THE HORMONAL REGULATION OF KISSPEPTIN AND NEUROPEPTIDE Y HYPOTHALAMIC NEURONS

by

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A thesis submitted in conformity with the requirements for the degree of Master's
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Abstract

Kisspeptin (encoded by Kiss1) is a hypothalamic neuropeptide that is directly regulated by sex steroids and directly stimulates gonadotropin-releasing hormone (GnRH) neurons. Kisspeptin cell models were established in order to facilitate future molecular analysis of kisspeptin. mHypoA-51 and mHypoA-63 cell lines were found to express kisspeptin, estrogen receptor α and β, substance P, but not tyrosine hydroxyase. Furthermore, estrogen decreased Kiss1 expression in both cell lines. Based on these results, it was concluded that mHypoA-51 and mHypoA-63 are representative of arcuate kisspeptin neurons. Accumulating evidence also indicates that kisspeptin indirectly stimulates GnRH neurons through afferent neurons. Kisspeptin receptor expression was detected in native neuropeptide Y (NPY) neurons. Using the mHypoE-38 cell line, kisspeptin was found to directly regulate NPY mRNA expression and secretion via the ERK1/2 and p38 MAPK pathways. This is the first evidence that kisspeptin directly stimulates NPY neurons to potentially exert indirect effects on GnRH neurons.
Acknowledgments

I would like to express my deepest thanks to my supervisor, Dr. Denise Belsham. Your personal mentorship over the years has allowed me to become a confident and passionate scientist. I am entirely grateful for both the academic and lifelong learning experiences you have provided me with. Thank you for making the past two years a truly rewarding and memorable period in my life. I would also like to thank my committee members, Dr. Michael Wheeler and Dr. David Lovejoy, for their constant support from day one. I am truly honoured to have been under the tutelage of such esteemed scientists.

To all members of the Belsham lab, past and present, thanks for the amazing ride. I will forever cherish the good times we had whether we were working late nights in the lab, having lunch on Baldwin Street, or touring the cities of France. In particular, I am truly grateful to Dr. Sandeep Dhillon, who guided me throughout my journey from performing experiments to writing my first manuscript. Thank you for everything.

Finally, I would like to thank my family for their unwavering love and instrumental guidance throughout every aspect of my life. I cannot imagine my world without you. To my mother, thank you for believing in me and never giving up.
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List of Abbreviations

AF-1 activating function-1
AF-2 activating function-2
AgRP agouti-related peptide
ARO anaplastic thyroid cancer
AR androgen receptor
ARC arcuate nucleus
AVPV anteroventricular paraventricular nucleus
BCA biocinchonicic acid
BSA bovine serum albumin
Ca$^{2+}$ calcium
CNS central nervous system
CNTF ciliary neurotrophic factor
cDNA complementary deoxyribonucleic acid
DPN 2,3-bis(4-Hydroxyphenyl)-propionitrile
DNA deoxyribonucleic acid
DAPI 4’-6-diamidino-2-phenylindole
DMSO dimethyl sulfoxide
DBD DNA-binding domain
DMEM Dulbecco’s modified eagle medium
estrogen 17β-estradiol
E embryonic day
EIA enzyme immunoassay
ER estrogen receptor
ERE estrogen response element
ERK extracellular-related kinase
FACS fluorescence-activated cell sorting
FBS fetal bovine serum
FITC fluorescein isothiocyanate
FSH follicle-stimulating hormone
GABA γ-aminobutyric acid
<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Full Form</th>
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<tbody>
<tr>
<td>GFAP</td>
<td>glial fibrillary acidic protein</td>
</tr>
<tr>
<td>GnRH</td>
<td>gonadotropin-releasing hormone</td>
</tr>
<tr>
<td>GPCR</td>
<td>G-protein couple receptor</td>
</tr>
<tr>
<td>hr</td>
<td>hour(s)</td>
</tr>
<tr>
<td>HPG</td>
<td>hypothalamic-pituitary gonadal axis</td>
</tr>
<tr>
<td>ICC</td>
<td>immunocytochemistry.</td>
</tr>
<tr>
<td>IHC</td>
<td>immunohistochemistry</td>
</tr>
<tr>
<td>IP3</td>
<td>inositol-(1,4,5)-triphosphate</td>
</tr>
<tr>
<td>icv</td>
<td>intracerebroventricular</td>
</tr>
<tr>
<td>ISH</td>
<td><em>in situ</em> hybridization</td>
</tr>
<tr>
<td>kb</td>
<td>kilobases</td>
</tr>
<tr>
<td>kDa</td>
<td>kilodaltons</td>
</tr>
<tr>
<td>Kiss1</td>
<td>kisspeptin gene</td>
</tr>
<tr>
<td>Kiss1r</td>
<td>kisspeptin receptor</td>
</tr>
<tr>
<td>KO</td>
<td>knock-out</td>
</tr>
<tr>
<td>LH</td>
<td>luteinizing hormone</td>
</tr>
<tr>
<td>LBD</td>
<td>ligand binding domain</td>
</tr>
<tr>
<td>MAPK</td>
<td>mitogen activated protein kinase</td>
</tr>
<tr>
<td>mRNA</td>
<td>messenger ribonucleic acid</td>
</tr>
<tr>
<td>ME</td>
<td>median eminence</td>
</tr>
<tr>
<td>NE</td>
<td>norepinephrine</td>
</tr>
<tr>
<td>NPY</td>
<td>neuropeptide Y</td>
</tr>
<tr>
<td>NSE</td>
<td>neuron specific enolase</td>
</tr>
<tr>
<td>NT</td>
<td>neurotensin</td>
</tr>
<tr>
<td>NTC</td>
<td>no template control</td>
</tr>
<tr>
<td>OVX</td>
<td>ovarectomized</td>
</tr>
<tr>
<td>PBS</td>
<td>phosphate buffered saline</td>
</tr>
<tr>
<td>RT-PCR</td>
<td>reverse transcriptase polymerase chain reaction</td>
</tr>
<tr>
<td>PVN</td>
<td>periventricular nucleus</td>
</tr>
<tr>
<td>PI3K</td>
<td>phosphatidylinositol 3-kinase</td>
</tr>
<tr>
<td>PLC</td>
<td>phospholipase C</td>
</tr>
<tr>
<td>POA</td>
<td>preoptic area</td>
</tr>
<tr>
<td>Acronym</td>
<td>Description</td>
</tr>
<tr>
<td>---------</td>
<td>-------------</td>
</tr>
<tr>
<td>POMC</td>
<td>pro-opiomelanocortin</td>
</tr>
<tr>
<td>PKC</td>
<td>protein kinase C</td>
</tr>
<tr>
<td>PPT</td>
<td>4,4’,4”’-(4-Propyl-[1H]-pyrazole-1,3,5-triyl)trisphenol</td>
</tr>
<tr>
<td>RFRP</td>
<td>RF (arginine-phenylalanine) amide related peptide</td>
</tr>
<tr>
<td>RNA</td>
<td>ribonucleic acid</td>
</tr>
<tr>
<td>siRNA</td>
<td>small interfering ribonucleic acid</td>
</tr>
<tr>
<td>SV40</td>
<td>simian virus 40</td>
</tr>
<tr>
<td>SEM</td>
<td>standard error of the mean</td>
</tr>
<tr>
<td>T-Ag</td>
<td>T-antigen</td>
</tr>
<tr>
<td>TBS-T</td>
<td>Tris-buffered saline with Tween</td>
</tr>
<tr>
<td>TH</td>
<td>tyrosine hydroxylase</td>
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Chapter 1

Introduction
1.1 Introduction

Kisspeptin is a key neuropeptide hormone involved in initiating puberty and maintaining reproductive function. Originally discovered to suppress metastasis, the role of kisspeptin in reproduction was first identified with the finding that mutations in the kisspeptin receptor, Kiss1r, were associated with idiopathic hypogonadotropic hypogonadism in human patients (1, 2). Kiss1r and Kiss1 (kisspeptin gene) knockout mice were found to have the same reproductively-impaired phenotype, with delayed pubertal development, infertility and reduced sexual behaviour (2-4). Additional studies revealed that kisspeptin regulates the reproductive axis by increasing gonadotropin secretion though the direct stimulation of gonadotropin-releasing hormone (GnRH) neurons. Specifically, studies have demonstrated that kisspeptin neurons project to GnRH neurons in the preoptic area (POA) and median eminence (ME) (5, 6), and 60-90% of GnRH neurons express Kiss1r (7). Kisspeptin has also been found to induce GnRH, luteinizing hormone (LH) and follicle-stimulating hormone (FSH) release (8-12), increase GnRH neuronal firing (13, 14), and stimulate GnRH mRNA expression and secretion in GnRH-secreting neuronal cell lines (15). Collectively, these studies provide strong evidence that kisspeptin exerts its control of the reproductive axis though the direct regulation of GnRH neurons.

Although it is well-established that GnRH neurons are regulated by sex steroid hormones, the precise mechanism by which this is achieved is poorly understood. With the emergence of kisspeptin as a critical regulator of GnRH neurons, it now seems likely that kisspeptin neurons integrate and conduct signals from sex steroids to GnRH neurons (16). Several studies have demonstrated that kisspeptin neurons located in the anteroventral periventricular nucleus (AVPV) mediate the positive effects of estrogen, while kisspeptin neurons located in the arcuate nucleus (ARC) mediate the inhibitory effects of estrogen to ultimately control GnRH neurons.
For instance, gonadectomy in mice significantly decreased Kiss1 mRNA expression in the AVPV and increased Kiss1 mRNA expression in the ARC. These changes could be reversed by sex steroid replacement (17, 18). Additional studies have investigated the estrogen receptor (ER) subtype involved in this differential response to estrogen. In female mice, estrogen regulation of Kiss1 expression in the AVPV and ARC is intact in ERβ knockout mice but not ERα knockout mice (17). The role of ERα was confirmed in studies showing that blockade of ERα completely abolishes the LH response to kisspeptin administration (20). Conversely, an ERα-specific agonist, but not an ERβ-specific agonist, mimics the Kiss1 mRNA response to estrogen within the ARC (21). Altogether, these results strongly indicate that estrogen acts through ERα, but not ERβ, to mediate both positive and negative regulation of Kiss1 expression in females. While the field of kisspeptin physiology has advanced considerably due to the wealth of knowledge provided by these in vivo studies, the molecular mechanisms underlying differential estrogen regulation of kisspeptin neurons are still largely unknown. Indeed, there has been a paucity of studies focusing on Kiss1 regulation at the molecular level due to the heterogeneity and inaccessibility of the hypothalamus. Thus, the first objective of this thesis was to establish kisspeptin-expressing neuronal cell lines from the murine adult hypothalamus in order to provide suitable models for these studies. Novel adult hypothalamic cell lines generated by Belsham et al. (22) were extensively characterized and evaluated herein in terms of expression profiles and hormonal responses. These characterization studies have become increasingly important as endogenous kisspeptin-expressing cell models have yet to be established.

To date, studies have largely focused on the direct regulation of kisspeptin on GnRH neurons. While this mechanism has been unequivocally demonstrated (5-15), the possibility that kisspeptin indirectly stimulates GnRH neurons through afferent neuronal networks has not been
excluded. In fact, the Kiss1r is expressed in other regions of the hypothalamus not associated with GnRH neurons (7, 23). One study found that blockade of fast synaptic transmission significantly reduced, but did not completely abrogate, GnRH neuronal responses to kisspeptin stimulation. In fact, kisspeptin was also found to increase the firing rate of non-GnRH neurons in the medial POA (24). These findings indicate that the effects of kisspeptin on GnRH neurons are both direct and indirect, and therefore, kisspeptin may indirectly regulate GnRH neuronal responses through an unidentified afferent neuronal network.

Neuropeptide Y (NPY) is another key hormone that is well established as a major regulator of the reproductive axis; therefore, the possibility that kisspeptin indirectly regulates GnRH neurons through the direct activation of NPY neurons was considered. NPY neurons in the ARC of the hypothalamus have been found to project to GnRH neurons (25, 26), and morphological evidence indicates co-localization of NPY receptors and GnRH neurons (26). Both in vivo and in vitro studies have demonstrated that NPY stimulates GnRH secretion (27-33), as NPY infusion into the third ventricle of ewes significantly elevated GnRH levels in the ME (27), and exposure of NPY to GT1-7 cells significantly increases GnRH secretion (29). Although the stimulatory effect of NPY on GnRH neurons has been well-documented, NPY can also have an inhibitory action on GnRH release, which is associated with changes in steroidal environment, stage of development and species (34-39). For instance, intrahypothalamic perfusion of NPY decreased mean GnRH levels in ovariectomized (OVX) rats, while the same NPY perfusion stimulated mean GnRH levels in intact rabbits (37). Altogether, NPY is a potent regulator of the reproductive axis with both positive and negative regulation demonstrated depending on the experimental conditions. The second goal of this thesis was to determine whether kisspeptin can directly regulate NPY synthesis and secretion, potentially resulting in GnRH regulation (Figure 1.1). It is not yet known whether NPY neurons in situ express Kiss1r,
Figure 1.1. Schematic illustration of the afferent neuronal control of GnRH neurons.

GnRH neurons situated within the hypothalamus are regulated by afferent neuronal inputs expressing kisspeptin, NPY, NT, dynorphin, neurokinin B, GABA, dopamine and NE, among several others. In particular, kisspeptin has recently emerged as a key regulator of the HPG axis, directly and potently stimulating the synthesis and release of GnRH. There is evidence that kisspeptin also acts via neurons afferent to the GnRH neuron to indirectly exert effects on reproduction. The identity of this afferent neuronal network, however, is unknown. In Aim II, it was hypothesized that kisspeptin directly regulates NPY neurons to indirectly activate GnRH neurons.
thus fluorescence-activated cell (FAC)-sorted NPY-GFP neurons from the hypothalamus of transgenic mice were used to explore this possibility. Due to the inherent cellular heterogeneity of the hypothalamus, hypothalamic NPY-secreting cell lines, mHypoE-38 and mHypoE-42, were used to further study the potential mechanisms involved in kisspeptin regulation of NPY neurons. Both cell lines have been previously characterized and have been found to be representative of NPY neurons (40, 41). The expression profiles, secretory events and intracellular signaling pathways activated by kisspeptin in these cell lines were evaluated. These studies will by key in revealing a potential mechanism through which kisspeptin indirectly stimulates GnRH secretion.

