THE HORMONAL CONTROL OF NEUROPEPTIDE Y AND GONADOTROPIN-RELEASING HORMONE HYPOTHALAMIC NEURONS

by

Sandeep S. Dhillon

A thesis submitted in conformity with the requirements for the degree of Doctor of Philosophy

Department of Physiology
University of Toronto

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2010

Abstract

The physiological mechanisms that control energy homeostasis are reciprocally linked to reproduction. However, the neuroendocrine circuitry that registers endocrine cues to direct homeostatic responses in energy balance and reproduction remain unknown. Neuropeptide Y (NPY) neurons have emerged as a key central target of estrogen and leptin that are capable of modulating both reproduction and energy balance. The hypothesis was generated that NPY neuronal subpopulations act as an integration centre to regulate the effects of estrogen and leptin on these important physiological processes through specific signaling pathways. Using hypothalamic cell lines that express the leptin receptor (Ob-R), estrogen receptor (ER) and NPY, this hypothesis was tested in three aims.

17β-estradiol (E2) was previously demonstrated to biphasically regulate NPY mRNA in the mHypoE-38 neuronal cell line; where 24 h E2 exposure induced NPY gene expression that our group proposed may be involved in the gonadotropin-releasing hormone (GnRH) preovulatory surge. E2 also acts as an anorexigenic hormone through unknown hypothalamic targets. E2 directly decreased NPY secretion in the mHypoE-42 and mHypoA-2/12 neuronal cell lines through ER-α. The anorexigenic action of E2 was
mediated through the energy sensing 5' AMP-activated protein kinase (AMPK) and the phosphoinositide-3-kinase (PI3K) pathway. NPY secretion was also decreased by leptin in mHypoA-59 and NPY-GFP cell models through AMPK- and PI3K-dependent mechanisms. Prolonged exposure to leptin in NPY-GFP cell lines prevented AMPK signaling and the leptin-mediated reduction in NPY secretion, indicating NPY neuronal resistance with prolonged leptin exposure. Leptin also stimulated NPY secretion in mHypoE-38 neurons, which was blocked by pharmacological inhibitors of the mitogen-activated protein kinase (MAPK) and PI3K pathways. Importantly, conditioned medium from the mHypoE-38 NPY neuronal cells induced GnRH transcripts in GT1-7 neurons, which was inhibited by Y1-receptor antagonists. Pharmacological inhibitors of the MAPK and PKA signal transduction pathways attenuated the NPY-mediated increase in GnRH transcription.

Based upon these findings, I propose NPY neurons in the hypothalamus consist of a heterogeneous population of neurons, and provide the first evidence of intrinsically different responses to function as physiological integrators for two different systems: NPY secretion can be suppressed to decrease food intake and induced to stimulate GnRH neurons.
Acknowledgments

I owe my deepest gratitude to Dr. Denise Belsham, who has been a significant presence in my life. I am indebted to Dr. Belsham whose patience, kindness and academic experience have allowed me to become the person I am today. Thank you Denise.

I would also like to thank my committee members, Dr. Isabella Canniggia and Dr. Theodore Brown. I have benefited greatly from their guidance, mentorship and encouragement, which have been instrumental to the completion of this degree.

I would like to thank the members of the Belsham lab for their valuable discussion, encouragement and making the lab such an enjoyable experience. I also want to specially thank Ginah Kim. You have become my best friend both inside and outside the laboratory. Thank you for always being there.

Finally, I would like to thank my family for their constant support, patience and love. I would not have made it here without them. This accomplishment is as much yours as it is mine.
Table of Contents

Acknowledgments ....................................................................................................................................... iv

Table of Contents ...................................................................................................................................... v

List of Tables ............................................................................................................................................... x

List of Figures ............................................................................................................................................... xi

List of Abbreviations ................................................................................................................................... xiv

Chapter 1 Relevant literature reviews ...................................................................................................... 1

1.1 Introduction .......................................................................................................................................... 2

1.2 Reproductive function .......................................................................................................................... 4

1.2.1 The hypothalamic-pituitary-gonadal axis ......................................................................................... 4

1.2.2 Gonadotropin-releasing hormone (GnRH) in the hypothalamus ...................................................... 6

1.2.3 Regulation of GnRH neurons ........................................................................................................... 7

