2 levels suppress Kiss1 gene expression, E2 augments KNDy GHSR expression, increases ghrelin sensitivity, and stimulates glutamate release leading differential modulation of the arcuate melanocortin circuit. This ghrelin induced KNDy activity reinforces the anorectic actions of E2 by exciting POMC tone and reducing ghrelin’s activation of NPY/AgRP neurons. These changes in melanocortin excitability alters downstream regulation, from the paraventricular hypothalamus, of sympathetic output controlling both food intake, energy expenditure, and thermoregulation.

In summary, both ghrelin and E2 have profound effects on energy balance and are known to interact at the level of ARC KNDy neurons. What is lacking is an understanding of the influence of ghrelin signaling in KNDy neurons on metabolic processes controlled by hypothalamic neurons. We hypothesize that the E2-induced GHSR expression increases KNDy neuronal sensitivity to ghrelin to suppress food intake and augment energy expenditure and activity in females during challenged states of energy balance (fasting/torpor). To examine the role of KNDy expression of GHSR in energy homeostasis, we will determine: 1) the effect of ovariectomy and E2 replacement on energy homeostasis; 2) the metabolic phenotype and locomotor behavior of intact Kiss1\(^{\text{Cre/EGFP, Ghsr}}\) female mice fed a low-fat or a high-fat diet; 3) the role of KNDy GHSR expression on fasting- and ghrelin-induced refeeding; and 4) the role of KNDy GHSR expression on thermoregulation.

4.3 Materials and Methods

4.3.1 Animals

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 (6-12 weeks of age) were housed under constant photoperiod conditions (12/12 h light/dark cycle) and maintained at a controlled temperature (25°C). Animals were given food and water *ad libitum*, unless noted otherwise. Animals were weaned at postnatal day 21 (PD21).

4.3.2 Production of $\text{Kiss}1^{\text{Cre/EGFP}, \text{Ghsr}^{\text{fl/fl}}}$ mice

To produce a selective GHSR knockout in KNDy neurons, we mated C57BL/6J-$\text{Kiss}1^{\text{tm1.1(cre/EGFP)}\text{Ste}}$J mice from Jackson Laboratory (#017701) to a floxed GHSR ($\text{GHSR}^{\text{fl/fl}}$) mouse strain obtained from our collaborator, Dr. Yuxiang Sun. The $\text{Kiss}1^{\text{Cre/EGFP}}$ allele expresses a Cre-EGFP fusion protein from the $\text{Kiss}1$ promoter and enhancer elements. Cre-mediated recombination will result in deletion of the Ghsr in the $\text{Kiss}$-expressing neurons in the offspring ($\text{Kiss}1^{\text{Cre+/Ghsr}^{\text{fl/fl}}}$). Genotype was determined by using PCR products of extracted DNA from ear clippings.

4.3.3 Chemical and diets

Estradiol benzoate (EB) was purchased from Steraloids (Newport, RI, USA)). Ketamine, Marcaine, and Rimadyl were purchased from Henry Schein Animal Health (Dublin, OH). EB was dissolved in 100% ethanol (Sigma) prior to dissolving in sesame oil (Sigma). Ethanol was allowed to evaporate for 24 hours prior to storage at 4°C until needed. Ghrelin was purchased from AnaSpec Peptides (Fremont, CA, USA) and was dissolved in saline prior to storage at -20°C until needed. For experiments 1 and 2, diets were purchased from Research Diets (New Brunswick, NJ): low-fat diet (LFD) (10% kcal fat; D12450B) and high-fat diet (HFD) (45% kcal fat; D12451).

4.3.4 Response to ovariectomy
4.3.4.1 Surgical Procedures

To elucidate the interactions of E2 and GHSR in KNDy neurons and the impact on energy balance (experiment 1), adult females (8-10 weeks of age) were bilaterally ovariectomized (OVX) under isoflurane anesthesia 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, females were separated into 2 treatment groups – oil vehicle and estradiol benzoate (EB). In order to reduce stressful injections during the 8 weeks of LFD, mice were passively orally dosed with oil vehicle or EB (300 μg/kg body weight) mixed with powdered, low-fat peanut butter daily (27). After sacrifice, uteri were weighed in order to confirm EB treatment was effective.

4.3.4.2 Food Intake and Body Weight and Body Composition

Food intake and body weight were measured weekly. Before and after diet, body composition was measured using a small rodent MRI (EchoMRI, Houston, Texas).

4.3.4.3 Metabolic and Meal Pattern Monitoring

Indirect calorimetry, food intake, and activity were measured using CLAMS chambers (Comprehensive Lab Animal Monitoring System, Columbus Instruments, Columbus, OH). Meal Patterns were measured using BioDAQ (Biological Data Acquisition, Research Diets, New Brunswick, NJ).

4.3.4.4 Glucose and Insulin Tolerance Tests

A glucose tolerance test (GTT) followed by an insulin tolerance test (ITT) was performed, each mouse was intraperitoneal (IP.)-injected with a bolus of glucose (2 g/kg) after a 5 h
fast. Glucose was measured in tail blood using an AlphaTrak glucometer (Zoetis, Parsippany, NJ). Glucose measurements were taken at times 0, 15, 30, 60, 90, and 120 min after injection. For the insulin tolerance test (ITT), mice were IP injected with insulin (0.75 U/kg body weight in sterile saline) after a 4 h fast. Glucose measurements were taken at times 0, 15, 30, 60, 90, and 120 min after insulin injection.

4.3.5 Diet-Induced Obesity

To elucidate the interactions of diet-induced obesity and GHSR in KNDy neurons and the impact on energy balance adult females (8-10 weeks of age) remained gonadally intact and were placed on either LFD or HFD for 8 weeks.

4.3.5.1 Food Intake and Body Weight and Body Composition

Food intake and body weight were measured weekly. Before and after diet, body composition was measured using a small rodent MRI (EchoMRI, Houston, Texas).

4.3.5.2 Metabolic and Meal Pattern Monitoring

Indirect calorimetry, food intake, and activity were measured using CLAMS chambers (Comprehensive Lab Animal Monitoring System, Columbus Instruments, Columbus, OH). Meal Patterns were measured using BioDAQ (Biological Data Acquisition, Research Diets, New Brunswick, NJ).

4.3.5.3 Glucose and Insulin Tolerance Tests

A glucose tolerance test (GTT) followed by an insulin tolerance test (ITT) was performed, each mouse was intraperitoneal (IP)-injected with a bolus of glucose (2 g/kg) after a 5 h fast. Glucose was measured in tail blood using an AlphaTrak glucometer (Zoetis, Parsippany, NJ). Glucose measurements were taken at times 0, 15, 30, 60, 90, and 120
min after injection. For the insulin tolerance test (ITT), mice were IP injected with insulin (0.75 U/kg body weight in sterile saline) after a 4 h fast. Glucose measurements were taken at times 0, 15, 30, 60, 90, and 120 min after insulin injection.

4.3.6 The effects of fasting and ghrelin on feeding behaviors
In another cohort of female experimental and control females, mice were single housed for 4 days prior to acclimation to the BioDAQ for 3 days. On day 4, 1h before lights out, half of the mice were fasted for 24h and the other half were fed ad lib. After 24h, food was returned to the fasted mice and feeding behavior was recorded, uninterrupted for the next 48h. On the following day, 1h before lights out, the previously fasted mice received an IP injection of saline, previously fed mice received an IP injection of ghrelin (1mg/kg) and feeding behavior was recorded for the next 48h.

4.3.7 Core body temperature recording
In another cohort of mice, core body temperature was recorded using temperature loggers (DST nano, Star-Oddi, Herfølge, Denmark) implanted abdominally, utilizing similar surgical methods for ovary removal. A pre-defined program was set to sample the temperature data every five minutes for baseline core body temperature. Mice were placed into the CLAMS and the after 24h acclimation, during the second night the ambient temperature was reduced from 25°C to 10°C for a period of 6h. Mice remained inside the CLAMS for an additional night and were removed the next day. At the end of the experiments, the loggers were retrieved, and the data were downloaded and analyzed.

4.3.8 Liver RNA Extraction and quantitative real-time PCR
To determine if the deletion of GHSR in Kiss-1 neurons altered the expression of GHSR or Kiss1 in the liver, we extracted RNA from the liver of 6 controls and 6 experimental
females. For collection of liver samples, mice were decapitated after an injection of ketamine at 1000h (100 µL of 100 mg/mL, IP) and the liver was extracted. Livers were transferred to RNALater (Life Technologies) and stored overnight at -80 °C until needed for extraction. Liver RNA was extracted using a Trizol extraction coupled with a Macherey-Nagel NucleoSpin kit (Bethlehem, PA). To extract the hepatic RNA, ~20 mg of each liver was used. MBH and liver RNA quantity and quality were determined using a NanoDrop-2000 spectrophotometer (ThermoFisher, Waltham, MA) (32).