The overall goal of this thesis was approached in two aims: 1) identify kisspeptin-synthesizing cell lines derived from the ARC and AVPV that can be differentially regulated by estrogen exposure to mediate the positive and negative steroidal influence on the reproductive axis, and 2) describe a novel, indirect pathway by which kisspeptin could potentially regulate the reproductive axis through NPY subpopulations afferent to GnRH neurons. Altogether, the studies presented in this thesis not only provide evidence for a novel kisspeptin pathway, but also establish valuable kisspeptin-expressing cell models, thereby serving to advance the growing field of kisspeptin physiology.

1.2 Reproductive Function

1.2.1 The hypothalamic-pituitary-gonadal (HPG) axis

Mammalian reproductive function is controlled by an intricate complex of hormonal messengers and feedback loops within the hypothalamic-pituitary-gonadal (HPG) axis (42-44) (Figure 1.2). At the pinnacle of the HPG axis are GnRH-expressing neurons, located in the medial POA of the hypothalamus. At puberty, these GnRH neurons initiate pulsatile secretion of
Figure 1.2. Schematic illustration of the hypothalamic-pituitary-gonadal axis.

GnRH neurons within the hypothalamus release GnRH in a pulsatile fashion into the hypophyseal portal system, which controls the synthesis and secretion of gonadotropin hormones, LH and FSH from the anterior pituitary gland. LH and FSH are released into systemic circulation and target the testes and ovaries to regulate steroidogenesis. The sex steroids, estrogen and testosterone, are in turn secreted into systemic circulation to exert positive and negative feedback effects on GnRH and gonadotropin levels at the hypothalamus and pituitary, respectively.
GnRH into the hypophyseal portal system. Pulsatile GnRH triggers gonadotrope cells within the anterior pituitary to stimulate the synthesis and secretion of the gonadotropin hormones, LH and FSH, into systemic circulation (44). LH and FSH target the gonads to trigger gametogenesis and steroidogenesis in both males and females. In the ovaries, LH promotes the synthesis of androgen precursors, induces ovulation of mature follicles and sustains the corpus luteum, while FSH stimulates the production of estrogen and promotes the development of immature follicles. In the testes, LH stimulates the synthesis of testosterone, and FSH supports the sustenance of Sertoli cells, which is important for sperm maturation (45-47). The steroidal hormones produced by the ovaries and testes are then released into systemic circulation and regulate hypothalamic GnRH and pituitary gonadotropin secretion through positive and negative feedback mechanisms (48, 49). This feedback loop is a highly orchestrated process and disruption to any component of the system can lead to a range of reproductive disorders. Thus, the precise regulation of hormone synthesis and secretion within the HPG axis is critical to the proper functioning of the reproductive system.

1.2.2 GnRH in the hypothalamus

GnRH is the master hormone regulating reproductive function. This decapeptide hormone is produced in peripheral tissues including the placenta, gonads and mammary glands, but is primarily localized in the hypothalamus (50-53). During rodent embryonic development, GnRH neurons first appear in the epithelium of the medial olfactory placode located outside of the central nervous system. GnRH neurons then migrate across the cribiform plate to enter the telencephalon and rostral diencephalon, ultimately reaching adult localization in the anterior hypothalamus (54). An estimated 400-1000 GnRH-expressing neurons are present in the adult mouse. The majority of these neurons are located within the medial POA, with a proportion found in a diffuse band extending from the diagonal band of Broca to the caudal portion of the
medial POA (55). This scattered network of neurons is believed to function in a synchronized manner as a GnRH pulse generator (56). The generation of pulsatile GnRH secretion at the ME is the central element governing reproductive function.

1.2.3 Afferent neuronal regulation of GnRH neurons

GnRH neurons form the final common pathway for the central control of reproduction, thus understanding the factors that control GnRH neurons is of fundamental importance. Afferent neuronal populations expressing kisspeptin (57), NPY (29, 30), neurotensin (NT) (58), dynorphin (59), neurokinin B (59), γ-aminobutyric acid (GABA) (60), dopamine (61) and norepinephrine (NE) (62), among several others, have been found to directly regulate the synthesis and release of GnRH. Two of these neuropeptides, kisspeptin and NPY, are of great significance to this thesis and are discussed in greater detail below.

1.2.3.1 Kisspeptin

Please refer to Section 1.2.4.1.

1.2.3.2 Neuropeptide Y

NPY is recognized as a key neuropeptide involved in the control of the HPG axis. Neuroanatomical evidence indicates that NPY neurons synapse directly with GnRH neurons, as NPY neurons in the ARC have been found to project to the cell bodies and presynaptic terminals of GnRH neurons (25, 26). The role of NPY in reproductive function is shown in NPY knockout mice, which are incapable of generating a functional LH surge (33). To further assess this role of NPY in the regulation of GnRH release, one study used a push pull cannula approach and measured GnRH levels after NPY infusion. It was found that the concentration of GnRH within the ME was significantly elevated in response to NPY (27). In a separate study, NPY exposure to the GnRH-expressing GT1-7 cells was found to significantly increase GnRH secretion (29).
Although the stimulatory effect of NPY on GnRH neurons has been well-documented, NPY can also have an inhibitory action on GnRH release, which is associated with changes in the steroidal milieu and developmental stage (34-39). In OVX rats, administration of NPY into the third ventricle leads to a decrease in plasma LH levels (38). Moreover, intrahypothalamic perfusion of NPY decreased GnRH levels in OVX rats, while the same NPY perfusion stimulated GnRH levels in intact rabbits (37). Overall, NPY is a potent regulator of the reproductive axis with both positive and negative regulation demonstrated depending on the experimental conditions.

1.3 Kisspeptin

1.3.1 Synthesis and Expression

The term kisspeptin refers to a family of peptides encoded by the Kiss1 gene. This gene is located on the long arm of human chromosome 1 at the locus 1q32 and contains 4 exons. The first two exons are not translated, while the third exon contains 38 non-coding bases, the translational start site and 100 translated bases, and the fourth exon consists of a further 332 translated bases, the translational stop site and the polyadenylation signal (63). The Kiss1 gene product is a 145-amino acid precursor protein, which includes a signal peptide and a 54-amino acid peptide termed kisspeptin-54. In the 145-amino acid precursor, the kisspeptin-54 sequence is surrounded by dibasic residues where furin or prohormone convertases are speculated to proteolytically cleave the propeptide (64). Kisspeptin-54 can be further truncated into 14, 13 or 10 amino acid C-terminal fragments (kisspeptin-14, -13 and -10). Collectively, these peptides are referred to as kisspeptin (Figure 1.3), and belong to a family of peptides called the RF-amide related peptides (RFRPs), which all share a common Arg-Phe-NH$_2$ motif at the C-terminus. As no obvious cleavage sites have been identified that would result in the generation of kisspeptin-14, -13 or -10, it is believed that kisspeptin-54 is unstable and may be degraded into the shorter
Figure 1.3. Schematic Illustration of the processing of Kiss1 to produce kisspeptins.

The primary peptide product of the Kiss1 gene (Kp145) is proteolytically cleaved to produce the kisspeptins, Kp54, Kp14, Kp13 and Kp10. All forms of kisspeptin are amidated at the C-terminal end and are capable of binding
products (64). All four peptides (kisspeptin-54, -14, -13 and -10) exhibit similar efficacy and affinity to Kiss1r, revealing the importance of the C-terminal end of the peptide in binding and activating the receptor (64). Although all four kisspeptin peptides are biologically active (65), the physiological relevance of the shorter peptides is still unknown.

Kisspeptin is a highly conserved peptide found within mammalian and non-mammalian vertebrates, and has been localized both centrally in the brain and in peripheral tissues. The following expression analyses will refer to that of the mouse, as detailed distribution patterns of kisspeptin has been mapped within this species. Kisspeptin mRNA and protein has been localized in hypothalamic nuclei including the ARC, AVPV and periventricular nucleus (PVN), with additional but more subtle expression in the anterodorsal POA (9, 17, 18, 66). The expression of kisspeptin in the hypothalamus is consistent with the role of kisspeptin in the HPG axis. Within regions of the murine brain outside of the hypothalamus, kisspeptin mRNA and protein has been detected in the medial amygdala, bed nucleus of the stria terminalis, subfornical organ, paraventricular thalamus, periaqueductal grey and locus coeruleus (9, 66, 67). In the periphery, kisspeptin mRNA and/or protein is found in: the syncytiotrophoblasts of the placenta; pancreatic islet cells; cells of the corpora lutea, interstitial gland and thecal layer of the ovary; vascular endothelial cells of the aorta, coronary artery and umbilical vein; as well as the kidney, liver, testes and small intestine (68-70). Although the role of kisspeptin within extrahypothalamic areas remains unclear, knowledge of the function of kisspeptin in the hypothalamus continues to expand.

1.3.2 The kisspeptin receptor, Kiss1r

The Kiss1r was first isolated as an orphan G-protein coupled receptor (GPCR) from a rat brain cDNA library using degenerate primers based on conserved sequences within the GPCR transmembrane domains (23). The Kiss1r maps to chromosome 19p13.3 and contains 5 exons,
which encodes a 398-amino acid protein sharing ~45% homology with galanin receptors, but with no functional interaction with galanin ligands (23, 65, 70). With a larger than predicted molecular weight of 75 kDa, it is suggested that the Kiss1r is post-translationally modified by glycosylation at three potential N-terminal sites or by palmitoylation at the C-terminus (23, 65).

The expression pattern of the Kiss1r overlaps with that of Kiss1, with the receptor expressed in the central nervous system, placenta, testis, ovary, pancreas, intestine, liver, pituitary, vasculature, spleen and lymph nodes (23, 64, 65, 70). In relation to its role in reproduction, Kiss1r is expressed within the POA, colocalized with approximately 60-90% GnRH neurons (7, 71). At the cellular level, GnRH neurons are currently the only neurochemically defined cell type expressing Kiss1r. Expression of Kiss1r has also been documented in the ARC, PVN, dorsomedial hypothalamic area and posterior hypothalamus (7, 23). In extra-hypothalamic areas, Kiss1r is found in the pons, midbrain, thalamus, hippocampus, amygdala, cortex and striatum (7, 23). Apart from the hippocampus, the physiological role of the Kiss1r outside the hypothalamus remains obscure.

1.3.3 Signaling pathways activated by kisspeptin

Initial studies of the intracellular signaling pathways activated by the Kiss1r utilized heterologous cell models expressing Kiss1r. These original studies demonstrated that activation of Kiss1r by kisspeptin is coupled to the G-protein $G_{q/11}$ to activate phospholipase C (PLC), leading to inositol-(1,4,5)-triphosphate (IP$_3$)-mediated intracellular Ca$^{2+}$ release and activation of protein kinase C (PKC) via diacylglycerol (64, 65, 70). Additionally, kisspeptin was found to stimulate arachidonic acid release and activate the mitogen-activated protein kinase (MAPK) pathways, extracellular signal-related kinase 1/2 (ERK1/2) and p38 (64, 72). Subsequent studies using cell models derived from different tissues revealed that the specific pattern of signaling kinases activated by kisspeptin/Kiss1r binding is dependent on cellular context (73-75). For
example, anaplastic thyroid cancer (ARO) cells demonstrated stimulation of ERK, but not p38 or Akt in response to kisspeptin treatment (73). In contrast, ERK and Akt were activated by kisspeptin in the GnRH-expressing GT1-7 and GN11 cell lines (15). In order to investigate the signaling cascades activated by kisspeptin in a more physiological context, blockade of signaling pathways was performed in hypothalamic explants (72). In this ex vivo system, it was shown that kisspeptin-stimulated GnRH secretion is mediated by PLC activation, increases in intracellular Ca$^{2+}$, and stimulation of the ERK1/2 and p38 MAPK pathways. This study, however, was completed in hypothalamic explants comprised of multiple cell types, and therefore the signaling mechanisms activated in potentially different sets of kisspeptin responsive neuronal systems could not be distinguished. Therefore, further studies utilizing endogenous Kiss1r-expressing cell models are required in order to provide a more complete understanding. A summary of the signaling pathways reported to be activated by kisspeptin/Kiss1r binding is found in Figure 1.4.

1.3.4 Role of kisspeptin in reproduction

The essential role of kisspeptin in reproduction was originally identified with the finding that mutations in the Kiss1r were associated with idiopathic hypogonadotropic hypogonadism in human patients (1, 2). This role for the Kiss1r was confirmed through the independent generation of three different transgenic mouse lines with mutations in the Kiss1r gene (2, 3, 10, 76, 77). These mice were complete phenocopies of affected humans, displaying infertility, low levels of sex steroids and gonadotropic hormones, and impaired pubertal development. Mice with genetic disruption of Kiss1 were subsequently generated and were also found to share this reproductively-impaired phenotype (77). Despite severe hypogonadotropism and sexual immaturity, Kiss1 mutant mice retain the ability to secrete gonadotropins after kisspeptin administration, and Kiss1r mutant mice retain a normal pituitary response to GnRH, show preserved hypothalamic GnRH content, and correct localization of GnRH neurons (2). These
Figure 1.4. Schematic illustration of the signal transduction pathways activated by kisspeptin/Kiss1r binding.