1.3 Energy homeostasis and reproductive function .................................................................................... 9

1.3.1 Hypothalamic nuclei associated with regulation of food intake ...................................................... 9

1.3.2 Energy homeostasis and reproductive function ............................................................................... 10

1.4 Neuropeptide Y ..................................................................................................................................... 13

1.4.1 Synthesis .......................................................................................................................................... 13

1.4.2 NPY receptors .................................................................................................................................. 14

1.4.3 Signaling pathways activated by NPY ............................................................................................ 14

1.4.4 NPY effects on energy homeostasis and reproduction ..................................................................... 15

1.5 Estrogen .................................................................................................................................................. 18

1.5.1 Synthesis and metabolism ................................................................................................................ 18

1.5.2 Estrogen receptors ............................................................................................................................ 19

1.5.3 Signaling pathways activated by estrogen ....................................................................................... 22

1.5.4 Effects of estrogen on reproduction and feeding behaviour ........................................................... 27
1.5.5 Estrogen-mediated regulation of NPY neurons ................................................................. 29

1.6 Leptin ...................................................................................................................................... 30
  1.6.1 Synthesis and metabolism ............................................................................................... 30
  1.6.2 Leptin receptors and signaling events ............................................................................. 31
  1.6.3 Effects of leptin on feeding behaviour and reproduction .................................................. 34
  1.6.4 Leptin-mediated regulation of NPY neurons ................................................................... 35
  1.6.5 Leptin Resistance ............................................................................................................. 37

1.7 Cell models .......................................................................................................................... 38
  1.7.1 GnRH-expressing GT1-7 neurons .................................................................................... 39
  1.7.2 Embryonic hypothalamic cell lines – mHypoE-xx ......................................................... 41
  1.7.3 Adult hypothalamic cell lines – mHypoA-xx ................................................................. 42
  1.7.4 NPY-GFP cell line ........................................................................................................ 43

1.8 Hypothesis and aims ........................................................................................................... 44

Chapter 2 ...................................................................................................................................... 47

Materials and methods ........................................................................................................... 47

2.1 Cell culture and reagents .................................................................................................... 48

2.2 Semi-quantitative RT-PCR ................................................................................................ 49
  2.2.1 One step RT-PCR ........................................................................................................ 49
  2.2.2 Two step RT-PCR ....................................................................................................... 49

2.3 Real-Time RT-PCR ............................................................................................................. 51

2.4 Enzyme Immunoassay ...................................................................................................... 51

2.5 Fluorescence-activated cell sorting (FACS) ..................................................................... 52

2.6 Radioactive Immunoassay ................................................................................................. 53

2.7 Western Blot Analysis ....................................................................................................... 53

2.8 Immunocytochemistry ...................................................................................................... 55

2.9 Co-culture .......................................................................................................................... 56
Chapter 3

17β-estradiol inhibits NPY secretion through membrane-associated estrogen receptor (ER)-α in clonal, immortalized hypothalamic neurons

3.1 Abstract

3.2 Introduction

3.3 Results

3.3.1 Expression of ER-α in FAC-sorted NPY-GFP neurons

3.3.2 Expression of the ER subtypes and other hypothalamic markers in mHypoE-42 and mHypoA-2/12 neurons

3.3.3 Regulation of NPY secretion by E₂ in mHypoE-42 and mHypoA-2/12 neurons

3.3.4 E₂-mediated regulation of NPY secretion is dependent on ER-α in the mHypoE-42 and mHypoA-2/12 neurons

3.3.5 E₂ decreases NPY secretion via membrane-bound ER-α

3.3.6 Inhibition of the PI3K and AMPK pathways affect the E₂-mediated regulation of NPY secretion

3.4 Discussion

Chapter 4

Leptin differentially regulates NPY secretion in NPY-expressing hypothalamic cell lines through distinct intracellular signal transduction pathways

4.1 Abstract

4.2 Introduction

4.3 Results

4.3.1 Expression of the Ob-R and other markers in mHypoE-38, mHypoE-42, mHypoA-59 and NPY-GFP neurons

4.3.2 Regulation of NPY secretion by leptin in mHypoE-38, mHypoE-42, mHypoA-59 and NPY-GFP neurons

4.3.3 Leptin increases NPY secretion in the mHypoE-38 neurons via PI3K and MAPK pathways
4.3.4 Leptin decreases NPY secretion in the mHypoA-59 and NPY-GFP neurons via AMPK and PI3K pathways .........................................................87