4.3.8.1 Reverse transcription and quantitative real-time PCR
As previously described (33), cDNA was synthesized from 0.5 µl of Superscript III transcriptase, 4 µl of 5x SS buffer, 1.25 µl of dNTP, 1 µl of 100 ng random hexamers, and 0.38 µl of RNasin in DEPC water to have a total volume of 20 µl. Reverse transcription was conducted using the following protocol: Incubation at 25 °C for 5 min, transcription at 50 °C for 60 min, denature at 70 °C for 15 min, and cooling for 4 min at 4°C. Each sample was diluted using nuclease-free water at a 1:20 dilution to a final concentration of 1.5 ng cDNA/µl). Quantitative real-time PCR (qPCR) was conducted using the primers found in Table 3. Four µl of cDNA was amplified by SSO Advanced (BioRad, Hercules, CA) Master Mix using standard protocols. Relative gene expression was determined using the δδCT method calculated by the geomean of reference genes Hprt and Gapdh (34, 35). Efficiencies were calculated as a percent efficiency and are approximately equal (90%–110% or one doubling per cycle). Positive, negative and water blank controls were included in the qPCR plate design. The geomean of the Cq values from each reference gene was used to calculate relative gene expression (36).

4.3.9 Statistical Analysis
All data were expressed as mean ± SEM. All data were analyzed using Statistica 7.1
software (StatSoft, Tulsa, OK, USA) and by a two-way (diet, genotype) or multi-factorial (diet or steroid, genotype, time) ANOVA followed by a post-hoc Holm Sidak’s test. GTT and ITT data were analyzed using repeated-measures, two-way ANOVA with a post-hoc Holm Sidak’s test. In all experiments, effects were considered significant at $\alpha \leq 0.05$.

4.4 Results

4.4.1 Experiment #1: Response to ovariectomy

4.4.1.1 Body weight gain and body composition

GHSR deletion in KNDy neurons did not alter body weight, body composition, or crude food intake. EB treatment reduced body weight gain, regardless of genotype (Figure 13a and b). EB treatment also reduced the percent change in fat mass and increased the percent change in lean mass from week zero to week 8 regardless of genotype (Figure 13c and d). There were no differences in crude cumulative food intake over 8 weeks of diet (Figure 13e). Overall, we observed only steroid effects on body weight and body composition.

4.4.1.2 Glucose and insulin tolerance tests

To determine the effects on glucose homeostasis in OVX in females with GHSR knocked out of KNDy neurons, we conducted glucose and insulin tolerance tests on all mice. We observed no differences in GTT or ITT with steroid treatment or genotype, however there was an effect of time for GTT (Figure 14a and c; $F(5, 140)=105.65$, $p=0.0000$) and ITT (Figure 14b and d; $F(5, 115)=30.860$, $p=0.0000$). Surprisingly, experimental females had an elevated fasting glucose in response to a 5h fast compared to controls, regardless of treatment (Figure 14e; $F(1, 23) = 5.349$, $p= 0.0300$). This difference in fasting glucose indicates that GHSR expression in hypothalamic KNDy neurons participates in the regulation of glucose homeostasis.
4.4.1.3 Locomotor Activity and Ingestive Behaviors in CLAMS

X-plane activity was increased in EB-treated experimental females during the night compared to the day as well as compared to EB-treated controls at night (Figure 15a; steroid: F(1, 56)=10.178, p=.00233; time: F(1, 56)=10.647, p=.00188). Hourly X-plane activity was elevated in EB-treated experimentals at 0100, 0200 and 0500 hours compared to EB-treated controls (Figure 15b; steroid: F(1, 28)=5.9072, p=.02174; time: F(23, 644)=11.835, p=0.0000; interaction of time*steroid: F(23, 644)=2.6751, p=.00004). Y-plane activity was also increased in EB-treated experimental females during the night compared to the day as well as compared to EB-treated controls at night (Figure 15c; steroid: F(1, 56)=10.678, p=.00185; time: F(1, 56)=16.230, p=.00017). Hourly Y-plane activity was affected by EB treatment in experimentals at hours 0100 and 0200 (Figure 15d; steroid: F(1, 28)=6.0720, p=.02014; time: F(23, 644)=16.453, p=0.0000; time*steroid: F(23, 644)=3.1487, p=.00000). These data indicate that GHSR expression in KNDy neurons play a critical role in locomotor activity on female mice.

EB-treated females consumed more food at night compared to the day. In addition, EB-treated controls consumed less then EB-treated experimental females at night (Figure 15e; steroid: F(1, 56)=9.4640, p=.00324; time: F(1, 56)=22.400, p=.00002). Although the not significant, a main effect of genotype was trending (F(1, 56)=3.5840, p=.06351). Furthermore, water intake was elevated only in EB-treated experimental females at night compared to the day (Figure 15f; steroid: F(1, 56)=4.9101, p=.03078; time: F(1, 56)=14.649, p=.00033). These data reveal the importance of GHSR expression in KNDy neurons in ingestive behavior.

4.4.1.4 Metabolic Parameters in the CLAMS

All females consumed more oxygen (V.O₂) during the night compared to the day. EB-treated females consumed more oxygen during the day and night compared to Oil-treated
females and EB-treated controls consumed more oxygen compared to experimentals during both the day and night. (Figure 16a; steroid: $F(1, 54)=113.10$, $p=.00000$; genotype: $F(1, 54)=21.103$, $p=.00003$; time: $F(1, 54)=54.115$, $p=.00000$; time*steroid: $F(1, 54)=6.8972$, $p=.01121$). Hourly V.O$_2$ consumption was elevated in EB-treated controls compared to EB-treated experimentals at hours 0000, 0300, 0700, 1200, 1600, 2100, 2200, and 2300. In addition, Oil-treated controls also exhibited an increase in V.O$_2$ compared to Oil-treated experimentals at hours 0000, 0600, 1100, 1200, 1500, 2000, 2200, and 2300 (Fig. 16b; steroid: $F(1, 28)=36.432$, $p=.00000$; genotype: $F(1, 28)=9.2728$, $p=.00502$; time: $F(23, 644)=35.816$, $p=0.0000$; time*steroid: $F(23, 644)=5.9374$, $p=.00000$).

All females produced more carbon dioxide (V.CO$_2$) during the night compared to the day. EB-treated females produced more V.CO$_2$ during the day and night compared to Oil-treated females. Furthermore, EB-treated controls produced more V.CO$_2$ compared to EB-treated experimentals during the night (Figure 16c; steroid: $F(1, 54)=131.76$, $p=.00000$; genotype: $F(1, 54)=11.051$, $p=.00160$; time: $F(1, 54)=89.700$, $p=.00000$; time*steroid: $F(1, 54)=15.595$, $p=.00023$). Similar to V.O$_2$ consumption, hourly V.CO$_2$ production in EB-treated control females was elevated at hours 0300, 0700, 1200, 2100, 2200, and 2300. Further, Oil-treated controls had an elevated V.CO$_2$ production compared to Oil-treated experimentals at 2000 hours (Figure 16d; steroid: $F(1, 27)=78.343$, $p=.00000$; genotype: $F(1, 27)=6.5286$, $p=.01656$; time: $F(23, 621)=41.501$, $p=0.0000$; time*steroid: $F(23, 621)=7.3378$, $p=0.0000$).

Respiratory exchange ratio (RER), a measure of substrate utilization (fat vs. carbohydrates) was higher in EB-treated females at night compared to the day (Figure 16e; steroid: $F(1, 51)=5.0672$, $p=.02872$; time $F(1, 51)=27.088$, $p=.00000$). Hourly RER was increased in EB-treated experimental females compared to Oil-treated experimentals
at hours 0000 and 1900, while Oil-treated controls revealed a reduced RER compared to EB-treated controls at hour 0200 (Figure 16f; time: $F(23, 391)=9.1637$, $p=0.0000$), although an effect of steroid was trending ($F(1, 17)=4.2759$, $p=.05423$).

All females exhibited elevated heat production, a measure of energy expenditure, at night compared to the day. Additionally, EB-treated females exhibited an increase in nighttime energy expenditure compared to Oil-treated females (Figure 16g; steroid: $F(1, 54)=38.796$, $p=.00000$; genotype: $F(1, 54)=5.9726$, $p=.01783$; time: $F(1, 54)=68.971$, $p=.00000$; time*steroid: $F(1, 54)=7.5442$, $p=.00816$). Hourly energy expenditure was elevated in EB-treated controls at hours 2200 and 2300 and Oil-treated experimental females exhibited reduced energy expenditure compared to Oil-treated controls at the 0600 hour (Figure 16h; steroid: $F(1, 27)=24.597$, $p=.00003$; time: $F(23, 621)=38.603$, $p=0.0000$; time*steroid: $F(23, 621)=5.5747$, $p=.00000$). These data support our hypothesis that without GHSR upregulation by E2 in females, metabolic measurements become dysregulated.

4.4.1.5 Meal Pattern Analysis

Food intake between days 1, 2, and 3 of data acquisition revealed Oil-treated controls had reduced food intake on day one and day two compared to day three and EB-treated controls consumed more on days two and three compared to day one (Figure 17a; steroid: $F(1, 28)=14.000$, $p=.00084$; time: $F(2, 56)=5.3649$, $p=.00738$; time*genotype: $F(2, 56)=6.5200$, $p=.00285$). Meal size (Figure 17b), meal frequency (Figure 17c) and meal duration (Figure 17d) were not significantly different, although EB-treated females consistently exhibited a larger meal size and longer meal duration compared to Oil-treated females, while experimental females consistently consumed meals less frequently compared to controls. Hourly food intake was elevated in EB-treated experimental and control females during 0000 and 2300 hours and elevated in EB-treated experimental
females during hour 2000 (Figure 17e; steroid: F(1, 28)=8.2844, p=.00757; time: F(23, 644)=15.529, p=0.0000; time*steroid: F(23, 644)=4.3282, p=.00000). Regardless of genotype, the average total ingested was increased in EB-treated females compared to Oil-treated females (Figure 17f). Taken together, deletion of GHSR in KNDy neurons potentially dysregulates input from KNDy to POMC and NPY neurons, altering feeding behavior.