Kisspeptin can activate a number of signaling pathways through activation of the G-protein coupled Kiss1r. Kisspeptin stimulates the G protein Gq/11 to activate phospholipase C (PLC), resulting in inositol-(1,4,5)-triphosphate (IP3)-mediated intracellular calcium release and activation of protein kinase C (PKC) via diacylglycerol. In addition, kisspeptin can activate the phosphatidylinositol-3-kinase 3-kinase (PI3K)/Akt pathway as well as the mitogen-activated protein kinase (MAPK) pathways, extracellular signal-related kinase 1/2 (ERK1/2) and p38.
observations suggest a primary defect in the GnRH pulse generator due to impaired signaling of an essential upstream regulator. On this basis, numerous studies were initiated to evaluate whether kisspeptin is able to regulate GnRH neurons and elicit GnRH release.

1.3.4.1 Kisspeptin-mediated regulation of GnRH neurons

The presence of both kisspeptin and its receptor in the hypothalamus and pituitary and their critical role in puberty and reproduction indicate that kisspeptin is key regulator of the HPG axis. In Kiss1 and Kiss1r knockout mice, LH and FSH levels are significantly lower than in wildtype mice, suggesting a role for kisspeptin in the control of gonadotropin secretion (2, 3, 10, 76, 77). Indeed, kisspeptin administration has been found to increase plasma LH levels in mice (9), rats (11), sheep (78), cows (79), primates (80) and humans (8, 81), and plasma FSH levels in mice (9), rats (12) and humans (8). Doses as low as 1 fmol and 100 fmol effectively stimulate LH and FSH release, respectively, making kisspeptin the most potent stimulator of gonadotropin secretion (12, 82). This control of gonadotropin levels by kisspeptin has been found to occur through pituitary gonadotropes and hypothalamic GnRH neurons; however, the role of kisspeptin on gonadotropes is debated. While some in vitro studies have shown an increase in LH secretion in response to kisspeptin treatment (83), in vivo findings indicate that kisspeptin is not involved in gonadotropin release (84). On the other hand, studies examining the effect of kisspeptin on hypothalamic GnRH neurons have been more conclusive. Approximately 60-90% of GnRH neurons express the Kiss1r (7, 71), and immunohistochemical (IHC) data demonstrate that greater than 85% of GnRH neurons exhibit increased levels of cfos in response to kisspeptin (71). In addition, close apposition between GnRH and kisspeptin neurons has been detected (5).

Subsequent studies found that the LH and FSH response to kisspeptin administration can be abolished by a GnRH receptor antagonist (9, 12, 82), and intracerebroventricular (icv) injection of kisspeptin was found to elicit GnRH release into the cerebrospinal fluid of sheep (10). Using
an *ex vivo* model system, kisspeptin was shown to elicit GnRH release from hypothalamic explants (72). Taken together, these studies convincingly demonstrate that the action of kisspeptin in the regulation of the gonadotropic axis occurs primarily at GnRH neurons (*Figure 1.1*).

In addition to having direct effects on GnRH neurons, kisspeptin has been found to have indirect effects through afferent neuronal networks to the GnRH neuron. Evidence for an indirect action of kisspeptin was first provided by the presence of Kiss1r in other regions of the hypothalamus not associated with GnRH (7, 23). Subsequently, kisspeptin was found to excite unidentified non-GnRH neurons in the presence and absence of estrogen. In order to distinguish between the direct and indirect effects of kisspeptin on GnRH neurons, fast synaptic transmission was blocked using GABA and glutamate inhibitors, and electrophysiological responses were measured. It was found that the firing rate of GnRH neurons was significantly reduced, but not completely abolished, in response to kisspeptin treatment, indicating that the excitatory action of kisspeptin on GnRH neurons is partially mediated through afferent neurons. Furthermore, this attenuated response occurred only in the presence of estrogen (24). These findings indicate that the effects of kisspeptin on GnRH are both direct and indirect, and therefore, kisspeptin may regulate GnRH indirectly through an unidentified afferent network.

### 1.3.4.2 Kisspeptin activation at puberty

As inactivating mutations of the Kiss1r result in impaired pubertal maturation, activating mutations of the Kiss1r, which causes prolonged activation of the receptor, can lead to precocious puberty (85, 86). These observations led to further investigation into the potential role of kisspeptin in switching on the HPG axis. Both Kiss1 and Kiss1r mRNA levels increase with puberty in several species including mice (13), rats (87) and primates (88, 89). In mice, it was found that this increase in Kiss1 mRNA is localized to the AVPV but not in the ARC (5, 13), and
Figure 1.1. Schematic illustration of the afferent neuronal control of GnRH neurons.

GnRH neurons situated within the hypothalamus are regulated by afferent neuronal inputs expressing kisspeptin, NPY, NT, dynorphin, neurokinin B, GABA, dopamine and NE, among several others. In particular, kisspeptin has recently emerged as a key regulator of the HPG axis, directly and potently stimulating the synthesis and release of GnRH. There is evidence that kisspeptin also acts via neurons afferent to the GnRH neuron to indirectly exert effects on reproduction. The identity of this afferent neuronal network, however, is unknown. In Aim II, it was hypothesized that kisspeptin directly regulates NPY neurons to indirectly activate GnRH neurons.
is associated with an increased sensitivity of GnRH neurons to kisspeptin. One study found that kisspeptin causes depolarization in only 27% of GnRH neurons in juvenile mice, which increases to 45% in prepubertal mice and 90% in adult mice (13). This increase in the responsiveness of GnRH neurons does not appear to be due to an increase in Kiss1r expression on GnRH neurons, as Kiss1r expression was similar between age groups, but rather a heightened sensitivity of Kiss1r, increased neuronal connectivity between kisspeptin immunoreactive fibers and GnRH neurons, along with elevated Kiss1 mRNA levels (5, 13). Further studies investigated the effect of exogenous kisspeptin injection to juvenile female rats (90) and juvenile primates (80). Both studies reported that chronic kisspeptin administration was sufficient to advance puberty, as detected by vaginal opening and uterine weight in the rats, an elevated gonadotropin levels in both species. These studies demonstrate that kisspeptin and its receptor facilitate pulsatile GnRH release and awakening of the reproductive axis, thus acting as gatekeepers to the development of puberty.

1.4 Estrogen

1.4.1 Synthesis and Expression

Estrogens are a group of steroid hormones that are involved in a diverse set of physiological processes including reproduction, sexual development, energy homeostasis, cardiovascular physiology, neuroprotection, neuronal growth and differentiation, mood and cognition (91-93). There are three major forms of estrogen found within the body: estrone, estriol and estradiol, which has two further subforms, 17β-estradiol and 17α-estradiol (91, 94, 95). Estriol, estrone and 17α-estradiol have a significantly lower ER binding affinity as compared to 17β-estradiol (96), except within the brain, where both 17α-estradiol and 17β-estradiol have high binding affinities (97). 17α-estradiol, however, is not present in systemic circulation and is
unaltered by ovariectomy, castration or adrenaolectomy, indicating production of 17α-estradiol primarily within the brain (98, 99). Therefore, 17β-estradiol (estrogen) is considered to be the most potent and predominant form of estrogen (95, 100).

The major source of systemic estrogen is the premenopausal ovary, where estrogen biosynthesis occurs within thecal and granulosa cells (91). All three forms of estrogen are derived from the C-19 androgen precursors, androstenedione and testosterone, which are produced within thecal cells and delivered to granulosa cells. Aromatase cytochrome p450, a microsomal enzyme of the p450 superfamily, then catalyzes the conversion to estrogens within the granulosa cells (96, 101). During pregnancy, the placenta becomes the major source of estrogen in humans and primates. In both males and females, smaller amounts of estrogen are produced by osteoblasts and chondrocytes, vascular endothelium, mesenchymal cells of adipose tissue, aortic smooth muscle and within the brain (93, 102). Estrogens act mainly through paracrine mechanisms at these sites (93, 102).

1.4.2 Estrogen receptors (ERs)

The biological actions of estrogen are mediated by three ER subtypes: ERα, ERβ and GPR30. ERα and β are classically recognized as estrogen receptors, and are members of the nuclear receptor superfamily (103, 104). In addition, these two receptor subtypes can mediate rapid non-genomic responses through membrane-associated ERs (98, 105). The gene structure of ERα and β is characteristic to members of the steroid receptor superfamily, consisting of: 1) the N-terminal A-B domain, containing activating function 1 (AF-1) that is involved in transcriptional activation; 2) the C region, a highly conserved DNA-binding domain (DBD) containing two zinc fingers; 3) the D region, which is referred to as the hinge region, linking the DNA-binding domain to the ligand-binding domain (LBD); and 4) the C-terminal E domain, which contains AF-2 and is responsible for ligand binding (106). Although ERα and β are
derived from separate genes, there is 90% homology in the DBDs and 53% homology in the LBDs (107, 108). Accordingly, ERα and β have been reported to have both unique and overlapping functions within the body (107-109).

GPR30, in contrast, has only recently been recognized as an estrogen receptor (110). Upon cloning of GPR30, the receptor was immediately identified to be a part of the GPCR family, as characterized by its seven membrane-spanning domains. Within the GPCR family, GPR30 belongs to the class A rhodopsin-like receptors, which can be further classed into the chemokine receptor-like 2 subfamily. Closely related GPCRs such as the angiotensin II 1A receptor and the interleukin 8A receptor share approximately 28% sequence homology with GPR30 (111, 112).

ERs display a widespread distribution through the body, with overlapping expression in certain tissues (98, 107). ERα and β can be found co-expressed within the epididymis, mammary glands, adrenal glands, bones, thyroid and brain, while ERα is expressed uniquely in the uterus, heart, kidney and liver, and ERβ is expressed uniquely in the ovary, prostate, gastrointestinal tract, bladder, lung and hematopoetic cells (98, 107). Expression analyses of GPR30 have been performed recently, with widespread expression reported in the ovary, heart, lung, liver and brain (113-115). At the cellular level, GPR30 has been detected at the plasma membrane and endoplasmic reticulum (114). Taken together, ERα, ERβ and GPR30 have each been established as endogenous receptors of estrogen, mediating the diverse biological actions of this important steroid hormone.

1.4.3 Role of estrogen in reproduction

Estrogen is one of the primary factors controlling the gonadotropic axis through both positive and negative feedback loops between the gonads and brain (46, 47, 116, 117) (Figure 1.2). In both males and females, estrogen exerts a negative feedback loop on hypothalamic
GnRH and pituitary gonadotropin levels (48, 118, 119). However, during the period prior to ovulation in females, this action is switched to a positive feedback mechanism for the generation of the GnRH/LH surge (116, 120, 121). For instance, ovariectomy of female rats in diestrus or early proestrus results in an increase in LH pulse frequency and amplitude, but ovariectomy during late proestrus reduces these parameters (122). Despite the vital role of estrogen in reproductive control, the precise mechanisms underlying this switch from negative to positive feedback are poorly understood. However, it is generally believed that both direct and indirect processes are involved. One study demonstrated a reduction in GnRH mRNA expression and secretion in response to estrogen treatment in the GT1-7 GnRH-expressing cell line, indicating that the direct action of estrogen on the GnRH neuron is inhibitory (118). On the other hand, in vivo studies in OVX mice and rhesus monkeys found that administration of a physiological dose of estrogen is sufficient to induce the GnRH/LH surge, suggesting that the direct inhibitory effect of estrogen on GnRH neurons can be overcome by estrogen-sensitive afferent neurons (123). In fact, a number of estrogen-sensitive afferent neurons whose fibres intimately contact GnRH neurons have been identified, including but not limited to neuronal populations expressing kisspeptin, NPY, NT, NE and glutamate (124). Elevated levels of estrogen could potentially stimulate the synthesis and release of these excitatory neuropeptides/neurotransmitters to ultimately overcome the direct inhibitory effects of estrogen on GnRH neurons. Taken together, the effects of estrogen on the HPG axis are believed to occur through both direct and indirect mechanisms, which have yet to be fully characterized.

1.4.3.1 Estrogen-mediated regulation of Kisspeptin neurons

The hypothalamic kisspeptin neuronal population has emerged as a key target of estrogen, and is a strong candidate for mediating the effects of estrogen on GnRH neurons. The role of sex steroids in the regulation of kisspeptin neurons was first revealed with the finding that
gonadectomy of male and female rats increased Kiss1 mRNA expression in the hypothalamus, and sex steroid replacement reversed this effect (11). Subsequent studies using \textit{in situ} hybridization (ISH) and immunocytochemistry (ICC) provided a higher anatomical resolution for expression analyses, revealing differential estrogen regulation of \textit{Kiss1} expression in distinct hypothalamic areas. In mice, Kiss1 mRNA expression was decreased specifically in the AVPV and PVN and increased in the ARC after gonadectomy. These changes could be reversed by sex steroid replacement (17, 18). Studies examining \textit{Kiss1} regulation across the rat ovarian cycle further demonstrated that ARC \textit{Kiss1} levels were increased at diestrus when estrogen levels are low, and decreased at proestrus when estrogen levels are high. In contrast, AVPV \textit{Kiss1} levels were increased on the afternoon of proestrus when estrogen is at peak levels (125). Altogether, these studies demonstrate that AVPV kisspeptin neurons mediate the positive effects of estrogen, while ARC kisspeptin neurons mediate the inhibitory effects of estrogen on the gonadotropic axis (\textbf{Figure 1.5}). Furthermore, recent studies in the mouse, goat and sheep have demonstrated that ARC kisspeptin neurons colocalize with neurokinin B (NKB) and dynorphin (Dyn), neuropeptides that are also essential in the control of reproduction (126-128). Increasing evidence indicates that this subpopulation of ‘KNDy’ neurons plays a key role in mediating sex steroid feedback control of GnRH neurons (126-128). Thus, ARC kisspeptin neurons may co-secrete kisspeptin, NKB and Dyn, and these three neuropeptides may work in concert to regulate the pulsatile release of GnRH.