4.3.5 AICAR directly stimulates NPY secretion in mHypoA-59 and NPY-GFP neurons .......................................................................................89

4.3.6 Leptin pre-treatment attenuates leptin-mediated phosphorylation of AMPK in NPY-GFP neurons ........................................................................89

4.3.7 Leptin pre-treatment attenuates the leptin-mediated decrease in NPY secretion in NPY-GFP neurons .................................................................93

4.4 Discussion .................................................................................................................................93

Chapter 5 ........................................................................................................................................103

Neuropeptide Y induces gonadotropin-releasing hormone gene expression directly and through conditioned medium from mHypoE-38 NPY neurons .................................................................103

5.1 Abstract ....................................................................................................................................104

5.2 Introduction ..............................................................................................................................104

5.3 Results .......................................................................................................................................106

5.3.1 Expression of NPY receptor subtypes in GT1-7 neurons and hypothalamic markers in mHypoE-38 neurons .........................................................106

5.3.2 Regulation of GnRH mRNA expression by NPY in GT1-7 neurons .................................107

5.3.3 Effect of NPY receptor agonists on cAMP activity ............................................................107

5.3.4 NPY rapidly phosphorylates PKA, ATF-1 and CREB in GT1-7 neurons ............................110

5.3.5 Inhibition of MAPK and PKA-C signaling pathways affects NPY-mediated regulation of GnRH mRNA expression in GT1-7 neurons.............113

5.3.6 Regulation of GnRH transcription by conditioned media from NPY-secreting mHypoE-38 neurons is mediated through the NPY Y1 receptor subtype ........................................................................113

5.4 Discussion ................................................................................................................................117

Chapter 6 ........................................................................................................................................125

Overall Discussion and Future Directions .....................................................................................125

6.1 Overall Discussion ....................................................................................................................126

6.2 Limitations ..............................................................................................................................134
6.3 Future directions of study .................................................................136
Chapter 7 – References .........................................................................140
List of Tables

Table 1.1 Peptides implicated in regulation of food intake .............................................11

Table 1.2 NPY receptors characteristics ........................................................................15

Table 1.3 Co-activators in estrogen receptor physiology ..............................................24

Table 2.1 Primer sequences ...........................................................................................49

Table 3.1 Expression of ER, NPY, Ob-R and AgRP in hypothalamic nuclei .................77
List of Figures

Figure 1.1 Schematic illustration of the hypothalamic-pituitary-gonadal axis.................5

Figure 1.2 Schematic illustration of the signal transduction mechanisms activated by NPY
----------------------------------------------------------------------------------------16

Figure 1.3 Schematic illustration of estrogen signaling mechanisms..............................22

Figure 1.4 Schematic illustration of leptin receptor signaling........................................32

Figure 1.5 Immortalization of hypothalamic cell models..............................................39

Figure 1.6 Schematic illustration of the objectives of the current thesis.........................45

Figure 3.1 Expression of ER-α mRNA in NPY-GFP neurons using RT-PCR ..................61

Figure 3.2 Expression of ERs and other hypothalamic markers in mHypoE-42 and mHypoA-2/12 neurons..........................................................................................62

Figure 3.3 Estrogen directly decreases NPY secretion in the mHypoE-42 and mHypoA-2/12 neurons.............................................................................................................64

Figure 3.4 Estrogen attenuates NPY secretion via ER-α in mHypoE-42 and mHypoA-2/12 neurons.............................................................................................................66

Figure 3.5 ER-α localized at the cell membrane with caveolin-1 protein.......................68

Figure 3.6 ER-α localized at the cell membrane is required for the estrogen-mediated decrease in the mHypoE-42 and mHypoA-2/12 neurons ...........................................69

Figure 3.7 PI3K inhibitor LY294002 and AMPK inhibitor Compound C attenuates NPY-mediated regulation of NPY secretion in mHypoE-42 neurons ...........................70

Figure 3.8 Model of the potential cellular signaling pathways involved in estrogen regulation of NPY secretion.................................................................78

Figure 4.1 Characterization of NPY-expressing hypothalamic cell models.................84
Figure 4.2 Leptin directly regulates NPY secretion in NPY-expressing hypothalamic cell lines