### 4.4.2 Experiment #2: Response to diet induced obesity

#### 4.4.2.1 Body weight gain and body composition

Females lacking GHSR in KNDy neurons are resistant to body weight gain on HFD, as opposed to control mice on a HFD (Figure 18a). At the end of the 8-week diet, experimental females weighed less than controls, regardless of diet (Figure 18b). Furthermore, after 8 weeks of diet, control females had a higher percent change in body fat (Figure 18c), while there were no differences in the change in percent of lean mass (Figure 18d). During weeks 5-8, control females on a LFD had greater cumulative energy intake (kCal) compared to controls on a HFD, and controls on both diets had a greater energy intake compared to experimental females. In addition, during weeks 6-8 experimental females on a LFD had greater cumulative energy intake compared to experimental females on a HFD (Figure 18e). Overall, deletion of GHSR in KNDy neurons reduced cumulative energy intake and abrogated body weight gain on HFD, indicating a crucial role in feeding behavior and adiposity.

#### 4.4.2.3 Glucose and insulin tolerance tests

To determine the effects of diet induced obesity in females with GHSR knocked out of KNDy neurons on glucose homeostasis, we conducted glucose and insulin tolerance tests on all mice. As a result of glucose injection (2 g/kg; IP), we observed an increase in the time it takes for glucose to return to baseline levels only in experimental females fed
a HFD, the glucose levels peaked around minute 30, as opposed to around minute 15 for the other females (Figure 19a; Time: F(5, 140)=102.03, p=0.0000). We observed no differences in the ITT (Figure 19b; Time: F(5, 115)=74.716, p=0.0000; Time*diet: F(5, 115)=4.1093, p=.00181). Additionally, there were no differences in the AUC analysis of GTT or ITT (Figure 19c and d). Surprisingly, experimental females had an elevated fasting glucose in response to a 5h fast compared to controls, regardless of diet (Figure 19e; genotype: F(1, 28)=7.806, p=0.0093). This data echoes our findings in that GHSR expression in hypothalamic KNDy neurons participates in the regulation of glucose homeostasis.

4.4.2.4 Locomotor Activity and Ingestive Behaviors in CLAMS

X-activity was increased in all females at night compared to the day (Figure 20a; time: F(1, 56)=54.127, p=.00000). Hourly X-activity was elevated in LFD-fed controls at 2000, 2200, and 2300 hours compared to experimental. Furthermore, controls on LFD had more X-activity during hours 2200 and 2300 (Figure 20b; Time: F(23, 644)=35.080, p=0.0000; time*genotype: F(23, 644)=1.7458, p=.01711). Y-activity was also increased in all females at night compared to the day, with the exception of LFD-fed controls (Figure 20c; diet: F(1, 56)=4.5673, p=.03697; time: F(1, 56)=26.224, p=.00000). Hourly Y-activity was also increased in LFD-fed controls compared to experimental at hours 1000, 2200, and 2300 and was increased in controls fed LFD compared to controls fed HFD during hours 1000, 2200, and 2300 (Figure 20d; time: F(23, 644)=19.068, p=0.0000; time*genotype: F(23, 644)=1.6800, p=.02465). This data shows that experimental females, regardless of diet, shared movement patterns with controls on a HFD, while controls on LFD appeared to move more. Control females fed LFD had greater energy intake (kCal) at night compared to the day (Figure 20e; time: F(1, 56)=23.066, p=.00001). Furthermore, there were no differences in water intake between females (Figure 20f; time:
Metabolic Parameters in the CLAMS

All females consumed more oxygen (V.O\textsubscript{2}) during the night compared to the day (Figure 21a; Time: F(1, 56)=89.747, p=.00000). Hourly V.O\textsubscript{2} consumption was no different between groups (Fig. 21b; time: F(23, 644)=73.269, p=0.0000). All females produced more carbon dioxide (V.CO\textsubscript{2}) during the night compared to the day (Figure 21c; time: F(1, 56)=86.938, p=.00000). Similar to V.O\textsubscript{2} consumption, hourly V.CO\textsubscript{2} production consumption was no different between groups (Figure 21d; time: F(23, 644)=72.433, p=0.0000; time*diet: F(23, 644)=1.6699, p=.02605).

Respiratory exchange ratio (RER), a measure of substrate utilization (fat vs. carbohydrates) was higher in LFD-fed females at night compared to the day. In addition, LFD-fed females exhibited an elevated RER compared to HFD fed females of the same genotype at night (Figure 21e; diet: F(1, 56)=41.512, p=.00000; time: F(1, 56)=25.032, p=.00001; diet*genotype: F(1, 56)=6.1682, p=.01603; diet*time: F(1, 56)=8.0857, p=.00622). Hourly RER was increased in both controls and experimentals fed LFD compared to HFD-females at hours: 0000-0600, 2200 and 2300. Hourly RER was increased in only experimentals fed LFD compared to HFD-fed experimentals at hours: 0700-1200. Furthermore, experimentals females fed LFD exhibited higher RER compared to controls on LFD at hours 0800, 0900, 2000, and 2100 (Figure 21f; diet: F(1, 28)=25.765, p=.00002; diet*genotype: F(1, 28)=4.1489, p=.05121; time: F(23, 644)=17.474, p=0.0000; time*diet: F(23, 644)=6.6722, p=0.0000; time*genotype: F(23, 644)= 1.8598, p=.00887). All females exhibited elevated heat production, a measure of energy expenditure, at night compared to the day (Figure 21g; genotype: F(1, 56)=11.992, p=.00103; time: F(1, 56)=106.01, p=.00000). Hourly energy expenditure was elevated in
HFD-fed controls compared to HFD-fed experimentals at the 1200 hour (Figure 21h; genotype: F(1, 28)=7.5977, p=.01017; time: F(23, 644)=74.264, p=0.0000). These data indicate that GHSR expression in KNDy neurons plays a critical role in the metabolic compensation to HFD.

4.4.2.6 Meal Pattern Analysis

Energy intake (kCal) between days 1, 2, and 3 of data acquisition revealed an increase in energy intake in LFD-fed controls between day one and day three (Figure 22a; time*diet: (trending) F(2, 56)=3.0018, p=.05776). There were no significant differences in meal size (kCal) between genotypes or diets (Figure 22b). Females fed LFD consumed meals less frequently compared to females fed HFD (Figure 22c; Controls p=.0282; Experimentals p=.0005). Inversely, females fed HFD spend less time-consuming meals compared to those fed LFD (Figure 22d Controls p= .0019; Experimentals p=.0024). Hourly energy intake was elevated in experimentals fed a HFD compared to controls fed a HFD at the 0200 hour (p=.0332). Furthermore, control females on both diets consumed more energy than experimentals during hours 1900 (LFD: p=.0216, HFD: p=<.0001) and 2000 (LFD: p=.0059, HFD: p=.0228) (Figure 22e; time: F(23, 644)=19.199, p=0.0000; time*genotype: F(23, 644)=2.5320, p=.00011). Overall, the average total ingested (kCal) was the same for both genotypes on both diets (Figure 22f). This data, taken together indicates that females on HFD consumed meals less often and for shorter durations.

4.4.3 Experiment #3: Fasting- and ghrelin-induced refeeding

4.4.3.1 Meal Pattern Analysis

Upon refeeding one hour before lights out, food intake increased rapidly after a 24h fast in control females compared to food intake before fasting and fed controls (Figure 23a). Experimental females, however, did not exhibit the same rapid increase in food intake as
the controls. Experimental females did consume more at the time of refeeding; however, the food intake was clearly blunted (Figure 23b and c). Across the 24h before fasting, 24h after fasting and 24h of *ad libitum* feeding, total food ingested, meal size, and meal duration was not different between groups (Figure 23d, f, and g). Meal frequency was elevated in fed experimental females compared to controls (Figure 23e). Overall, fasting did not have the same effect in females lacking GHSR expression in KNDy neurons as it did with controls.

Unexpectedly, ghrelin injection (1mg/kg, IP) one hour before lights out did not have the same effect as fasting in females. All females, regardless of injection, showed a similar increase in food intake during the night (Figure 24a-c). Furthermore, across the 24h before ghrelin injection, 24h after ghrelin injection and 24h of after saline injection, no differences were observed in the total amount food ingested, meal frequency, meal size, or meal duration (Figure 24d-g).