Differences between the involvement of ER$\alpha$ and $\beta$ in estrogen-mediated regulation of kisspeptin neurons have also been investigated. In female mice, the majority of kisspeptin neurons in the ARC, AVPV and PVN express ER$\alpha$ and ~25-40% express ER$\beta$ (17). In male mice, ~90% of kisspeptin neurons in the ARC express ER$\alpha$ and ~65% express the androgen receptor (AR) (18). The role of each of the ER subtypes in the regulation of kisspeptin neurons
Kisspeptin is an important mediator of the sex steroid feedback loop that regulates hypothalamic GnRH neurons. Kisspeptin activates Kiss1r-expressing GnRH neurons, resulting in GnRH secretion, which in turn stimulates LH and FSH secretion and the production of sex steroids from the gonads. Estrogen upregulates Kiss1 mRNA expression in kisspeptin neurons from the anteroventral periventricular nucleus (AVPV), and downregulates Kiss1 mRNA expression in kisspeptin neurons from the arcuate nucleus (ARC). Therefore, the hypothalamic kisspeptin neuronal population provides a mechanism whereby estrogen can exert both positive and negative feedback effects on the reproductive axis.
has been studied through analysis of transgenic mice with specific mutations in these receptor subtypes. In male ERα knockout (KO) and ARKO mice, regulation of ARC Kissl expression by testosterone still occurs, indicating that both receptor types mediate this regulation (18). In female mice, estrogen regulation of Kissl expression in the AVPV and ARC is intact in ERβKO but not ERαKO mice (17). This finding was confirmed in studies showing that blockade of ERα completely abolishes the LH response to kisspeptin administration (20), and the ERα agonist, 4,4',4"-(4-Propyl-[1H]-pyrazole-1,3,5-triyl)trisphenol (PPT), but not the ERβ agonist, 2,3-bis(4-Hydroxyphenyl)-propionitrile (DPN), mimics the Kissl mRNA response to estrogen within the ARC (21). Thus, these results strongly indicate that estrogen acts through ERα to mediate both positive and negative regulation of Kissl expression in females.

1.5 Cell Models

The physiological system implicated in reproductive function is situated predominantly in the hypothalamus. However, the cellular events and transcriptional regulation underlying reproductive control are poorly understood due to the complex circuitry of the hypothalamus and the lack of appropriate cell models (129-131). Classical in vivo approaches have been instrumental in establishing synaptic connectivity between distinct hypothalamic nuclei and the functional purpose of numerous neuropeptides and neurotransmitters (132). However, in vivo techniques cannot firmly establish the direct action of an agent on specific hypothalamic neurons and cellular events (118). Non-transformed primary hypothalamic cultures are difficult to maintain, have a short life span and represent a heterogeneous population of neurons and glial cells. For this reason, researchers have turned to immortalized, clonal cell lines, which represent an unlimited, homogeneous neuronal population that can be manipulated using a number of molecular techniques (133-135) (Figure 1.6a). In particular, cell lines have proven to be
indispensable in the study of gene structure, gene expression, secretory events and characterizing the molecular mechanisms responsible for regulating gene expression (136). The use of cell models has contributed profoundly to our understanding of how peripheral factors regulate transcriptional and secretory events that may ultimately contribute to the control of reproduction.

1.5.1 Embryonic hypothalamic cell lines, mHypoE-xx

In order to study the cellular mechanisms and direct regulation of specific hypothalamic neurons, researchers have turned to immortalized cell models. Cell lines representative of the central nervous system (CNS) have previously been established from neuroblastomas (Neuro2A) and pheochromocytomas (PC12) (137); however, these models are not truly representative of fully differentiated CNS neurons. As of 2004, few cell models existed that were representative of the hypothalamus and proved to be useful in understanding the molecular biology of specific peptidergic hypothalamic cells (133-135). The paucity of cell models available to study the specific biology of neuroendocrine cells prompted the Belsham group to generate an array of immortalized cell models from the hypothalamus (136). Cells were immortalized using retroviral gene transfer of the oncogene SV40 T-antigen into primary hypothalamic cell culture from mice at embryonic days 15, 17 and 18 (40). The resulting mixed population of cells was further subcloned to obtain homogeneous cell lines (Figure 1.6b). Altogether, Belsham et al. have established 38 embryonic, clonal, hypothalamic cell lines. These cell lines are designated as mHypoE-‘clone number’. Each cell line possesses a neuronal phenotype with clearly defined perikarya and neurites; however, the precise cell morphology of each cell line appears to be distinct from one another, indicating that they represent unique cell types. Detailed
Figure 1.6. Schematic illustration of the advantages and immortalization of hypothalamic cell models.

(A) Classical in vivo approaches have been instrumental in establishing synaptic connectivity between distinct hypothalamic nuclei and the functional purpose of numerous neuropeptides and neurotransmitters. However, the inherent complexity of the neuronal circuitry comprising the hypothalamus creates an unwelcoming environment to study the direct regulation of neuropeptides and cellular events by neuromodulators. Additionally, the paucity of specific neuronal cell lines that are responsible for key physiological effects such as feeding and reproductive function, calls on new tools and methods to study the exact hormonal regulation of distinct hypothalamic cell populations. Non-transformed hypothalamic primary cultures are difficult to maintain, have scarce functional peptidergic neurons and are comprised of a heterogeneous cell population. For this reason, researchers have turned to immortalized, clonal, hypothalamic cell populations. (B) Our group has developed an array of cell lines from mouse embryonic and adult hypothalamus and rat embryonic hypothalamus. Hypothalamic primary culture was transformed using the SV40 T-antigen and then treated with genetin to select for retroviral infected neurons. Cultures were then subcloned to ensure homogeneous cell populations were obtained. A full description of the immortalization methodology used are available in the corresponding manuscripts. Figure adapted from Dhillon SS 2010.
characterization studies indicate that the cells express neuronal cell markers, including neuron-specific enolase and neurofilament protein, but do not express glial fibrillary acidic protein. The cells also express markers of neurosecretory machinery, such as syntaxin, exhibit dense core granules that are indicative of secretory neurons, and demonstrate an intracellular calcium response after potassium chloride depolarization. Importantly, a number of these cell lines have been found to express neuropeptides and receptors involved in reproductive control, providing suitable models to study the intracellular signaling cascades, transcriptional mechanisms and the secretory events involved in reproductive function (41, 138). Two of these cell lines, mHypoE-38 and mHypoE-42, were utilized in the studies presented in this thesis. Further details regarding characterization of these cell models are provided in Section 3.2.

1.5.2 Adult hypothalamic cell lines, mHypoA-xx

In order to understand the cellular mechanisms involved in reproduction function within fully differentiated hypothalamic neurons, the Belsham group utilized a novel method to immortalize neurons from the adult mouse (22). In order to infect cells with the SV40 T-antigen, cells must be dividing. Thus, adult hypothalamic primary culture was treated with ciliary neurotrophic factor (CNTF), a nerve growth factor that induces cell proliferation and neurogenesis (22). Cells were then retrovirally infected with SV40 T-antigen in a similar protocol used for the immortalization of embryonic neurons. In total, over 50 adult mouse cell lines have been established, labeled as mHypoA-‘clone number’. Similar to the embryonic cell models, these adult cell lines express mature neuronal markers, exhibit neuronal morphology, and have been characterized for the expression of various neuropeptides and receptors. These cell models will be key to understanding the physiology of the mature neuron. Together, cell lines present an optimal model for the study of neuroendocrine gene regulation that has not been possible in the whole brain. In the studies presented within this thesis, two adult hypothalamic
cell lines were used: mHypoA-51 and mHypoA-63. Further characterization of these cell lines is found in Section 3.1.

### 1.6 Hypothesis and Aims

The hypothalamic kisspeptin system is a critical component to reproductive function. Numerous studies have shown that estrogen differentially regulates kisspeptin neurons within the AVPV and ARC in order to exert its effects on GnRH neurons; however, the molecular mechanisms underlying this differential effect of estrogen is currently unknown due to the unavailability of appropriate cell models. Furthermore, kisspeptin has been reported to indirectly activate GnRH neurons, but the afferent neuronal network by which this occurs has yet to be identified.

Thus, for Aim 1 of this thesis, it was hypothesized that hypothalamic kisspeptin neurons can be differentially regulated by estrogen treatment depending on whether the cell line originated from the AVPV or the ARC. These studies were completed using four adult hypothalamic cell lines identified to synthesize kisspeptin mRNA and protein. In Aim 2 of this thesis, it was hypothesized that kisspeptin may act through indirect pathways to stimulate GnRH neuronal cell populations. Specifically, this hypothesis examined the effect of kisspeptin on NPY synthesis and secretion and the activity of MAPK and/or PI3K signal transduction pathways. These studies were completed using two embryonic hypothalamic cell models, the mHypoE-38 and mHypoE-42 cell lines. Each aim is outlined below and in Figure 1.7.

**Aim I:** This aim evaluates the expression and synthesis of key neuropeptides, receptors and markers and determines the Kiss1 mRNA response to estrogen in adult hypothalamic cell lines in order to identify bona fide kisspeptin-expressing cell lines. These results are described in Section 3.1.
**Aim II:** This aim assesses the expression of Kiss1r on NPY neurons, investigates the direct regulation of NPY mRNA expression and secretion by kisspeptin, and determines the cellular signaling cascades mediating kisspeptin regulation of NPY synthesis and secretion. These results are presented in Section 3.2.
Figure 1.7. Schematic illustration depicting the current aims of this thesis.

The studies of this thesis are separated into two specific aims. Aim I establishes the first available kisspeptin-expressing cell models from the hypothalamus. Aim II evaluates the direct regulation of hypothalamic NPY neurons by kisspeptin, and determines the cellular pathways involved in this effect. Both aims will be carried out using cell lines derived from the embryonic or adult mouse hypothalamus.
Chapter 2
Methods and Materials
2.1 Cell culture and reagents

Immortalized cells were cultured in a monolayer in Dulbecco's Modified Eagle Medium (DMEM) (Invitrogen Life Technologies, Burlington, ON, Canada), supplemented with 5% fetal bovine serum (FBS) (Hyclone Laboratories Inc., Logan, UT) and 1% penicillin/streptomycin (Invitrogen Life Technologies, Burlington, ON) and maintained at 37 °C in 5% CO₂, as previously described (40). 17β-estradiol was obtained from Sigma-Aldrich (Oakville, ON) and dissolved in ethanol (10 nM final concentration). Kiss-10 peptide was purchased from Phoenix Pharmaceuticals (Burlingame, CA, USA) and dissolved in water (10 nM final concentration). The primary kisspeptin antibody was gifted by Dr. Alain Caraty. The MEK1/2 inhibitor U0126 and the PI3K inhibitor LY294002 were obtained from Cell Signaling Technology Inc. (Danvers, MA, USA). The p38 inhibitor SB239063 and PKC inhibitor GF109203X were obtained from Tocris Bioscience (Ellisville, Missouri, USA). All inhibitors were dissolved in dimethylsulfoxide (DMSO) (Sigma-Aldrich, Oakville, ON, Canada) at a final concentration of 25 µM (U0126 and LY294002) or 10 µM (SB239063 and GF109203X), and applied to neurons 1 hr before kiss-10 treatment. The G protein β (Gβ) and phopho-p38 antibodies were purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA), and the phospho-ERK1/2 and phospho-Akt antibodies were purchased from Cell Signaling Technology.

2.2 One-step RT-PCR

Each cell line was analyzed for the expression of specific markers by RT-PCR. Total RNA was isolated using the guanidinium thiocyanate phenol chloroform extraction method, and assessed for purity and concentration using spectrophotometric analysis (Ultraspec3000, Amersham Pharmacia Biotech, USA). RNA samples were amplified using a one-step RT-PCR Kit (Qiagen, Mississauga, ON, Canada) as per the manufacturer’s instructions. PCR was
conducted according to the following: 95°C for 30 s, 60°C for 30 s, and 72°C for 1 min (40 cycles). A total of 200 ng of RNA template from each cell line was used for each reaction. All PCR-amplified products were visualized on 2% agarose gels containing ethidium bromide (final concentration of 0.05 mg/mL), under ultraviolet light. A 50 bp DNA Ladder (Fermentas Life Science, Burlington, ON) was used to determine product size. The primer sequences, amplicon sizes and annealing temperatures are listed in Table 2.1.

2.3 Quantitative RT-PCR

In Section 3.1 and 3.2, cells were grown to 80% confluence and cell culture medium was replaced with DMEM containing 1% FBS and 1% penicillin/streptomycin for a minimum of 4 h prior to treatment with 10 nM kiss-10, 10 nM estrogen or vehicle alone for 4, 8 and 24 hr at 37°C. Total cellular RNA was isolated by the guanidium isothyiocyanate phenol chloroform extraction method. cDNA was made using the Applied Biosystems High Capacity cDNA Reverse Transcriptase Kit (Foster City, CA, USA). Quantitative analysis of NPY, AgRP, Kiss1r and ghrelin mRNA expression was completed using a SYBR green mix containing 0.3X Sybr green dye, 1X ROX, 1X buffer, 3 mM MgCl₂, 0.2 mM dNTP, and 0.5 U Platinum Taq (all from Invitrogen Life Technologies, Burlington, ON). Analysis of NT mRNA expression was completed using Taqman Universal PCR Master Mix (Applied Biosystems). 100 ng of template was used per reaction and run on the Applied Biosystems Prism 7000 real-time PCR machine. The SYBR primer sequences, amplicon sizes and annealing temperatures are listed in Table 2.1. Gene-specific TaqMan primers for NT were purchased from Applied Biosystems. Real-time RT-PCR values were normalized to histone at the corresponding time points.

Primers used for both one-step PCR and quantitative PCR were designed using an online primer design tool, PrimerBLAST. When possible, primers were designed to flank an intron to
Table 2.1 List of primers used for one-step and quantitative RT-PCR

<table>
<thead>
<tr>
<th>Gene</th>
<th>Primer Sequence</th>
<th>Amplicon Size (bp)</th>
<th>Annealing Temp (°C)</th>
</tr>
</thead>
</table>
| NSE   | S: ggaccagctggcactct  
|       | AS: tgcagggccgccccactttg | 380 | 60 |
| GFAP  | S: ctaggacctggacagagaaac  
|       | AS: cctgttagtggcagatgcgtag | 621 | 60 |
| T-Ag  | S: agaggaaattttgacgctaa  
|       | AS: ctaaacacagcatgacactaa | 433 | 60 |
| NPY   | S: taggttaacaagcgaatggg  
|       | AS: acatggaagaggtgttcaagc | 342 | 60 |
| AgRP  | S: agggtcagaaggcctgacaccag  
|       | AS: tgaagaagcggcacagtagaagt | 252 | 60 |
| POMC  | S: taggtgtgtgagcgtggtgc  
|       | AS: cagtcagggcgctgttcacgct | 149 | 60 |
| NT    | S: ataggaatgaaccttcagctg  
|       | AS: gtaggagccctcccttgat | 498 | 60 |
| Ghrelin | S: agcatgctctggtgatgacatg  
|       | AS: aggcatgctctggttacagt | 329 | 60 |
| Erα   | S: aatccattgactgacagccag  
|       | AS: gaattcattgctacatctccctec | 344 | 57 |
| Erβ   | S: gaatccattgacctccactcgtcag  
|       | AS: gaatccacacctctctctgtcacat | 332 | 60 |
| Kiss1r| S: tgggtccccctgttctgctga  
|       | AS: cagctggaacacagtcacata | 356 | 60 |
| Kiss1 | S: agggaccagaacaccactccagaca  
|       | AS: attgatgctcacttggctcag | 336 | 60 |
| TH    | S: ttcagagggagggagatggcact  
|       | AS: ttgctcagagatgtgccacatta | 329 | 60 |
| Sub P | S: tgcggcttccactcactctctctgtctg  
|       | AS: agccttaaacagggccaacctt | 194 | 55 |
| Kiss1 SYBR | S: agctggtctctctctctctgt  
|       | AS: gcattaaagggccactct | 127 | 60 |
| NPY SYBR | S: aatcattgctctggtgatgacag  
|       | AS: cagctggaacacagtcacata | 74 | 60 |
| AgRP SYBR | S: cgagggtcttggtgtgagcccag  
|       | AS: agagttctgctgaggttacag | 69 | 60 |
| Kiss1r SYBR | S: catctgccagagctgagatccac  
|       | AS: cagctgccagagctgagatccac | 75 | 60 |
| Ghrelin SYBR | S: ggaggagttggagatgtgaggtt  
|       | AS: ggcctggcccagagttgtc | 85 | 65 |
| Histone SYBR | S: eggtttcagagt gcagctatt  
|       | AS: atctttcagactcgaagccatag | 72 | 60 |
control for DNA contamination. All PCR products were run on an agarose gel to verify the molecular size of the PCR product and sequenced to confirm identity.

2.4 Enzyme immunoassay (EIA)

In Section 3.1, mHypoA-48, -49, -50, 51, -55 and -63 cells were grown to 90% confluence and cell culture medium was replaced with serum-free DMEM containing 1% penicillin/streptomycin for a minimum of 4 h prior to treatment with 60 mM KCl or vehicle (water) for 15 min at 37°C. KCl stimulates depolarization and a large influx of Ca$^{2+}$ to induce exocytosis (139), and was therefore used to test for kisspeptin secretion. Media was collected in duplicate and the concentration of kisspeptin was analyzed using a kisspeptin enzyme immunoassay (EIA) kit (Phoenix Pharmaceuticals Inc., Burlingame, CA) according to the manufacturer’s protocol.

In Section 3.2, mHypoE-38 and -42 cells were grown to 90% confluence and cell culture medium was replaced with serum-free DMEM containing 1% penicillin/streptomycin for a minimum of 4 h prior to treatment with 10 nM kiss-10 or vehicle alone for 1 hr at 37°C. 60 mM KCl treatments for 15 min were completed as a positive control for secretion. In the pharmacological inhibitor studies, mHypoE-38 and -42 cells were treated with kiss-10 (10 nM) or vehicle in the presence of absence of pharmacological inhibitors directed against the PI3K, MEK1/2 or p38 pathways. Inhibitors were applied for 1 hr prior to kiss-10 treatment. Media was collected in triplicate after 15 min (KCl) or 1 h (kiss-10) and the concentration of NPY was analyzed using an NPY-specific EIA kit (Phoenix Pharmaceuticals Inc., Burlingame, CA) according to the manufacturer’s protocol.
2.5 Western blot analysis

In Section 3.2, mHypoE-38 and -42 cells were grown to 90% confluence, serum-starved for 4 h and then treated with kisspeptin (10 nM) or vehicle. The cells were washed with ice-cold PBS and harvested at 5, 15, 30 and 60 min using a 1X lysis buffer (20 mM Tris HCl, 150 mM NaCl, 1 mM Na₂EDTA, 1 mM EGTA, 1% Triton, 2.5 mM sodium pyrophosphate, 1 mM β-glycerophosphate, 1 mM Na₃VO₄, 1 mM leupeptin) (Cell Signaling Technology Inc.) supplemented with 1 mM PMSF and phosphatase inhibitor cocktail (Sigma–Aldrich Canada Ltd.). Cell lysates were centrifuged at 14,000 rpm for 10 min at 4°C and the supernatant was stored at -80°C. Concentration of the protein was measured using a biocinchoninic acid (BCA) protein assay kit (Pierce Biotechnology, IL). 30 ug of protein was resolved on an 8% SDS-polyacrylamide gel and blotted overnight onto immuno-blot PVDF membrane (Bio-rad Laboratories, Hercules, CA). The blots were blocked with 5% bovine serum albumin (BSA) (Sigma Aldrich) in Tris-buffered saline with 0.1% Tween (TBS-T) for 30-60 min, washed three times with TBS-T and incubated overnight at 4°C with primary antibodies phospho-ERK1/2 (1:1000), phospho-p38 (1:1000), phospho-Akt (1:1000) and Gβ (1:5000). Blots were then washed three times with TBS-T and incubated with horseradish peroxidase-labeled secondary goat anti-rabbit antisera (1:5000, Cell Signaling Technology) or sheep anti-mouse (1:5000, Cell Signaling Technology) for 1-2 h at room temperature. Membranes were visualized with enhance chemiluminescence (ECL kit, GE Healthcare, UK) on the Kodak Imager 2000R.

For western blot studies, phospho-protein levels were normalized to the loading control protein, Gβ. Although total protein normalization is ideal for these studies, previous studies have found that Gβ is a reliable indicator of loading status (41, 140-146). In addition, vehicle-treated samples display moderate, but not significant, changes in phosphorylation of ERK1/2 and p38 over time (Figure 3.7 and 3.8). In order to conclude true changes in the phosphorylation levels of
these proteins, cells were split concurrently and time-matched vehicle-treated controls were included.

2.6 Immunocytochemistry (ICC)

In Section 3.1, cells were plated on four-well chamber slides (BD Biosciences) and grown overnight to 90% confluence in DMEM supplemented with 5% FBS and 1% penicillin/streptomycin. Cells were then fixed with 4% paraformaldehyde for 25 min, washed (all washings for ICC experiments were performed three times with PBS) and permeabilized with 0.5% Triton-X-100 in 1% BSA-PBS for 60 min at room temperature. After washing, cells were incubated with primary antibody for 48 hr at 4°C, washed again, followed by incubation with secondary antibody at room temperature for 60 min. Wells containing no antibody and antibody preadsorbed with kiss-54 were used as negative controls. The primary kisspeptin antibody was used at a 1:1000 1% BSA-PBS dilution, and FITC-conjugated donkey anti-rabbit IgG secondary antibody was used at a 1:200 BSA-PBS dilution (Jackson Immuno Research, Westgrove, PA). After washing cells, gaskets were removed from chamber slides and mounted with ProLong Gold antifade reagent with DAPI (Invitrogen). Fixed cells were visualized with a deconvoluted microscope (Carl Zeiss) using Axiovision digital image processing software.

2.7 Fluorescence-activated cell sorting (FACS)

The NPY-GFP mice (strain B6.FVB-Tg(Npy-hrGFP)1Lowl/J generated by Dr. Bradford Lowell, Beth Israel Deaconess Medical Center, Boston, MA, and available through The Jackson Laboratory, Bar Harbor, ME) were housed under standard vivarium conditions in LD 12:12 light cycle with food and water available ad libitum. All procedures were conducted in accordance with the regulations of the Canadian Council on Animal Care and approved by the University of Toronto Animal Care Committee. NPY-GFP transgenic mouse hypothalamii from
10-20 wk old mice were dissected individually and stored in BSA. Cells were dispersed by trituration through a 21 gauge needle and passed through 40 µM filter tubes. Cells were sorted on a BD FACS Aria cell sorter (Becton Dickinson, Franklin Lakes, NJ) with a 100-micron nozzle tip and sheath pressure at 20 psi with a purity greater than 95%. NPY-GFP cells were sorted on GFP fluorescence after gating to remove cell aggregates. All FACS was performed in the Faculty of Medicine Flow Cytometry Facility, University of Toronto.

2.8 Statistical Analysis

Data was presented as the mean ± standard error of the mean (SEM) and analyzed using SigmaStat Software. Statistical significance was determined using a Student’s t test (secretion studies, KCl and kisspeptin treatment), one-way ANOVA followed by a post hoc Student Newman-Keuls test (inhibitor studies), or two-way ANOVA followed by a post hoc Bonferroni test (mRNA expression studies, kisspeptin treatment; protein phosphorylation studies). Experiments were performed on three to eight separate occasions. Data was considered statistically significant when p < 0.05.
Chapter 3

Results

3.1 Kisspeptin cell models from the hypothalamus

Kim GL and Belsham DD

Contributions:

• GLK completed the experiments
• DDB provided scientific input, direction and funding

3.2 Kisspeptin directly regulates NPY synthesis and secretion via the ERK1/2 and p38 MAPK signaling pathways in NPY-secreting hypothalamic neurons

Manuscript in press within Endocrinology

Citation:

Kisspeptin directly regulates NPY synthesis and secretion via the ERK1/2 and p38 MAPK signaling pathways in NPY-secreting hypothalamic neurons.


Contributions:

• GLK completed the experiments and wrote the manuscript
• SSD completed experiments for figure 3.3 and edited the manuscript
• DDB edited the manuscript and provided scientific input, direction and funding
3.1 Kisspeptin cell models from the ARC

3.1.1 Characterization of kisspeptin cell models

Belsham et al. have recently established an array of cell lines derived from the adult mouse hypothalamus (22), which were used for the following studies. Forty-six adult male and female cell lines were screened for expression of Kiss1 mRNA using RT-PCR, and varying levels of expression was found in select cell lines (Figure 3.1a). The mHypoA-51 and mHypoA-63 cell lines were selected for further characterization based on their strong relative levels of Kiss1 mRNA. Notably, screening of embryonic hypothalamic cell lines (40) for Kiss1 mRNA expression revealed low relative levels of expression in comparison to adult cell lines (data not shown). This observation is in accordance with the increase in Kiss1 expression that occurs throughout development (11, 89). Based on evidence that kisspeptin neurons in the ARC co-express substance P (147) and kisspeptin neurons in the AVPV co-express tyrosine hydroxylase (TH) (148), co-expression studies of these neuropeptides were performed in order to differentiate which kisspeptin neuronal population each cell line represents. It was found that TH mRNA expression was absent while substance P mRNA expression was present in both cell lines, indicating that the mHypoA-51 and mHypoA-63 lines are representative of ARC kisspeptin neurons. Both cell lines were also confirmed for the presence of ERα and β mRNA expression (Figure 3.1b). Lastly, the mHypoA-51 and mHypoA-63 neurons were found to synthesize kisspeptin protein using ICC (Figure 3.1c), and basally secrete kisspeptin as shown by an EIA (Figure 3.1d). Thus far, these findings suggest that the mHypoA-51 and mHypoA-63 cell lines are models of ARC kisspeptin neurons.
Figure 3.1. Expression and secretion analysis of the mHypoA-51 and mHypoA-63 cell models.

(A) Expression of Kiss1 mRNA was analyzed in adult female (first row) and adult male (second row) hypothalamic cell lines using one-step RT-PCR. RNA isolated from each cell line was used as a template for RT-PCR with primers specifically designed to amplify the gene of interest. RNA from mouse hypothalamus (Hypo) was used as a positive control and reactions containing no RNA template (NTC) were used as a negative control. (B) Expression of ERα, ERβ, TH and substance P mRNA was analyzed in the mHypoA-51 and mHypoA-63 cell lines using one-step RT-PCR following a similar protocol outlined in (A). (C) Representative ICC images of mHypoA-51 and mHypoA-63 cells using FITC-conjugated antibodies (green) and counterstained with DAPI (blue). Cells incubated with primary kisspeptin antibody preadsorbed with kisspeptin peptide or no primary kisspeptin antibody were used as negative controls. (D) Basal secretion of kisspeptin from the mHypoA-51 and mHypoA-63 cell lines using a kisspeptin-specific EIA.
3.1.2 Estrogen regulates Kiss1 mRNA expression in hypothalamic neurons

Several studies demonstrate that estrogen differentially regulates Kiss1 mRNA expression in hypothalamic kisspeptin neurons depending on the location of the kisspeptin neuron within the hypothalamus, i.e. ARC or AVPV (17, 18, 125). Thus, in order to more precisely determine which kisspeptin neuronal population each cell line represents, time course studies were performed in the mHypoA-51 and mHypoA-63 cells and Kiss1 mRNA levels were measured in response to estrogen. After performing estrogen treatment time courses measuring Kiss1 mRNA levels after 1, 2, 4, 8 and 24 hr, initial observations suggested that regulation of Kiss1 mRNA occurs at 4, 8 or 24 hr. Based on these observations, the mHypoA-51 and mHypoA-63 cells were treated with estrogen (10 nM) and harvested after 4, 8 and 24 h. Kiss1 mRNA expression was significantly decreased after 4 hr estrogen treatment in both the mHypoA-51 (vehicle 2.71±1.06 vs. kisspeptin 0.34±0.04) and mHypoA-63 cells (vehicle 3.60±1.07 vs. kisspeptin 0.92±0.49), as shown by quantitative RT-PCR (Figure 3.2). This down-regulation of Kiss1 mRNA in response to estrogen agrees with in vivo data demonstrating that estrogen decreases Kiss1 mRNA in the ARC. Taken together, these studies identify the mHypoA-51 and mHypoA-63 lines as ARC kisspeptin cell models.