### 4.4.4 Experiment #4: Thermoregulation

#### 4.4.4.1 Metabolic Parameters in the CLAMS in response to overnight cold stress

Experimental females consumed more oxygen (V.O2) during the night at both 10 and 25 ºC compared to the day. In addition, experimental females consumed more V.O2 at 10 ºC compared to 25 ºC at night, while control females only consumed more V.O2 at 10 ºC at night compared to the day (Figure 25a; time: F(2, 30)=29.932, p=.00000). Hourly V.O2 consumption was no different between groups (Fig. 25b; time: F(47, 470)=29.534, p=0.0000). Experimental females produced more carbon dioxide (V.CO2) during the night at both 10 and 25 ºC compared to the day. Furthermore, experimental females produced more V.CO2 at 10 ºC compared to 25 ºC at night, while control females only produced more V.CO2 at 10 ºC at night compared to 25 ºC at night (Figure 25c; time: F(2,
Similar to V.O₂ consumption, hourly V.CO₂ production consumption was no different between groups (Figure 25d; time: F(47, 470)=28.245, p=0.0000).

Respiratory exchange ratio (RER), a measure of substrate utilization (fat vs. carbohydrates) was closer to 1.0 for all females, indicating the utilization of carbohydrates instead of fat for energy. Only control females exhibited an increase in RER between day and both nights at 10 and 25 °C (Figure 25e; time: F(2, 30)=27.231, p=.00000). Hourly RER was not different between groups (Figure 25f; time: F(2, 30)=27.231, p=.00000). All experimental females exhibited elevated heat production, a measure of energy expenditure, both nights at 10 and 25 °C compared to the day. Furthermore, both control and experimental females produced more heat during the night at 10 °C compared to 25 °C (Figure 25g; genotype: F(1, 30)=5.2124, p=.02968; time: F(2, 30)=28.229, p=.00000). Hourly energy expenditure was elevated in experimental females compared to controls one hour before lights on (Figure 25h; time: F(47, 470)=28.701, p=0.0000). Hourly core body temperature (Tc) was no different between experimental or control females (Figure 25i).

4.4.4.2 Locomotor Activity in CLAMS in response to overnight cold stress

X-plane activity was not significantly different between controls and experimentals; however, there was a main effect of genotype on X-plane activity in that experimental females moved more than controls (Figure 26a; genotype: F(1, 30)=5.7932, p=.02245; time: F(2, 30)=3.2916, p=.05100). Hourly X-plane activity was elevated experimental females compared to controls 3-4 hours into the dark period (Figure 26b; time: F(47, 470)=7.8575, p=0.0000; time*genotype (trending): F(47, 470)=1.3658, p=.05939). Furthermore, wheel count activity was not significantly different between females (Figure 26c; time: F(2, 27)=3.3772, p=.04909 and 26d; time: F(47, 376)=11.376, p=0.0000).
These data, taken together reveal the importance of GHSR expression in KNDy neurons regarding thermoregulation. Experimental females appear to experience elevated metabolic activity as well as ambulatory activity in order to maintain the same core body temperature as control females, indicating a dysregulation in thermoregulation.

4.4.5 Liver gene expression

Because experimental females exhibited a higher fasting glucose compared to controls, we wanted to determine if we had inadvertently deleted Ghsr from kisspeptin expression cells in the liver. Ghsr expression was not detectable in either controls or experimental females (data not shown), these findings are corroborated in multiple publications (37, 38). More recent publications have found that liver Ghsr expression does increase to detectable values after a 24h fast, which we did not do (39, 40). These findings indicate that the differences in glucose levels after a 5h fast are due, in part, to the central deletion of GHSR from KNDy neurons and not a peripheral deletion.

4.5 Discussion

There is a gap in our understanding of the influence of E2 on ghrelin signaling in KNDy neurons and how they impact metabolic processes controlled by hypothalamic neurons. It is imperative to understand the inner workings of these circuits that regulate energy balance, especially as the world faces growing complications due to the popularity of extreme dieting as well as the overwhelming number of people dealing with the effects of obesity. Ghrelin, E2, and KNDy neurons work together to control neighboring neurons that regulate energy homeostasis. A recent study from our lab found that both negative (24 h fast and 30% caloric restriction) and positive (diet induced obesity) states of energy balance differentially impact the expression of ARC KNDy neuropeptides and their receptors. We demonstrated that E2 can both augment and oppose the effects of positive
or negative energy states on KNDy neuropeptides and receptors (27). In addition, we found that E2 increases Ghsr expression by 6-fold in KNDy (Tac2-GFP) neurons (27). In a follow up study, using electrophysiology, we found that ghrelin inhibits the M-current, a KCNQ potassium channel current, in KNDy (Tac2-GFP) neurons, making them more easily excitable by ghrelin. Furthermore, we found that E2 increases KNDy neuronal sensitivity to ghrelin (28). Therefore, KNDy neurons synergistically coordinate energy balance with levels of both E2 and ghrelin.

One pathway for KNDy neurons to control energy homeostasis is interacting with the melanocortin circuitry by directly depolarizing POMC neurons through kisspeptin and/or glutamate release and directly or indirectly hyperpolarizing NPY/AgRP neurons through glutamate release and/or through an enhancement of inhibitory GABA-ergic tone (13, 14). Furthermore, POMC and NPY/AgRP neurons modulate KNDy activity (13, 22, 41-43), thus producing a neuronal network of at least three cell types that respond differently to steroids, nutrients, and peripheral peptide hormones to modulate energy homeostasis.

Based on this information, we conducted a study to examine the importance of E2-induced GHSR expression in KNDy neurons in the maintenance of energy balance and thermoregulatory responses to temperature stress. We hypothesized that E2 increases KNDy sensitivity to ghrelin in females by augmenting Ghsr expression to diminish the orexigenic input of ghrelin to NPY neurons by enhancing the activity of POMC neurons, via glutamate, and to control thermogenesis via activation of sympathetic tone from POMC innervation into the PVH and via the release of neurokinin B in the POA, respectively.

We found that deleting GHSR in ARC KNDy neurons, regardless of steroid-treatment or diet, resulted in a more elevated blood glucose level in response to a 5h fast compared to control females. This in conjunction with the lack of GHSR expression in the liver indicates that the result of elevated fasting glucose in experimental females is due to
a central effect, not a peripheral artifact. POMC neurons are known to play a critical role in the control of blood glucose levels, particularly in females. Glucose tolerance tests performed in POMC knockout female mice found that these mice were glucose intolerant, the same could not be said for POMC knockout males (44).

Furthermore, ghrelin has been identified as a critical mediator of glucose homeostasis (45). Ghrelin administration increases blood glucose levels and reduces insulin levels, although the opposite effects have also been indicated (46). Recently, the differential effects of ghrelin on glucose homeostasis has been found to be dependent on the heteromerization between GHSR and the somatostatin receptor 5, both of which are G-protein-coupled receptors (GPCRs) (47). By deleting GHSR in KNDy neurons, and therefore altering the heteromerization between GHSR and somatostatin receptor in addition to altering the input from KNDy neurons, which in turn reduces POMC neuronal excitability and downstream results in the observed increase in fasting blood glucose levels. As both POMC and NPY/AgRP neurons play role in glucose homeostasis and hepatic glucose production (48), activation of the KNDy-melanocortin circuit by ghrelin in females with high E2 (proestrus) would increase hepatic glucose production ensuring more energy is available for elevated locomotor activity and energy expenditure.

High levels of E2 are known to increase activity levels in female mice, however, in our experimental females, the effect of EB-treatment was magnified compared to controls, indicating that GHSR expression in KNDy neurons does play some role in activity. We know that both POMC and NPY neurons project to the preoptic area (POA) as well as the ventromedial hypothalamus (VMH) where there is melanocortin-4-receptor (MC4R) expression. It was recently discovered that when E2 is high, input to the MC4R in the VMH increases activity in female mice (25). At the same time, we understand that KNDy neurons have increased ghrelin receptor expression when E2 is high (36), providing more input to POMC neurons which are known to project to and activate VMH and POA MC4R
receptor expressing neurons via α-MSH, altering energy expenditure and activity in females (49). This could explain why during periods of food scarcity (high ghrelin) a female in proestrus (high E2) would have more sensitive KNDy neurons, enhancing the activation of POMC neurons, leading to an increase in release of β-endorphin and cessation of reproduction (11). Furthermore, EB-treated experimental females exhibited a reduced V.O2 consumption and V.CO2 production compared to EB-treated controls. RER and heat were elevated by EB-treatment in both controls and experimentals; however, there was greater increase in controls, indicating dysregulation of E2’s influence on metabolism, substrate utilization, and energy expenditure due to the lack of GHSR in KNDy neurons.

We also found that EB-treated controls consumed less then EB-treated experimental females at night, this is a clear indication of the reduced input to POMC neurons in experimental females compared to controls. Under fed conditions, control and experimental females exhibited similar meal patterns and feeding behavior in the BioDAQ. However, the hourly data shows an increase in ingestive behavior at night, which is consistent with our findings from the metabolic chambers. On a HFD, meal patterns and energy intake were mostly not affected by genotype, only diet. Meal duration was reduced by HFD and meal frequency was increased, indicating mice on a HFD were eating for shorter periods of time, but were returning to the food hopper more frequently. The reduced meal duration, possibly due the high energy density, indicates mice were reaching satiety at a faster rate. This would also explain the reduced body weight and adiposity compared to controls, as previous work has shown that HFD does not change the total caloric intake, but it does alter meal patterns (50). Meal duration and size have been found to be correlated with body weight gain and adiposity (51). The increase in meal frequency could be due to the high palatability of the HFD as well as HFD-induced
increase in the number of ghrelin precursor-expressing cells in the mouse stomach, enhancing the drive to consume food (52).

Moreover, on HFD, experimental females were resistant to body weight gain and increased adiposity and had a slower glucose clearance rate. In addition, unlike in the E2-treated OVX females, activity in experimental females was not elevated compared to controls and metabolic parameters were no different (V.O2, V.CO2, RER, and energy expenditure). Only HFD reduced RER, which indicates the utilization of fat over carbohydrates for energy, a common effect of HFD. This data is supported by previous findings in which aged GHSR null mice had lower body weight and reduced adiposity; however, they also found elevated metabolic rates (V.O2 and V.CO2), which we did not, likely due to the HFD. Furthermore, they saw no differences in food intake or locomotor activity (53). As GHSR is only deleted in kisspeptin-expressing neurons in our model, our hypothesis is that the differences observed in body weight and adiposity were due the reduced meal duration on a HFD resulting from the GHSR deletion in KNDy neurons as total food intake, activity, and metabolic rates were no different. This hypothesis is supported by recent data suggesting a role for POMC and NPY/AgRP neurons in mediating the central control of adiposity by leptin (54). Leptin receptor activation in both POMC and NPY neurons plays a critical role in the sympathetic innervation of adipose tissue. When leptin receptors are deleted from POMC and NPY neurons, innervation of brown adipose tissue is reduced by 50% and in white adipose tissue is reduced by 30% (54). While leptin receptor activation in ARC neurons is not the focus of our study, we can postulate that the lack of ghrelin-induced KNDy activation may interfere with the sympathetic activation by melanocortin neurons leading to changes in adiposity. Likewise, consuming HFD is known to increase serum leptin concentration and reduce CNS leptin receptor expression. Therefore, due to the HFD, a reduction in POMC/NPY leptin receptor expression can reduce adipose tissue innervation as well as brown adipose tissue.
thermogenesis. In addition, GHSR deletion has also been shown to enhance thermogenic capacity in brown adipose tissues, an endpoint which we did not explore, this could also explain the resistance to HFD body weight gain and adiposity (55). Further experiments combining methods from thermoregulation experiments and HFD and measuring brown adipose tissue thermogenesis would be necessary to determine if this is true.

While E2-treated experimental females consumed more food than controls, intact experimental females did not respond to a 24h fast with an increase in food intake as compared to controls. Upon refeeding, controls have a clear surge in food intake immediately upon the return of food, while experimental females do not exhibit the same robust spike in food intake, which again is an indication of the importance of ghrelin signaling in KNDy neurons in controlling food intake in females. The lack of food intake in control females after ghrelin injection could be due to an insufficient quantity of ghrelin injected to elicit a response, although other publications (56) also used 1mg/kg, IP of ghrelin and did see an increase in food intake in wild type male mice using a different source of ghrelin. Additional experiments using higher doses (3 – 10 mg/kg) would be required to clearly determine the role of KNDy Ghsr expression in ghrelin-induced hyperphagia. Moreover, fasting provides additional cues to the hypothalamus during fasting, while ghrelin injection does not. For example, when mice are fasted several hormone levels change, such as ghrelin, leptin, insulin, and corticosterone (57). In addition, gene expression in the hypothalamus also changes, including NPY gene expression that increases after fasting (58). The physiological cues that arise due to fasting would provide more of a drive toward food intake than a single ghrelin injection alone, which could account for the differences in ingestive behavior between fasting and ghrelin injected females.

Kisspeptin neurons have been found to be involved in other homeostatic processes including thermoregulation, activity (25) and bone remodeling (15, 16). In fact,
current studies have confirmed that neurokinin B, expressed in KNDy neurons, mediated hot flushes in response to reduced E2. In this study, optogenetic activation of KNDy axon terminals in the preoptic area of the hypothalamus increased heat dissipation and vasodilation in the tail skin of mice, which was abolished by a neurokinin B receptor antagonist (17, 18). Additionally, in mice, which undergo torpor due to food scarcity and low ambient temperatures, ghrelin administration lowers core body temperature and enhances torpor. Interestingly, the ghrelin-induced torpor response is eliminated by ablation of the ARC (59). Fasting, which typically stimulates ghrelin, induces torpor in mice and rats housed in cool environments (60, 61). Ghrelin regulates the thermoregulatory response to cold stress through time-dependent changes in the circadian rhythm of hypothalamic neurosecretory neurons (61). Our thermoregulation study reveals that deletion of GHSR in KNDy neurons modestly increased energy expenditure and induced greater activity and wheel running in experimental females experiencing a short-term cold stress while maintaining core body temperature. These data indicate that experimental females moved more and produced more heat compared to controls to maintain the same core body temperature during cold stress. We hypothesize that it is due to suppressed Neurokinin B release from KNDy neurons in addition to the reduction in ghrelin input to KNDy neurons in our experimental females, resulting in a different thermogenic regulatory response to a short-term cold stress.

In conclusion, by deleting GHSR expression from ARC KNDy neurons, we have revealed a novel central pathway for the control of metabolism in female mice. E2 upregulates GHSR expression in ARC KNDy neurons, which, in turn, increases KNDy sensitivity to ghrelin (28). The activation of the KNDy-melanocortin circuit reduces food intake and increases activity and metabolism. As experimental females exhibited reduced metabolic rates when E2-treated, decreased adiposity and delayed glucose clearance on HFD and elevated activity and energy expenditure when cold stressed, we also conclude
that the interaction of E2 and ghrelin in KNDy neurons increases metabolic rates and alters the activation of the downstream circuits that control sympathetic tone, glucose homeostasis, and adiposity. This conclusion is supported by our findings (28) and by other studies characterizing the KNDy-POMC- NPY/AgRP circuit (14, 22, 30, 62). We hypothesize that by increasing excitatory input, via kisspeptin or glutamate, to POMC neurons while simultaneously inhibiting NPY/AgRP neurons, activation of KNDy neurons by ghrelin tilts the melanocortin balance towards POMC activation in females. GHSR expression plays a major role in adiposity and glucose homeostasis, without it, these aspects of energy balance become disrupted. Furthermore, experimental females are more sensitive to cold stress and increase activity to maintain the same core body temperature as controls. Collectively, these data suggest that GHSR activation in KNDy neurons modulates metabolism, activity, glucose homeostasis and thermoregulation illustrating one of many novel mechanisms for E2 and ghrelin to control KNDy neurons and their physiological functions.

4.6 Acknowledgement
The authors have no acknowledgements.

4.7 Statement of Ethics
The research presented in the manuscript was ethically conducted in accordance with institutional guidelines based on National Institutes of Health standards and all animal experiments were performed with Institutional Animal Care and Use Committee approval at Rutgers University.

4.8 Funding Sources
This work was supported by the US Department of Agriculture-National Institute of Food and Agriculture (NJ06195) and the National Institutes of Health (R21ES027119; P30ES005022; R01MH123544), RCLR Small Grant FY-21, and the Rutgers University ONE Nutrition Initiative.

4.9 Author Contributions

KC & TAR conceived and designed the project; YS provided the Ghsr$^{fl/fl}$ mice; NB provided the Tc data loggers; KC, DK, and SD performed the experiments; KC & TAR analyzed and interpreted data and drafted the manuscript.
References

30. Santollo J, Eckel LA. The orexigenic effect of melanin-concentrating hormone (MCH) is influenced by sex and stage of the estrous cycle. Physiol Behav. 2008;93:842-50.


Figures

Table 3. List of primers for liver qPCR

Figure 13. Body weight gain and body composition, and cumulative food intake for Oil- and EB-exposed control and experimental (KO) female mice fed LFD for 8 weeks. (A) Weekly body weight gain and (B) Week 8 body weight. (C) Percent change in fat mass and (D) lean mass. (E) Cumulative food intake over 8 weeks on LFD. For A: Single lowercase letters denote a steroid effect within controls only, single lowercase letters with * denote a steroid effect within only experimentals only, two lowercase letters denote a steroid effect in control and experimentals respectively. For B-D: Capped lines with lowercase letter denotes a steroid effect (a = P<.05; b= P<.01; c= P<.001; d = P<.0001). Data are represented as mean ± SEM and n=8 mice per group. Data were analyzed with two-way ANOVA with post hoc Holm-Sidak’s multiple comparison test.

Figure 14. Glucose and Insulin tolerance tests. (A) Glucose tolerance test (GTT) and (B) Insulin tolerance test (ITT) for Oil- and EB-exposed control and experimental (KO) female mice fed LFD for 8 weeks. Area under the curve (AUC) analysis for GTT (C) and ITT (D) females. (E) Fasting (5 h) glucose. * denotes significance between genotype (*= P<.05). Data are represented as mean ± SEM and n=8 mice per group. Data were analyzed with two-way ANOVA with post hoc Holm-Sidak’s multiple comparison test.

Figure 15. Average daytime, nighttime, and hourly X-activity, Y-activity, Food Intake, and Water Intake in Oil- and EB-exposed control and experimental (KO) female mice fed LFD for 8 weeks. (A) Daytime and nighttime total X-activity (counts), (B) Hourly X- activity, (C) Daytime and nighttime total Y-activity (counts), (D) Hourly Y-activity. (E)
Daytime and nighttime food and (F) water intake. Uppercase letters denote significance between genotypes. * above capped lines denote significance between time periods within treatment and genotype (a, * = P<.05; b, ** = P < .01). Data are represented as mean ± SEM and n=8 mice per group. Data were analyzed with two-way ANOVA with post hoc Holm-Sidak’s multiple comparison test.