3.2 Kisspeptin directly regulates NPY synthesis and secretion via the ERK1/2 and p38 MAPK signaling pathways in NPY-secreting hypothalamic neurons

3.2.1 Expression of Kiss1r mRNA in flow cytometry-isolated NPY-GFP neurons

The Kiss1r is expressed not only in the GnRH neuron, but also in other regions of the hypothalamus (7, 23). The function of Kiss1r in these regions is currently unknown. In an attempt to determine whether in vivo NPY neurons express Kiss1r, native NPY-GFP neurons were isolated from the hypothalamus of the transgenic mouse using fluorescence-activated cell
Figure 3.2. Estrogen directly decreases Kiss1 mRNA expression in the mHypoA-51 and mHypoA-63 cell lines.

mHypoA-51 and mHypoA-63 cell lines were serum starved for 4 hr before treatment with 10 nM estrogen or vehicle alone over a 24 hr timecourse. Kiss1 mRNA expression was determined using quantitative RT-PCR and levels were normalized to histone H3. n=3-4 independent experiments. Data are shown as mean±SEM. *p<0.05, **p<0.01.
sorting (FACS), and total RNA was isolated and subjected to RT-PCR using primers specific to the Kiss1r (Figure 3.3). Total RNA from mouse hypothalamus was used as a positive control and reactions containing no RNA template were used as a negative control for one-step RT-PCR reactions. These results indicate that native NPY neurons in the mouse hypothalamus express the kisspeptin receptor Kiss1r, and may therefore respond to kisspeptin in a physiological setting.

3.2.2 Expression of Kiss1r mRNA and hypothalamic markers in NPY-secreting hypothalamic cell lines

Studying the direct regulation of specific neurons in the hypothalamus is difficult due to the inherent cellular heterogeneity and complexity of neuronal circuits within. Thus, in order to optimally study the direct regulation of NPY neurons by kisspeptin, clonal, immortalized, murine hypothalamic cell lines, mHypoE-38 and -42, were employed. The generation (40) and characterization (40, 41) of these cells has been previously reported. Briefly, cells were immortalized through the retroviral transfer of the SV40 T-antigen oncogene and subcloned until a homogeneous cell population was obtained. Each cell line displays neuronal morphology and has a distinct phenotypic profile (Figure 3.4a). Using RT-PCR, both lines were confirmed for the presence of neuron-specific enolase (NSE) and T-antigen and the absence of glial fibrillary acidic protein (GFAP). Further screening for hypothalamic neuropeptides and receptors determined that both cell lines express mRNA for NPY, agouti-related peptide (AgRP), ghrelin, ERα and β, but not proopiomelanocortin (POMC). NT mRNA expression was found in the mHypoE-42 but not the mHypoE-38 cell line. Importantly, both cell lines express Kiss1r mRNA, predicting that these lines are responsive to kisspeptin and are therefore suitable models to investigate the direct regulation of NPY mRNA expression and secretion by kisspeptin (Figure 3.4b). These cell lines also secrete NPY basally and in response to KCl-induced depolarization, as shown by an NPY-specific EIA (Figure 3.4c).
Figure 3.3. Expression of Kiss1r mRNA in NPY-GFP neurons.

Expression of Kiss1r mRNA in NPY-GFP neurons isolated from the hypothalamus of the transgenic mouse using fluorescence-activated cell sorting (FACS). RNA harvested from NPY-GFP neurons was used as a template for one-step RT-PCR with primers specifically designed to amplify Kiss1r. RNA from mouse hypothalamus (Hypo) was used as a positive control, and a reaction containing no RNA template (NTC) was used as a negative control for one-step RT-PCR reactions. Amplicon size: Kiss1r, 314bp.
Figure 3.4. Characterization of the mHypE-38 and mHypE-42 cell models.

(A) Representative phase-contrast micrographs of clonal, immortalized, murine, hypothalamic cell lines, mHypE-38 and mHypE-42. Each cell line displays neuronal morphology and has a distinct phenotypic profile. (B) Expression of neuronal markers, neuropeptides and receptors was analyzed in both cell lines using RT-PCR. Positive controls using hypothalamus RNA and negative controls containing no RNA template were performed and validated for each gene. Listed in the table is the presence (+) or absence (-) of specific genes. Neuron-specific enolase, NSE; T-antigen, T-Ag; glial fibrillary acidic protein, GFAP; neuropeptide Y, NPY; agouti-related peptide, AgRP; pro-opiomelanocortin, POMC; neurotensin, NT; ghrelin; Kiss1r; estrogen-receptor α, ERα; estrogen receptor β, ERβ. (C) mHypE-38 and mHypE-42 cells were treated with 60 mM KCl or vehicle. Media was collected 15 min after treatment and the concentration of NPY was analyzed using a NPY-specific enzyme immunoassay. Data are shown as mean±SEM. n=3-6 independent experiments. * p=0.003.
3.2.3 Kisspeptin regulates NPY mRNA and secretion in the mHypoE-38 cells

Currently, functions of Kiss1r-expressing non-GnRH neurons in the hypothalamus remain unclear. Kiss1r mRNA expression was detected in FAC-sorted native NPY-GFP cells and two NPY-secreting hypothalamic cell lines. Thus, the hypothesis that kisspeptin directly regulates NPY synthesis and secretion was tested using the NPY-secreting mHypoE-38 and -42 cell lines. Cells were treated with the kiss-10 peptide (10 nM) and harvested after 4, 8 and 24 h. Using quantitative RT-PCR, we found that NPY mRNA expression was significantly increased by kiss-10 treatment at the 24 h timepoint in the mHypoE-38 cells (vehicle 0.74±0.09 vs. kisspeptin 1.29±0.12). NPY mRNA expression was not significantly regulated by kiss-10 at any timepoint in the mHypoE-42 cells (Figure 3.5a). Subsequently, rapid regulation of NPY secretion by stimulation with kisspeptin was investigated. It was found that kiss-10 treatment (10 nM) for 1 h significantly stimulated NPY secretion in the mHypoE-38 cells (vehicle 1.00±0.05 vs. kisspeptin 1.26±0.07), but not the mHypoE-42 cells, as determined by an NPY-specific EIA (Figure 3.5b). These findings suggest that kisspeptin can act directly on the NPY neuron to rapidly induce NPY secretion and increase NPY mRNA over long-term exposure.

3.2.3.1 Kisspeptin does not regulate AgRP, ghrelin, NT or Kiss1r mRNA in the mHypoE-38 and mHypoE-42 cells

Kisspeptin-mediated regulation of other important hypothalamic neuropeptides and receptors was studied in the mHypoE-38 and -42 cell lines. Cells were treated with kiss-10 over a 24 h timecourse, and it was found that AgRP, ghrelin, NT and Kiss1r mRNA expression were not significantly regulated by kisspeptin in both the mHypoE-38 and -42 cells (Figure 3.6). Because treatment with kisspeptin in the mHypoE-42 neurons did not produce a significant effect in any of the genes examined, the functionality of the kisspeptin receptor in this cell line
Figure 3.5. Kisspeptin regulates NPY mRNA expression and secretion in the mHypoE-38 cell line.

(A) mHypoE-38 and mHypoE-42 cells were treated with kisspeptin (10 nM) over a 24 h time-course. NPY mRNA expression was determined using real-time RT-PCR and levels were normalized to histone H3. n=3-8 independent experiments. (B) mHypoE-38 and mHypoE-42 cells were treated with kisspeptin (10 nM) for 1 h. Concentration of NPY in the media was measured using an EIA. NPY secretion levels set to 1.0. n=3-8 independent experiments. Data are shown as mean±SEM. *p<0.05.
Figure 3.6. Kisspeptin-mediated regulation of other hypothalamic neuropeptides in the mHypoE-38 and mHypoE-42 cell lines.

mHypoE-38 and mHypoE-42 cells were treated with kisspeptin (10 nM) over a 24 h timecourse. (A) AgRP, (B) Kiss1r, (C) ghrelin, and (D) NT mRNA expression were determined using real-time RT-PCR and levels were normalized to histone H3. n=3 independent experiments. Data are shown as mean±SEM.
was investigated. The mHypoE-42 cells were treated with 10 nM kiss-10 and protein was harvested at the 5, 15, 30 and 60 min timepoints. Using western blot analysis, it was found that ERK1/2 phosphorylation was significantly activated after 5 min (vehicle 1.00±0.02 vs. kisspeptin 2.04±0.04), indicating that Kiss1r is indeed functional in this cell line (Figure 3.7).

3.2.4 Kisspeptin phosphorylates ERK1/2 and p38 in the mHypoE-38 cells

Next, the signaling pathways activated by kisspeptin was examined in an endogenous Kiss1r-expressing cell line. The mHypoE-38 neurons were treated with 10 nM kiss-10 over a 60 min timecourse, and it was found that phosphorylation of the ERK1/2 and p38 signaling kinases were significantly activated at the 15 and 60 min timepoints, respectively, as shown by western blot analysis (ERK1/2: vehicle 1.00±0.12 vs. kisspeptin 1.61±0.14; p38: vehicle 1.00±0.05 vs. kisspeptin 1.93±0.12). Phosphorylation of Akt was also investigated, but was not significantly altered by kiss-10 treatment (Figure 3.8).

3.2.5 The ERK1/2 inhibitor U0126 and p38 inhibitor SB239063 attenuate kisspeptin-mediated induction of NPY mRNA expression and secretion

In order to determine if the MEK-ERK and the p38 signaling pathways mediate the regulation of NPY by kisspeptin, the MEK-specific (U0126) and p38-specific (SB239063) inhibitors were used. The mHypoE-38 cells were pre-treated with the inhibitors for 1 h, followed by co-treatment with inhibitors and 10 nM kiss-10 for 24 h. RNA was then harvested and analyzed using quantitative RT-PCR. It was determined that both U0126 (vehicle 0.63±0.27 vs. kisspeptin 0.59±0.15) and SB239063 (vehicle 0.74±0.07 vs. kisspeptin 0.78±0.17) significantly attenuated the kisspeptin-mediated induction of NPY mRNA expression (vehicle 0.74±0.09 vs. kisspeptin 1.29±0.12) (Figure 3.9a). Next, the involvement of the MEK-ERK and p38 pathways in the kisspeptin-mediated regulation of NPY secretion was investigated. Similar to the mRNA
Figure 3.7. Kisspeptin activates the ERK1/2 signaling kinase in the mHypoE-42 cell line.

mHypoE-42 neurons were serum starved for 4 h before treatment with kisspeptin (10 nM) over a 1 h timecourse. At the indicated timepoints, cell lysates were harvested and subjected to SDS-PAGE. Western blot analysis was performed with enhanced chemiluminescence using phospho-specific antibodies directed against ERK1/2. Results shown are relative to control protein Gβ (set to 1.0) and expressed as mean±SEM. n=3 independent experiments. *p<0.05.
mHypoE-38 neurons were serum starved for 4 h before treatment with kisspeptin (10 nM) over a 1 h time-course. At the indicated timepoints, cell lysates were harvested and subjected to SDS-PAGE. Western blot analysis was performed with enhanced chemiluminescence using phospho-specific antibodies directed against (A) ERK1/2, (B) p38, and (C) Akt. Results shown are relative to control protein Gβ (set to 1.0) and expressed as mean±SEM. n=3 independent experiments. *p<0.05.

Figure 3.8. Kisspeptin phosphorylates ERK1/2 and p38 in the mHypoE-38 cell line.
expression studies, the mHypoE-38 cells were pre-treated with inhibitors for 1 h, followed by co-treatment with inhibitors and 10 nM kiss-10, and media from the cells was collected 1 h after kiss-10 treatment. Using an NPY-specific EIA, we found that both U0126 (vehicle 1.01±0.05 vs. kisspeptin 0.98±0.04) and SB239063 (vehicle 1.17±0.02 vs. kisspeptin 1.17±0.07) prevented the induction of NPY secretion by kisspeptin (vehicle 1.00±0.05 vs. kisspeptin 1.26±0.07) (Figure 3.9b). The effect of inhibitors of the PI3K/Akt (LY294002) and PKC (GF109302X) pathways were also investigated for both mRNA expression and secretion studies; however, the kisspeptin-mediated increase in NPY mRNA expression and secretion were not significantly attenuated by inhibition of these pathways (Figure 3.9c). Altogether, these results indicate that the MEK-ERK and p38 MAPK pathways are essential in the regulation of NPY mRNA expression and secretion by kisspeptin.
Figure 3.9. The ERK1/2 inhibitor U0126 and p38 inhibitor SB239063 attenuate kisspeptin-mediated regulation of NPY mRNA expression and secretion in the mHypoE-38 cell line.