Figure 16. Average daytime and nighttime V.O2, V.CO2, RER, and Energy Expenditure in Oil- and EB-exposed control and experimental (KO) female mice fed LFD for 8 weeks. (A) Daytime vs nighttime Oxygen consumption (V.O2, ml/min/kg), (B) Hourly Oxygen consumption (V.O2, ml/min/kg) (C) Daytime vs nighttime Carbon dioxide production (V.CO2, ml/min/kg and (D) Hourly Carbon dioxide production (V.CO2, ml/min/kg). (E) Daytime vs nighttime Respiratory exchange ratio (RER, V.CO2/V.O2) and (F) Hourly Respiratory exchange ratio (RER, V.CO2/V.O2). (G) Daytime vs nighttime Energy expenditure (kCal/hr) and (H) Hourly Energy expenditure (kCal/hr). For A, C, E, and G: * above capped lines denote significance between time periods within treatment and genotype. Lowercase letters denote a steroid effect within genotype and time period. Uppercase letters denote significance between genotypes within the same treatment. For B, D, F, and H: Lowercase letters denote a steroid effect within controls only, lowercase letters with * denote a steroid effect within experimentals only. Uppercase letters denote significance between genotypes within the same treatment (A, a, * = P<.05; B, b, ** = P < .01; C, c, *** = P < .001; D, d, **** = P < .0001). Data are represented as mean and n=8 mice per group. Data were analyzed with two-way ANOVA with post hoc Holm-Sidak’s multiple comparison test.

Figure 17. Meal pattern analysis in Oil- and EB-exposed control and experimental (KO)
female mice fed LFD for 8 weeks. (A) Average food ingested per day (grams) (B) Average meal size (grams) (C) Meal Frequency (D) Average meal duration (E) Average hourly food intake, and (F) Total food ingested over 72h. For A: lowercase letters above the columns denote significance in comparison to day 1 and uppercase letters above the columns denote significance in comparison to day 2. For E: Uppercase letters denote significance between genotypes within the same treatment. Lowercase letters denote a steroid effect within controls only, single letters with * denote a steroid effect within only experimentals only. For F: Lowercase letters over capped lines denote a steroid effect within genotype. Data are represented as mean ± SEM and n=8 mice per group. Data were analyzed with two-way ANOVA with post hoc Holm-Sidak’s multiple comparison test.

Figure 18. Body weight gain, body composition, and cumulative food intake for intact control and experimental (KO) female mice fed LFD or HFD for 8 weeks. (A) Weekly body weight gain and (B) Week 8 body weight. (C) Percent change in fat mass and (D) lean mass. (E) Cumulative energy intake over 8 weeks. For A and E: Lowercase letters denote a diet effect, uppercase letters denote a genotype effect. For B-D: * over capped lines denote a main effect of genotype (a, * = P<.05; b, **= P<.01; c = P<.001; d = P<.0001). Data are represented as mean ± SEM and n=8 mice per group. Data were analyzed with two-way ANOVA with post hoc Holm-Sidak’s multiple comparison test.

Figure 19. Glucose and Insulin tolerance tests. (A) Glucose tolerance test (GTT) and (B) Insulin tolerance test (ITT) for intact control and experimental (KO) female mice fed LFD or HFD for 8 weeks. Area under the curve (AUC) analysis for GTT (C) and ITT (D) females. (E) Fasting (5 h) glucose levels in whole blood. * denotes significance between genotype, * over capped lines denote an overall effect of genotype (* = P<.05; **= P<.01). Data are represented as mean ± SEM and n=8 mice per group. Data were analyzed with
two-way ANOVA with post hoc Holm-Sidak’s multiple comparison test.

**Figure 20. Average daytime, nighttime, and hourly X-activity, Y-activity, Energy Intake, and Water Intake** in intact control and experimental (KO) female mice fed LFD or HFD for 8 weeks. (A) Daytime and nighttime total X-activity (counts), (B) Hourly X-activity, (C) Daytime and nighttime total Y-activity (counts), (D) Hourly Y-activity. (E) Daytime and nighttime energy and (F) water intake. Uppercase letters denote significance between genotypes. Lowercase letters denote significance between diet. * above capped lines denote significance between time periods within treatment and genotype (a, *= P<.05; b, ** = P <.01; c, ***= P<.001; d, **** = P<.0001). Data are represented as mean ± SEM and n=8 mice per group. Data were analyzed with two-way ANOVA with post hoc Holm- Sidak’s multiple comparison test.

**Figure 21. Average daytime and nighttime V.O2, V.CO2, RER, and Energy Expenditure** in intact control and experimental (KO) female mice fed LFD or HFD for 8 weeks. (A) Daytime cs nighttime Oxygen consumption (V.O2, ml/min/kg), (B) Hourly Oxygen consumption (V.O2, ml/min/kg) (C) Daytime vs nighttime Carbon dioxide production (V.CO2, ml/min/kg and (D) Hourly Carbon dioxide production (V.CO2, ml/min/kg). (E) Daytime vs nighttime Respiratory exchange ratio (RER, V.CO2/V.O2) and (F) Hourly Respiratory exchange ratio (RER, V.CO2/V.O2). (G) Daytime vs nighttime Energy expenditure (kCal/hr) and (H) Hourly Energy expenditure (kCal/hr). For F, and H: Lowercase letters denote a diet effect, two lowercase letters denote diet differences between controls, followed by experimentalss. Single lowercase letters denote significance between experimentalss only. Uppercase letters denote significance between genotypes within the same treatment. For A, C, E, and G: * above capped lines denote significance
between time periods within treatment and genotype. Lowercase letters denote a diet effect within genotype and time period (a, * = P<.05; b, ** = P <.01; c, ***= P<.001; d, **** = P<.0001). Data are represented as mean ± SEM and n=8 mice per group. Data were analyzed with two-way ANOVA with post hoc Holm- Sidak’s multiple comparison test.

**Figure 22. Meal pattern analysis** intact control and experimental (KO) female mice fed LFD or HFD for 8 weeks. (A) Average energy intake per day (kCal) (B) Average meal size (kCal) (C) Meal Frequency (D) Average meal duration (E) Average hourly energy intake, and (F) Total kCal ingested over 72h. For A: lowercase letters above the columns denote significance in comparison to day 1. For B, C, D, and F: Lowercase letters over capped lines denote a diet effect within genotype. For E: Lowercase letters denote a diet effect within controls only, Uppercase letters denote a genotype effect on LFD, uppercase letters with * denote a genotype effect on HFD only (a, * = P<.05; b, ** = P <.01; c, ***= P<.001; d, **** = P<.0001). Data are represented as mean ± SEM and n=8 mice per group. Data were analyzed with two-way ANOVA with post hoc Holm-Sidak’s multiple comparison test.

**Figure 23. Meal pattern analysis** intact control and experimental (KO) female mice on normal chow diet. (A) Average hourly food intake for control females 24h before fasting, 24h after fasting, and fed normally. (B) Average hourly food intake for experimental females 24h before fasting, 24h after fasting, and fed normally. (C) Average hourly food intake for control and experimental females 24h after fasting. (D) Total food ingested over 24h before fasting, 24h after fasting, and fed normally. (E) Meal Frequency Average 24h before fasting, 24h after fasting, and fed normally. (F) Average meal size (grams) 24h before fasting, 24h after fasting, and fed normally. (G) Average meal duration 24h before
fasting, 24h after fasting, and fed normally. Lowercase letters over capped lines denote a diet effect within genotype. Lowercase letters denote significance between time periods. Lowercase letters above capped lines denote significance between genotypes within the same time period (a= P<.05; b= P <.01; c= P<.001; d= P<.0001). Data are represented as mean ± SEM and n=14 controls and n=8 experimental mice per group. Data were analyzed with two-way ANOVA with post hoc Holm-Sidak’s multiple comparison test.

**Figure 24. Meal pattern analysis** intact control and experimental (KO) female mice on normal chow diet. (A) Average hourly food intake for control females 24h before ghrelin injection (1mg/kg), 24h after ghrelin, and control saline injection. (B) Average hourly food intake for experimental females 24h before ghrelin injection (1mg/kg), 24h after ghrelin, and control saline injection. (C) Average hourly food intake for control and experimental females 24h after ghrelin injection. (D) Total food ingested over 24h before ghrelin injection (1mg/kg), 24h after ghrelin, and control saline injection. (E) Meal Frequency Average 24h before ghrelin injection (1mg/kg), 24h after ghrelin, and control saline injection. (F) Average meal size (grams) 24h before ghrelin injection (1mg/kg), 24h after ghrelin, and control saline injection. (G) Average meal duration 24h before ghrelin injection (1mg/kg), 24h after ghrelin, and control saline injection. Lowercase letters over capped lines denote a diet effect within genotype. Lowercase letters denote significance between time periods (a= P<.05; b= P <.01; c= P<.001; d= P<.0001). Data are represented as mean ± SEM and n=14 controls and n=8 experimental mice per group. Data were analyzed with two-way ANOVA with post hoc Holm-Sidak’s multiple comparison test.

**Figure 25. Average daytime and nighttime V.O2, V.CO2, RER, and Energy Expenditure** in intact control and experimental (KO) female mice during 6h overnight cold
stress (10°C). (A) Daytime vs nighttime Oxygen consumption (V.O₂, ml/min/kg), (B) Hourly Oxygen consumption (V.O₂, ml/min/kg) (C) Daytime vs nighttime Carbon dioxide production (V.CO₂, ml/min/kg) and (D) Hourly Carbon dioxide production (V.CO₂, ml/min/kg). (E) Daytime vs nighttime Respiratory exchange ratio (RER, V.CO₂/V.O₂) and (F) Hourly Respiratory exchange ratio (RER, V.CO₂/V.O₂). (G) Daytime vs nighttime Energy expenditure (kCal/hr) and (H) Hourly Energy expenditure (kCal/hr). (I) Core body temperature (Tc). For A, C, E, and G: Lowercase letters denote significance within genotype compared to the day, uppercase letters denote significance within genotype, between night at 10°C and 25°C. For B, D, F, and H: * denote significance between genotype. (A, a,* = P<.05; B, b, ** = P <.01; C, c, ***= P<.001; D, d, **** = P<.0001). Data are represented as mean ± SEM and n=6 mice per group. Data were analyzed with two-way ANOVA with post hoc Holm-Sidak’s multiple comparison test.

**Figure 26.** Average daytime, nighttime, and hourly X-activity and Wheel Counts in intact control and experimental (KO) female mice during 6h overnight cold stress (10°C). (A) Daytime and nighttime total X-activity (counts), (B) Hourly X-activity, (C) Daytime and nighttime total Wheel activity (counts), (D) Hourly Wheel activity. * denote significance between genotype (* = P<.05). Data are represented as mean ± SEM and n=6 mice per group. Data were analyzed with two-way ANOVA with post hoc Holm-Sidak’s multiple comparison test.
### Table 3. List of primers for liver qPCR.

<table>
<thead>
<tr>
<th>Gene</th>
<th>Accession #</th>
<th>Forward Primer</th>
<th>Reverse Primer</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ghsr</td>
<td>NM_177330</td>
<td>CAGGGACCAGAACCACAAA</td>
<td>AGCCAGGCTCGAAAGACTC</td>
</tr>
<tr>
<td>Kiss1</td>
<td>NM_178260</td>
<td>TGATCTCAATGGCTTTTG</td>
<td>CTCTTGACATAACCGCGAT</td>
</tr>
</tbody>
</table>

*Ghsr*, growth hormone secretagogue receptor and *Kiss-1*, kisspeptin
**Figure 14.**

**A.**

GTT

![Graph showing glucose levels over time for different conditions.]

**B.**

ITT

![Graph showing glucose levels over time for different conditions.]

**C.**

GTT

![Bar graph showing AUC values for different conditions.]

**D.**

ITT

![Bar graph showing AUC values for different conditions.]

**E.**

Fasting Glucose

![Bar graph showing fasting glucose levels for different conditions.]

*Significance indicated.*
Figure 16.

A. 

B. 

C. 

D. 

E. 

F. 

G. 

H.
Figure 17.
Figure 18.
Figure 19.
Figure 20.
Figure 21.

A. 

B. 

C. 

D. 

E. 

F. 

G. 

H. 

---

Figure 21.

A. 

B. 

C. 

D. 

E. 

F. 

G. 

H.
Figure 22.

A. Ingested per day

B. Meal Size

C. Meal Frequency

D. Meal Duration

E. Hourly Energy Intake

F. Total Ingested
Figure 23.
Figure 24.
Figure 25.

A. Control Experimental

B. Intact Experimental

C. Control Experimental

D. Intact Experimental

E. Control Experimental

F. Intact Experimental

G. Control Experimental

H. Intact Experimental

I. Control Experimental
Figure 26.

A. X Ambulatory (counts)

B. X Ambulatory

C. Wheel (counts)

D. Wheel Counts
CHAPTER 5: SUMMARY
5. Summary

Estrogen signaling is a primary central regulator of reproduction and energy balance and ghrelin signaling is a critical modulator in the coordination between the two. In the current studies, we elucidate the influence of E2 on GHSR signaling in KNDy neurons in the ARC of the hypothalamus and the critical and understudied role it plays in modulating LH pulsatility, arcuate gene expression, the metabolic response to ovariectomy and to diet-induced obesity, and the thermoregulatory response to cold stress. Our data clearly shows the necessity of ghrelin receptor expression (GHSR) in KNDy neurons and the impact it has on reproduction and energy balance.

Prior to these experiments being conducted, early work indicated that Ghsr expression increases 6-fold in KNDy neurons when E2 is elevated (1). These experiments led us to investigate the sensitivity of KNDy neurons to ghrelin with high and low E2. Results from this work show that KNDy neurons are indeed more sensitive to the M-current inhibitory effect of ghrelin, making neurons more easily excitable (2), a mechanism commonly utilized in other hypothalamic neurons (3, 4). The next phase of investigation led to the development of a Kiss1-specific GHSR knockout mouse model utilizing Cre-lox technology. With this novel model, we were able to further investigate the physiological importance of Ghsr expression in KNDy neurons and the regulation by E2.

Based on previous work in this field, we know that KNDy neurons play a major role in reproduction, especially in females. We know that ARC kisspeptin neurons have also been found to directly sense steroid (E2) feedback (5). Furthermore, when ARC KNDy neurons were specifically targeted and stimulated using optogenetic techniques, this provided additional evidence that synchronous activation of ARC KNDy neurons can generate pulsatile LH release (6). Together these observations point to a hypothesis that KNDy neurons form an interconnected network that is modulated by its neuropeptides
(kisspeptin, neurokinin B, and dynorphin) in addition to negative feedback by E2 to alter LH pulsatility. In the current study, have found that when it comes to reproduction, deleting the GHSR in KNDy neurons alone is not sufficient to alter fertility, age of puberty or estrous cyclicity. Furthermore, we determined that KNDy regulation of LH pulsatility is strongly influenced by the interaction of E2 and Ghsr expression in these neurons, as ghrelin-injected control females exhibited a temporary cessation of LH pulsatility, while experimental females did not experience the same robust cessation. We were also able to confirm previous findings (1) that E2-induced increase in Ghsr expression in the ARC is indeed due to the increase in KNDy neurons and ghrelin injection was also able to significantly alter KNDy gene expression, revealing that reproduction and LH pulsatility are regulated by an interaction of both ghrelin and E2.

Not only does the interaction of E2 and Ghsr in KNDy neurons play a critical role in reproduction, but also energy balance. Previous studies report both POMC and NPY/AgRP neurons receive direct glutamatergic input from ARC KNDy neurons in mice (7), therefore KNDy, acting through POMC and NPY/AgRP neurons comprise the proposed key neurocircuit in coordinating energy status with reproduction (8). In our current study, we found that our experimental (KO) females treated with E2 had reduced metabolic rates. On HFD, experimental females were resistant to diet-induced obesity exhibiting less adiposity and delayed glucose clearance. Experimental females also respond to short-term cold stress overnight with increased activity and energy expenditure.

Future experiments would need to be conducted to further explore our hypothesis. These experiments would include repeating methods with OVX and E2 replacement and HFD at the same time to determine the effect of high E2 with diet-induced obesity and the implications on feeding, metabolism, glucose homeostasis, reproduction, LH pulsatility and gene expression. On the other hand, combining OVX and E2 replacement with chronic caloric restriction would also allow us to determine the physiological effects
(feeding, metabolism, glucose homeostasis, reproduction, LH pulsatility and gene expression) of high E2 and high levels of ghrelin together, especially during cold stress as it would give us a better understanding on what is occurring during states of torpor in female mice. Furthermore, we would perform additional electrophysiology experiments to determine the role of POMC and NPY/AgRP neurons in this neurocircuitry by simultaneously patching KNDy and POMC neurons and exploring the connectivity and response to ghrelin perfusion. We would also explore the effects on bone remodeling with our experimental model and the impact that E2 and ghrelin have on bone structure.

Alternative strategies include exploring the differential effects of developmental deletion of GHSR at different timepoints throughout the life of the animal using Ghsr\textsuperscript{fl/fl} mouse lines coupled with adeno-associated virus (AAV) delivery (icv) of kisspeptin driven Cre recombinase during discrete developmental timepoints. We would also explore alternative routes (icv, etc.) and concentrations (3-10mg/kg) of ghrelin administration for the LH and feeding studies. Finally, we will repeat these experiments with male mice to determine if androgens (testosterone, dihydrotestosterone) and E2 also enhances Ghsr expression in KNDy neurons and what effect it may have on energy balance and reproduction in males.