(A) mHypoE-38 cells were pretreated with U0126 (25 μM) or SB239063 (10 μM) for 1 h and then cotreated with kisspeptin (10 nM) for 24 h. NPY mRNA expression was determined using real-time RT-PCR and levels were normalized to histone H3. n=3-8 independent experiments. (B) mHypoE-38 cells were pretreated with U0126 (25 μM) or SB239063 (10 μM) for 1 h and then cotreated with kisspeptin (10 nM) for 1 h. Concentration of NPY in the media was measured using an EIA. NPY secretion levels set to 1.0. n=3-8 independent experiments. Data are shown as mean±SEM. *p<0.05. (C) Studies outlined in (A) and (B) were repeated for inhibitors of the PI3K/Akt (LY294002; 25 μM) and PKC (GF109203X; 10 μM) pathways. Data are listed in the table.
Chapter 4
Discussion
4.1 Discussion

4.1.1 Kisspeptin cell models from the ARC

Kisspeptin has emerged as a key regulator of the HPG axis through its potent stimulation of GnRH release (5-15). Although this essential role of kisspeptin in reproductive function has been well established, there has been a paucity of studies focusing on the regulation of the hypothalamic Kiss1 gene due to the unavailability of appropriate cell models. The first aim of this thesis was to establish kisspeptin cell models from the adult mouse hypothalamus to study the direct regulation of Kiss1 gene expression. Immortalized, clonal, hypothalamic cell lines were screened for the expression of relevant neuropeptides, receptors and markers. In addition, cell lines were examined for the Kiss1 mRNA response to estrogen exposure in order to identify kisspeptin cell models representative of AVPV and/or ARC kisspeptin neurons. Altogether, a range of molecular techniques was utilized to thoroughly characterize two kisspeptin-expressing cell models, mHypoA-51 and mHypoA-63.

Several reports demonstrate that hypothalamic Kiss1 expression is developmentally regulated, with expression reaching peak levels at puberty. In male and female rats, hypothalamic Kiss1 mRNA expression declines during the pre-pubertal stage (day 20 postpartum), and sharply increases thereafter with the highest expression at puberty (day 45 and day 30 in males and females, respectively) (11). This phenomenon was also confirmed in male and female primates (89). In the present study, an array of immortalized, clonal, hypothalamic cell lines from the adult (i.e. post-pubertal) mouse hypothalamus were used to establish kisspeptin-expressing cell models. Screening of these cell lines, which represent a mixture of hypothalamic cell types, demonstrated strong relative expression of kisspeptin mRNA in select cell lines using RT-PCR. In particular, the mHypoA-51 and mHypoA-63 cells strongly expressed kisspeptin mRNA, and were also found to synthesize and basally secrete kisspeptin protein. In
contrast, screening of hypothalamic cell lines derived from the embryonic mouse revealed low relative levels of kisspeptin mRNA (data not shown). This expression analysis is in accordance with in vivo studies that demonstrate increased levels of hypothalamic Kiss1 in post-pubescent animals.

Kisspeptin neurons are primarily localized in two discrete regions in the hypothalamus, the AVPV and ARC (9, 17, 18), and each of these kisspeptin subpopulations have been demonstrated to co-express specific neuropeptides and neurotransmitters. In female mice (148), though not in rats (149), kisspeptin neurons in the AVPV have been found to co-express TH, an enzyme required for dopamine synthesis. Alternatively, kisspeptin neurons in the infundibular nucleus of humans (the homologue of the ARC in rodents) have been found to co-express substance P (147), a member of the tachykinin family. The mHypoA-51 and mHypoA-63 cell lines were evaluated for expression of these two markers, and it was found that both cell lines express substance P, but not TH. This co-expression of substance P indicates that the mHypoA-51 and mHypoA-63 cell lines are characteristic of ARC kisspeptin neurons. Within the ARC of adult female mice, virtually all (99.8%) Kiss1-expressing cells express ERα mRNA and 25% express ERβ mRNA (17). Thus, expression analysis of ERα and β mRNA was performed and expression of both receptor subtypes was found in the mHypoA-51 and mHypoA-63 cell lines, further indicating that these hypothalamic neuronal cell lines are of ARC origin. Importantly, expression of the ERs also suggests that these cell lines may be responsive to estrogen, and were therefore investigated for regulation of Kiss1 mRNA expression in response to estrogen treatment. Studies in a number of species have shown that both AVPV and ARC kisspeptin neurons are targets of estrogen. During pro-oestrus in the female rat when estrogen levels are high, there is a peak in the number of Kiss1-expressing neurons in the AVPV and a nadir in the number of Kiss1-expressing neurons in the ARC (125, 150). Similarly, OVX mice have reduced
levels of Kiss1 mRNA in the AVPV and elevated levels in the ARC, and these changes can be eliminated by the reintroduction of estrogen (17, 18). These studies demonstrate that estrogen upregulates kisspeptin neurons in the AVPV, and downregulates kisspeptin neurons in the ARC, thereby providing a link between the positive and negative feedback effects of estrogen on GnRH neurons. In light of these findings, Kiss1 mRNA expression was measured in response to estrogen treatment in the mHypoA-51 and mHypoA-63 cell lines. Both cell lines displayed a significant decrease in Kiss1 levels after 4 hr estrogen exposure, providing strong evidence that the mHypoA-51 and mHypoA-63 neurons are indeed representative of ARC kisspeptin neurons.

Overall, based on evidence that kisspeptin neurons in the ARC coexpress substance P but not TH, and exhibit reduced Kiss1 mRNA in response to estrogen, the present studies indicate that the mHypoA-51 and mHypoA-63 cell lines are representative of ARC kisspeptin neurons. These kisspeptin cell models will be key to studying the cellular events underlying estrogen regulation of kisspeptin neurons. Furthermore, there is considerable ambiguity over the location of the mouse Kiss1 promoter due to the unavailability of endogenously synthesizing kisspeptin cell lines. Thus, the mHypoA-51 and mHypoA-63 cells will also enable a precise analysis of the hypothalamus-specific 5’-regulatory region of the Kiss1 gene. Overall, these cell lines are invaluable for the mechanistic analysis of endogenous Kiss1 gene regulation and kisspeptin secretion. These studies have not been possible in the whole brain and will lead to a better understanding of how kisspeptin neurons are controlled directly by hormones and neuropeptides.

4.1.2 Kisspeptin directly regulates NPY synthesis and secretion via the ERK1/2 and p38 MAPK signaling pathways in NPY-secreting hypothalamic neurons

Kisspeptin is an important reproductive neuropeptide that positively regulates the HPG axis through activation of GnRH neurons (8-15). However, the kisspeptin-mediated regulation of GnRH synthesis and secretion is only partially regulated by direct activation of Kiss1r on GnRH
neurons, as described by Pielecka-Fortuna and colleagues (24). The objective of the second aim of this thesis was to identify a component of the indirect regulation of GnRH neurons by kisspeptin stimulation. NPY neurons are found to innervate GnRH cell bodies and terminals (25, 26), and numerous studies have confirmed that NPY stimulates the reproductive axis (27-30). For the first time, Kiss1r mRNA expression was detected in native NPY-GFP neurons FAC-sorted from the hypothalamus of the NPY-GFP transgenic mouse, indicating that hypothalamic NPY neurons may respond to kisspeptin in an in vivo setting. Thus, it was hypothesized that NPY neurons may contribute to the afferent neuronal network through which kisspeptin indirectly stimulates the reproductive axis. Taking an in vitro approach, the direct effect of kisspeptin on NPY mRNA expression and secretion was determined in the putative reproductive NPY neuron, the mHypoE-38 cell line.

Previously, the Belsham group characterized the murine-derived embryonic, NPY-secreting cell lines mHypoE-38 and mHypoE-42 (41). This study identified the mHypoE-38 cell line as representative of a reproductive NPY neuron, as long-term estrogen exposure resulted in a 4-fold increase in NPY mRNA expression that is postulated to be involved in the GnRH reproductive hormone surge (Figure 4.1a). A subsequent study also found that conditioned media containing NPY synthesized from the mHypoE-38 cells significantly stimulated GnRH mRNA expression in the GT1-7 cell line, a GnRH neuronal model (138) (Figure 4.2). In contrast, the mHypoE-42 cell line was identified as representative of a feeding-related NPY neuron, as treatment with estrogen steadily repressed NPY mRNA expression to potentially exert anorexigenic effects (41) (Figure 4.1b). In the present studies, the mHypoE-38 and mHypoE-42 cell lines were further characterized. Importantly, both cell lines demonstrated the presence of Kiss1r mRNA and thus represent natural targets of kisspeptin. Therefore, the effect of kisspeptin on NPY mRNA expression and secretion was examined in both cell lines. Treatment with
Figure 4.1. Estrogen Regulates NPY mRNA in a Biphasic Manner in NPY-Expressing mHypoE-38 Neurons, but Only Represses NPY mRNA in mHypoE-42 Neurons.

mHypoE-38 (A) and mHypoE-42 (B) neurons were serum starved for 12–16 h before treatment with either 10 nM E2 or vehicle alone over a 72-h time course. At the indicated time points, total RNA was extracted and used as a template for real-time RT-PCR with primers specifically designed to amplify NPY or AgRP. Real-time RT-PCR products were amplified on an ABI Prism 7000. NPY and AgRP mRNA levels were quantified using the ∆∆CT method and normalized to the internal control (actin). All results shown are relative to corresponding control mRNA levels (set to 1.0) at each time point and are expressed as mean ± SEM (n = 3 independent experiments). *, P < 0.05; **, P < 0.01; ***, P < 0.005. Titolo et al. 2006 (41).

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Coordinate regulation of neuropeptide Y and agouti-related peptide gene expression by estrogen depends on the ratio of estrogen receptor (ER) alpha to ERbeta in clonal hypothalamic neurons

Titolo D, Cai F and Belsham DD

Figure 4.2. Conditioned media from NPY-expressing mHypoE-38 neurons increase GnRH mRNA expression in GT1-7 cells.

(A) mHypoE-38 neurons were serum starved for 4 h before treatment with KCl or vehicle for 1 h. NPY secretion was analyzed using an NPY-specific enzyme immunoassay (EIA). Results shown are relative to control (set to 1) and expressed as mean ± SEM (n = 9 independent experiments). (B) mHypoE-38 neurons were treated with vehicle or KCl (60 mM) for 15 min and conditioned media (CM) was collected and desalted. GT1-7 cells were treated with vehicle, NPY (100 nM) or conditioned media from mHypoE-38 neurons treated for 4 h. Neurons were also treated with desalted KCl alone and KCl alone in standard growth medium. RNA was collected and GnRH mRNA levels were quantified using real-time RT–PCR. GnRH mRNA levels were normalized to the internal control 18S. Results shown are relative to the corresponding vehicle treated mRNA levels (set to 1.0) and are expressed as mean ± SEM (n = 4 independent experiments). *P < 0.05, **P < 0.001. Dhillon et al. 2009 (134).

Figure and legend reproduced with permission:
Neuropeptide Y induces gonadotropin-releasing hormone gene expression directly and through conditioned medium from mHypoE-38 NPY neurons
Dhillon SS, Gingerich S and Belsham DD
Regul Pept 156(103):96-103 (7 August 2009)
kisspeptin revealed a ~70% induction in NPY mRNA expression after 24 h, and a ~30% increase in NPY secretion after 1 h in the mHypoE-38 neurons. Kisspeptin did not regulate NPY mRNA expression or secretion in the mHypoE-42 neurons. This effect of kisspeptin in the mHypoE-38 cells corroborates the previous characterization of this cell line as a putative reproductive NPY neuron. Interestingly, the kisspeptin-mediated increase in NPY secretion found in the mHypoE-38 neurons secreted a comparable amount of NPY (~1.1 ng/mL) that was previously demonstrated to directly stimulate GnRH transcription in GT1-7 neurons (138). These are the first known findings that indicate that kisspeptin can directly stimulate NPY neurons. Although the present thesis does not include data measuring the effect of NPY on GnRH synthesis or secretion, based on previous studies by the Belsham group and others, it is believed that the kisspeptin-mediated stimulation of NPY neurons results in NPY activation of GnRH neurons.

In support of the current findings, one recent study demonstrated close apposition of kisspeptin fibres and NPY neurons using double-label fluorescent IHC (151). This study further showed that infusion of kisspeptin into the third ventricle of OVX ewes results in an approximate 2-fold increase in NPY mRNA expression within the arcuate nucleus, in terms of both level of expression per cell and number of cells detected. These ewes were also found to have an associated increase in LH levels. Although these findings in combination with the present study are strongly supportive of a kisspeptin-NPY pathway, the exact physiological implications of this pathway on the gonadotropin axis remain to be determined. Current literature suggests that a substantial part of kisspeptin action on GnRH neurons is conducted via the direct activation of GnRH neurons, as the majority of GnRH neurons (~70%) express Kiss1r (7) and kisspeptin is the most potent stimulator of gonadotropin release known to date (82). Likewise, NPY is less potent than kisspeptin in stimulating LH release (28), and may have inhibitory effects on the gonadotropic axis under certain conditions (34-39). Therefore, it is suggested that the proposed
indirect actions of kisspeptin on GnRH neurons via afferent NPY neurons play a more complementary role to the direct stimulatory effect of kisspeptin on GnRH release. Nonetheless, future studies examining the gonadotropin response to kisspeptin administration after blocking the NPY response (e.g. with a NPY antagonist) will be highly important in establishing the physiological relevance of this pathway.

Interestingly, a reciprocal action of NPY on Kiss1 gene expression has been documented. Correlational studies have shown that NPY knockout mice have significantly reduced hypothalamic Kiss1 mRNA levels (152), while fasting-induced increases in hypothalamic NPY mRNA levels is associated with an estrogen-dependent decrease in Kiss1 mRNA expression in the anteroventral periventricular nucleus (153). In order to establish a direct effect of NPY on kisspeptin synthesis, one study found that treatment of NPY in a hypothalamic cell line resulted in an increase in Kiss1 mRNA expression. Furthermore, NPY fibres have been found in close contact with kisspeptin neurons using double-label IHC (151). Although further studies examining the direct effect of NPY on kisspeptin and kisspeptin on NPY are required, it seems likely that a reciprocal action exists between these two reproductive neuropeptides, thus adding another layer of control in the precise regulation of GnRH neurons.