We conclude that in the ARC, high levels of E2 (proestrus or OVX/EB replacement) increase Ghsr expression in KNDy neurons. Ghrelin activates GHSR to inhibit the M-current, a KCNQ potassium channel current, in KNDy (Tac2-GFP) neurons, via a PLC-PKA pathway. Thus, KNDy neurons are more excited by ghrelin, which we found in Chapter 2 (2). In addition, we have found that Ghsr expression in KNDy neurons participates in the control of LH secretion when fasting or after ghrelin injection and modulates the expression of KNDy neuropeptides in interaction with E2 (Chapter 3). Finally, we have found that the interaction of E2 and ghrelin in KNDy neurons increases metabolic rates and alters the activation of the downstream circuits that control
sympathetic tone, glucose homeostasis, thermoregulation, and adiposity (Chapter 4). These studies indicate that exploring these neurocircuits and coupled mechanisms of energy balance may be critical to our understanding of associated dysfunction in other physiological systems in human health such as infertility.
References


6. Appendices

6.1 Sex differences in regulation of Ghsr expression in Tac2-EGFP neurons

6.1.1 Introduction
To determine if Ghsr expression is similarly regulated by testosterone in ARC KNDy (Tac2) neurons, we repeated previous established experiments which discovered that Ghsr expression in increased by 6-fold by E2 treatment in females (1).

6.1.2 Materials and Methods

6.1.2.3 Animals
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 (6-12 weeks of age) were housed under constant photoperiod conditions (12/12 h light/dark cycle) and maintained at a controlled temperature (25°C). Animals were given food (LabDiet 5V75) and water ad libitum. Animals were weaned at postnatal day 21 (PD21). Sexually mature Tac2-EGFP males were used for all cell harvesting experiments. Genotype was determined by using PCR products of extracted DNA from ear clippings. Tac2-EGFP mice were generated by mating either positive EGFP males to positive EGFP females or positive EGFP males to WT EGFP females.

6.1.2.4 Surgical Procedure
To elucidate the interactions of testosterone and KNDy (Tac2) neurons, adult males were gonadectomized (GDX) under isoflurane anesthesia 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 GDX, males were separated into 2 treatment groups – oil and testosterone propionate (TP); n = 6 mice per group (10 single cells and 3 pools of 5 per mouse). TP was purchased
from Steraloids (Newport, RI, USA) and dissolved in ethanol prior to mixing in sesame oil. Ethanol was allowed to evaporate off for 24h prior to storage. TP was stored at 4°C until needed. Animals were injected subcutaneously (s.c.) at 1000 h on post-GDX day 5 with 100 μg of TP or oil-vehicle. On post-GDX day 6, the same dose of TP or oil was injected at 1000 h. On post-GD day 7, mice were rapidly decapitated, and the brain was prepared for cell harvesting.

6.1.2.5 Cell harvesting of dispersed Tac2 neurons

We collected Tac2-GFP neurons to determine cell-type specific changes in gene expression similar to our previous publication (2). Briefly, animals were sedated with ketamine (100 ml of 100 mg/ml stock, IP) and decapitated. Brains were transferred to a vibratome containing cold, oxygenated aCSF and sliced into 250 mM 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 papain solution (40 min at 30 °C) and washed with low calcium aCSF followed by regular aCSF. The ARC was triturated 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 3 pools of 5 Tac2 neurons from each animal.

Harvested single cells and pools were reverse transcribed as previously described (3, 4). 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 MgCl2, 0.625 mM dNTPs (Clontech), 15 U Rnasin, 400 ng anchored oligo(d)T (Life Technologies), 100 ng random hexamers (Promega), 10mM 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 pool or 5 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 (4, 5). Primers for single cell PCR are the same as those used with ARC tissue qPCR (Chapter 3; Table 2), with the exception of Tac2: F: 50 TCTGGAAGGATGGCTGAAAGTG-3'; R: 50-GTAGGGAGGGAGCCAACAG-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.

6.1.3 Results

Single Tac2 neurons were harvested from GDX oil- and TP-treated males to determine if co-expression of Tac2 and Ghsr is regulated by TP, as it is in females regulated by E2. Tac2 gene expression was detected in 8 of 10 single cells for both oil- and TP-treated males. Ghsr was not detected in any single cells (Figure1a). In pools of single Tac2-GFP neurons a trending reduction in Tac2 gene expression was observed with TP treatment (Figure 1b; P= .0696). Similar to single cell analysis, in pools, Ghsr was also not detected (data not shown). This data suggests that Ghsr expression in KNDy (Tac2) neurons is sexually dimorphic (not expressed in males) and is not regulated by steroids (TP) in males.
References


Figures

Figure 1. GHSR signaling in Tac2 neurons from GDX male mice is not regulated by TP. (A) Representative gel is single-cell PCR amplification products in oil- and TP-treated Tac2 neurons. a = artificial CSF; -RT = without reverse transcriptase (B) Tac2 gene expression in Tac2-GFP neuron pools (5 cells per pool). No Ghsr expression was detected in Tac2-GFP pools. Data were analyzed by Student’s t-test.
Figure 1.

A.

<table>
<thead>
<tr>
<th></th>
<th>Oil-treated</th>
<th>TP-treated</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Tac2 - 302 bp</td>
<td>Tac2 - 302 bp</td>
</tr>
<tr>
<td></td>
<td>GHSR - 122 bp</td>
<td>GHSR - 122 bp</td>
</tr>
</tbody>
</table>

B.

**Tac2**

0.0696

- Oil
- TP

Fold Change

- Oil
- TP
6.2 Ghrelin-induced c-Fos expression

6.2.1 Introduction

To determine if expression of c-Fos, an early immediate gene, is altered by GHSR deletion in KNDy neurons, we performed immunocytochemistry in intact control and experimental females injected with either ghrelin or saline. We expected experimental females to show reduced c-Fos expression in KNDy neurons specifically, with unaltered expression in neighboring cells, like NPY neurons.

6.2.2 Materials and Methods

6.2.2.3 Animals

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 (10-12 weeks of age) were housed under constant photoperiod conditions (12/12 h light/dark cycle) and maintained at a controlled temperature (25°C). Animals were given food and water ad libitum, unless noted otherwise. Animals were weaned at postnatal day 21 (PD21).

6.2.2.4 Immunocytochemistry

Mice were fasted for 5h and injected with either ghrelin (1mg/kg IP) or saline 90 minutes prior to perfusion. Mice were briefly anesthetized with isoflurane then perfused with 0.9% saline followed by 4% paraformaldehyde. After perfusion, brains were removed and stored in 4% paraformaldehyde for 24h, then switched to 4% paraformaldehyde containing 20% Sucrose until needed.

Free-floating, dual-labeled chromogen immunocytochemistry was undertaken as reported previously (1). Briefly, 40µM thick coronal brain sections, taken through the mediobasal hypothalamus were cut on a cryostat at -16°C (Leica) and stored in a cryoprotectant solution until needed. Cryoprotectant was first washed off of slices with Tris-buffered saline (TBS). Sections were treated with 3% hydrogen peroxide for 10 min
to quench endogenous peroxidase activity and then washed in TBS. For the first immunolabeling, sections were incubated for 24h at 4°C in rabbit polyclonal primary antisera directed against c-FOS in TBS containing 0.3% Triton X-100 and 0.25% BSA and 2% normal goat serum. Sections were then incubated in biotinylated anti-rabbit immunoglobulins (Vector Laboratories) at 1:200 for 90 min at room temperature. After subsequent washing in TBS, the sections were incubated in Vector Elite avidin–peroxidase (Vector Laboratories) at 1:100 for 90 min at room temperature. Immunoreactivity was revealed using glucose-oxidase, nickel-enhanced diaminobenzidine hydrochloride that resulted in a black precipitate within the nucleus of the labeled cell. For the second immunolabeling, sections were washed in 3% hydrogen peroxide to quench any remaining peroxidase, washed in TBS, and then incubated in a polyclonal rabbit anti-NKB antiserum containing 2% goat serum for 24h at 4°C. Sections were then incubated in peroxidase-labeled anti-rabbit immunoglobulins (1:200; Vector Laboratories) for 4 h at room temperature. Immunoreactivity was then revealed using, diaminobenzidine hydrochloride without nickel to generate a brown precipitate within the cytoplasm. Polyclonal rabbit antisera directed against c-FOS (1:1000; abcam; CA#190289) and neurokinin B (1:1000; novus bio; NB300-201SS).

6.2.2.5 Immunocytochemistry analysis

Sections were examined using an Olympus FSX microscope using bright-field microscopy. Analysis of the c-Fos-labeled cells was undertaken by counting the number of NiDAB-labeled cells (black) within the DAB-labeled region (NKB; brown) of the arcuate. Statistical analysis was undertaken with two-way ANOVA and post hoc Holm-Sidak’s multiple comparison test.

6.2.3 Results

Unfortunately, NKB staining was not optimal and was obstructed at the single cell level by the c-Fos staining (Figure 1a). Therefore, we were unable to count double-labeled
neurons, only c-Fos labeled cells within the arcuate nucleus and associated with the light brown staining of NKB. With this in mind, we found a significant reduction in c-Fos expression in experimental females compared to controls (Figure 1b; p=0.0230). Follow up experiments will be performed to further optimize NKB staining for more conclusive results.

**References**
Figures

Figure 1. GHSR KO and c-Fos expression in the ARC (A) Representative images of arcuate coronal sections with c-Fos (black) and NKB (brown) staining. All images are imaged at 10X. (B) Number of c-Fos positive cells. n=5-6 mice per treatment. Data are represented as mean ± SEM; n=5-6 mice per group. Data were analyzed with two-way ANOVA with post-hoc Holm-Sidak’s multiple comparison test.
Figure 1.

A. Control vs. Experimental

Saline

Ghrelin

B. c-Fos

# c-Fos positive cells

Saline

Ghrelin

Control

Experimental