Molecular studies have reported that kisspeptin activates the G protein Gq/11, resulting in activation of PLC as well as the MAPK and PI3K/Akt pathways (15, 57, 64, 65, 70, 73, 75, 154, 155). However, these studies were either completed in heterologous cell models transfected with Kiss1r (64, 65, 70, 73, 75, 155), or the GT1-7 and GN11 GnRH neuronal cell models (15). Alternatively, the NPY-secreting mHypoE-38 neurons that endogenously express the Kiss1r were utilized to examine the pathways activated by kisspeptin. Using western blot analysis, it was found that kisspeptin treatment significantly increased phosphorylation of ERK1/2 after 15 min, and p38 after 1 h in the mHypoE-38 cells. Kisspeptin did not significantly change the
phosphorylation of Akt in these cells. These results are in agreement with those initially reported for Kiss1/Kiss1r signaling using heterologous cell models. However, the present data also highlight a potential divergence of Kiss1/Kiss1r signaling in NPY and GnRH neurons. Kisspeptin stimulates ERK1/2 and p38 in both NPY and GnRH neurons; however, kisspeptin also activates Akt specifically in GnRH neurons (15).

Additional studies further investigated the specific intracellular signaling cascades responsible for the stimulatory effects of kisspeptin on GnRH neurons. Using pharmacological inhibitors against key pathways involved in Kiss/Kiss1r signaling, these studies have confirmed that the MAPK, particularly ERK1/2 and p38, and PI3K/Akt pathways mediate kisspeptin-stimulated GnRH release in both in vitro GnRH cell models (15) and ex vivo hypothalamic explants (72). In contrast, the present study examined the signaling events involved in the regulation of NPY synthesis and secretion by kisspeptin. It was found that the 24 h kisspeptin-mediated induction of NPY mRNA expression was significantly attenuated by the MAPK inhibitor U0126 and the p38 inhibitor SB239063. The role of these pathways in kisspeptin-stimulated NPY secretion was further assessed. Similarly, U0126 and SB239063 abolished the 1 h kisspeptin-mediated increase in NPY secretion. The PI3K inhibitor LY294002 did not significantly reduce kisspeptin-induction of NPY mRNA expression or secretion (data not shown). Taken together, these results demonstrate that the ERK1/2 and p38 MAPK pathways, but not the PI3K pathway, are essential in the kisspeptin-mediated increase in NPY synthesis and secretion, which is in contrast to the pathways mediating kisspeptin stimulation of GnRH. As a result, it is postulated that kisspeptin can regulate the reproductive axis both directly at the GnRH neuron and indirectly at the NPY neuron through integration of intrinsically different pathways.

The mHypoE-38 and -42 cells were found to express AgRP, ghrelin and NT, which are involved in the control of both feeding and reproduction (156-161). Therefore, the possibility
that kisspeptin regulates the expression of these neuropeptides was investigated in the putative feeding-related NPY neuron, mHypoE-42, and reproductive NPY neuron, mHypoE-38. The effect of kisspeptin on Kiss1r mRNA expression was also studied in order to determine the ability of kisspeptin to regulate its own receptor. Both cell lines were treated with kisspeptin over a 24 h timecourse and it was found that kisspeptin did not alter AgRP, ghrelin, NT or Kiss1r mRNA expression in the mHypoE-42 cells, or AgRP, ghrelin or Kiss1r mRNA expression in the mHypoE-38 cells. Since kisspeptin was not found to regulate any of these genes including NPY in the mHypoE-42 cells, the functionality of the Kiss1r receptor in these cells was tested. It was found that phosphorylation of ERK1/2 was significantly increased with kisspeptin exposure, indicating that Kiss1r is indeed functional in this cell line. The downstream effect of Kiss1r activation in this cell line, however, has yet to be determined.

Several studies have reported expression of Kiss1r in non-GnRH neurons of the hypothalamus (7, 23); however, neither the identity nor the function of these hypothalamic Kiss1r-expressing neurons has yet to be determined. The present study demonstrates that NPY neurons from the mouse hypothalamus express Kiss1r, and that kisspeptin directly increases NPY mRNA expression and secretion in an NPY-secreting cell line via the ERK1/2 and p38 MAPK signaling pathways (Figure 4.3). Thus, evidence is provided that kisspeptin directly regulates NPY neurons, suggesting that the indirect effects of kisspeptin on GnRH neurons may occur at least partially through the activation of afferent NPY neurons. This study provides significant insight into the mechanisms underlying indirect kisspeptin regulation of the reproductive axis.

In summary, the research performed in both aims of this thesis represent novel studies that widen the scope of kisspeptin physiology. First, hypothalamic cell lines from the adult mouse were thoroughly characterized in order to establish the first available kisspeptin-
Figure 4.3. Schematic illustration summarizing the findings of Aim II.

In Aim II, a mechanism by which kisspeptin indirectly activates GnRH neurons was described. Native NPY neurons from the mouse hypothalamus were found to express the Kiss1r, suggesting that kisspeptin directly regulates NPY neurons in a physiological setting. Using the mHypoE-38 hypothalamic cell line, it was found that kisspeptin directly induces NPY mRNA expression and secretion through activation of the ERK1/2 and p38 MAPK signaling pathways. These findings suggest that kisspeptin directly regulate NPY neurons, potentially resulting in NPY regulation of GnRH neurons.
expressing cell models. These cell models will be invaluable for studying the mechanisms underlying the effect of estrogen on *Kiss1* expression, and for dissecting the structure of the *Kiss1* gene. Secondly, the direct effect of kisspeptin on NPY synthesis and secretion was investigated, and the intracellular signaling pathways involved in this effect were described. These studies represent the first line of evidence that kisspeptin directly regulates NPY synthesis and secretion, thus providing a putative mechanism whereby kisspeptin indirectly activates GnRH neurons. Taken together, the studies presented in this thesis provide a better understanding of the mechanisms involved in kisspeptin action on the HPG axis. Delineation of these mechanisms is important as kisspeptin is widely being considered as a promising new treatment for infertility disorders (162).

4.2 Limitations

In this thesis, hypothalamic kisspeptin cell models were established and a direct effect of kisspeptin on NPY synthesis and secretion was demonstrated. However, the experimental model and pharmacological inhibitors used within these studies impose limitations that must be considered to avoid overstating conclusions. In order to test the specific hypotheses posed in this thesis, clonal cell lines were used as a model. While this system allows for the determination of direct hormonal effects on a specific neuronal population by eliminating indirect influences, clonal cell lines only represent a single neuronal cell type and therefore conclusions cannot be extended to all neuronal cell types. Along this line, further caution must be taken when gathering conclusions from cell line data, as these cells lack the complex neuronal architecture and hormonal milieu observed in the intact hypothalamus.

The cell lines employed in the second aim of this thesis were developed from the embryonic hypothalamus and may or may not be an accurate representation of adult
hypothalamic neurons. Specifically, these cell lines were developed from the embryonic hypothalamus at embryonic day 15 (E15), E17 and E18 (40). Although the majority of hypothalamic neurons are formed throughout E10 to E16 (163), the fetal hypothalamus is still developing at E17, and therefore fetal hypothalamic neurons have yet to form the complex synaptic connections found in the adult hypothalamus. As a result, embryonic cell lines may display functional differences from fully-differential adult neurons. Despite this, a number of studies have found that the expression profiles and hormonal responses of these embryonic cell lines are similar to native adult neurons (136, 138, 140-145, 164-167). In addition, the specific embryonic cell lines used in the presented studies, mHypoE-38 and mHypoE-42, were previously characterized and were found to function in a similar manner to adult NPY neurons (41). Thus, although there are numerous advantages to using cell lines, including the unlimited supply, easy maintenance, and capability of executing a focused analysis of precise molecular processes, there are several considerations that must examined before drawing conclusions from the resulting data.

One final limitation that cell lines impose is the effect of the immortalizing factor on the biology of the cell. The factor used to immortalize the cell lines employed in this thesis was the SV40 T-antigen (T-Ag) oncogene, which can interfere with cellular processes at several levels. The Belsham group is currently conducting preliminary studies examining the effect of T-Ag on key neuropeptides, receptors and signaling kinases in multiple immortalized cell lines. Using shRNA knockdown of T-Ag, these studies have demonstrated that T-Ag expression increases the basal activity of the Akt, Jak2 and STAT3 signaling kinases. Importantly, the expression of key genes of interest, including NPY, was not altered by T-Ag expression in the mHypoE-38 cells (Belsham DD 2010, unpublished data). Based on these data, the western blot studies performed within this thesis utilized serum starvation and low glucose conditions in order to reduce the
basal activity of these signaling kinases. In a separate study, it was found that only 379 genes (out of a total 22,600 probes) were changed by T-Ag expression in an embryonic fibroblast cell line. The majority of these genes were involved with nucleotide synthesis and proliferation (168). Thus, immortalized, clonal cell lines are valuable tools for dissecting complex cellular pathways and determining the direct actions of neuromodulators on specific neuronal populations. However, due to the limitations posed by the nature of cell lines, scientists must be conscientious of these limitations when both designing and drawing conclusions from these studies.

In this thesis, conclusions were derived from the use of pharmacological inhibitors. Although inhibitors exhibit specificity for the intended target protein, inhibitors can also have effects on other signaling pathways. For example, U0126 can potentially inhibit calcium/calmodulin-dependent kinase 1, and LY294002 can potentially inhibit glycogen synthase kinase 3 and polo-like kinase 1 (169). As a result, the potential effects of inhibitors on other cellular pathways limit the conclusions that can be drawn. To corroborate data produced from the use of inhibitors, RNA interference or the over-expression of dominant negative proteins could be used. Although these alternate methods are also accompanied by their own drawbacks such as inadequate construct expression and transfection difficulties, the high specificity of these methods would allow for more decisive conclusions.

4.3 Future Directions

The first aim of this thesis established cell models that represent ARC kisspeptin neurons through the analysis of cellular gene expression and hormonal responses. The development of these models opens a new avenue for the molecular analysis of Kiss1 gene structure and gene regulation in endogenously synthesizing kisspeptin neurons. Specifically, the mHypoA-51 ad mHypoA-63 cell lines could be used to study the non-genomic signal transduction pathways
activated by estrogen to down-regulate *Kiss1* expression. These molecular studies are difficult
due to the heterogeneous nature of the hypothalamus and have therefore not yet been completed.

With respect to the genomic actions of estrogen, a recent study showed that estrogen up-
regulation of *Kiss1* in the AVPV requires ‘classical’ estrogen response element (ERE)-dependent
signaling, and estrogen down-regulation of *Kiss1* in the ARC requires ‘non-classical’ ERE-
independent signaling (170). This study used a ‘non-classical’ ER knock-in mouse model, which
was created by crossing ERαKO mice with mutant mice that have an ERα allele that cannot bind
ERE, and found that estrogen regulation of *Kiss1* was abolished in the AVPV but not in the
ARC. Although this study provided significant insight into the mechanism for differential
estrogen regulation of *Kiss1* in the AVPV and ARC, use of the mHypoA-51 and mHypoA-63
cell lines would allow for a more detailed mechanistic analysis. For instance, identification of the
specific co-regulators or co-activators as well as the non-ERE regulatory site involved in ERE-
independent signaling could be carried out in the mHypoA-51 and mHypoA-63 ARC kisspeptin
neurons. In addition, the contribution of ERα could be confirmed in these cell lines by examining
the *Kiss1* response to estrogen after knockdown of ERα using small interfering RNA (siRNA).

Of particular importance, the location of the *Kiss1* transcriptional start site and promoter
region is still unknown. This analysis has been performed in non-*Kiss1*-expressing cell lines, and
have therefore produced inconclusive results (Colledge WH, unpublished data). The mHypoA-51
and mHypoA-63 cell lines, on the other hand, are suitable models for this analysis and may lead
to successful location of the *Kiss1* promoter. This undertaking would be useful for the generation
of tissue-specific *Kiss1* knockout mice, which would be greatly beneficial to an overall
understanding of kisspeptin physiology.

The second aim of this thesis described an indirect mechanism by which kisspeptin could
regulate the gonadotropic axis. Using hypothalamic cell lines, it was found that kisspeptin
directly stimulates NPY synthesis and secretion through activation of MAPK signaling pathways, potentially resulting in NPY activation of GnRH neurons. Furthermore, Kiss1r expression was detected in native NPY neurons, a finding that future studies could confirm by demonstrating NPY and Kiss1r co-localization using IHC. Although these studies were requisite in order to provide evidence of a direct pathway between kisspeptin and NPY neurons, the exact physiological relevance of the proposed kisspeptin-NPY-GnRH pathway should be explored in vivo. For instance, studies examining the LH response to kisspeptin after administration of a NPY antagonist should be undertaken. This future study is necessary to establish the physiological significance of the kisspeptin-NPY-GnRH pathway.

In order to further substantiate the data regarding kisspeptin to NPY neuronal communication, experiments could be conducted by treating the NPY-secreting cell line, mHypoE-38, with conditioned media containing kisspeptin synthesized from the kisspeptin-secreting mHypoA-51 or mHypoA-63 cell line. This study in combination with the use of a Kiss1r antagonist would determine whether any other neuropeptides or factors release from these neurons can contribute to the activation of NPY neurons. Moreover, these experiments could be compared to studies involving co-culture of the mHypoE-38 neurons with the mHypoA-51 or mHypoA-63 neurons. Using cell culture inserts (BD Biosciences), this method allows the formation of synaptic connections between the two different cell types. Together, these studies will help define whether synaptic connections are necessary, or whether only the secretory products of the kisspeptin neuron are critical for NPY gene expression and secretion. Overall, the completion of the studies outlined above would significantly advance the current understanding of Kiss1 gene structure and gene regulation, and strengthen the finding that kisspeptin directly acts on NPY neurons to indirectly control reproductive function.
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