Figure 4.3 Leptin increases NPY secretion in the mHypoE-38 cell lines via PI3K and MAPK pathways

Figure 4.4 Leptin decreases NPY secretion in the NPY-GFP cell line via AMPK and PI3K pathways

Figure 4.5 Leptin decreases NPY secretion in the mHypoA-59 cell line via AMPK and PI3K pathways

Figure 4.6 AICAR increases NPY secretion in the NPY-GFP and mHypoA-59 cell lines

Figure 4.7 Prolonged leptin exposure prevents the leptin-mediated decrease in phospho-AMPK in the NPY-GFP cell line

Figure 4.8 Prolonged leptin exposure prevents the leptin-mediated decrease in NPY secretion in the NPY-GFP cell line

Figure 4.9 Model of the potential cellular signaling pathways involved in leptin regulation of NPY secretion

Figure 5.1 Expression of NPY Y1, Y2 and Y4 receptor mRNA transcripts in GT1-7 neurons

Figure 5.2 NPY-mediated regulation of GnRH gene expression in GT1-7 neurons

Figure 5.3 NPY Y1 or Y4 receptor-mediated cAMP activity in the GT1-7 neurons

Figure 5.4 NPY activates signal transduction second messengers in GT1-7 neurons

Figure 5.5 NPY Y1 antagonist BIBP-3226, MEK and PKA inhibitors attenuate NPY-mediated regulation of GnRH mRNA levels in GT1-7 neurons
Figure 5.6 Conditioned media from NPY-expressing mHypoE-38 neurons increases GnRH mRNA expression in GT1-7 cells and can be blocked by the NPY Y1 antagonist BIBP-3226 ..........................................................116

Figure 5.7 Neuron-specific gene promoter for GnRH..................................................122

Figure 5.8 Model of the potential cellular signaling pathways involved in NPY regulation of GnRH mRNA expression .................................................................124

Figure 6.1 Overall findings and future directions..........................................................133
## List of Abbreviations

<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>AC</td>
<td>adenylyl cyclase</td>
</tr>
<tr>
<td>AF-1</td>
<td>transactivating function-1</td>
</tr>
<tr>
<td>AF-2</td>
<td>transactivating function-2</td>
</tr>
<tr>
<td>AgRP</td>
<td>agouti-related peptide</td>
</tr>
<tr>
<td>AICAR</td>
<td>5-aminoimidazole-4-carboxamide ribonucleotide</td>
</tr>
<tr>
<td>AMP</td>
<td>adenosine monophosphate</td>
</tr>
<tr>
<td>AMPK</td>
<td>5' AMP-activated protein kinase</td>
</tr>
<tr>
<td>AP-1</td>
<td>activator protein-1</td>
</tr>
<tr>
<td>ARC</td>
<td>arcuate nucleus</td>
</tr>
<tr>
<td>ATP</td>
<td>adenosine-5’-triphosphate</td>
</tr>
<tr>
<td>AVPV</td>
<td>anteroventricular paraventricular nucleus</td>
</tr>
<tr>
<td>Bp</td>
<td>base pairs</td>
</tr>
<tr>
<td>BSA</td>
<td>bovine serum albumin</td>
</tr>
<tr>
<td>cAMP</td>
<td>cyclic adenosine monophosphate</td>
</tr>
<tr>
<td>cDNA</td>
<td>complementary deoxyribonucleic acid</td>
</tr>
<tr>
<td>CNS</td>
<td>central nervous system</td>
</tr>
<tr>
<td>CPON</td>
<td>C-terminal of neuropeptide Y</td>
</tr>
<tr>
<td>CRE</td>
<td>cAMP response element</td>
</tr>
<tr>
<td>CREB</td>
<td>cAMP response element binding protein</td>
</tr>
<tr>
<td>CRH</td>
<td>corticotropin-releasing hormone</td>
</tr>
<tr>
<td>CT</td>
<td>cycle threshold</td>
</tr>
<tr>
<td>DBD</td>
<td>DNA-binding domain</td>
</tr>
<tr>
<td>DMEM</td>
<td>Dulbecco’s modified eagle medium</td>
</tr>
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</table>
DMN  dorsomedial nucleus
DMSO  dimethyl sulfoxide
DNA  deoxyribonucleic acid
DPN  2,3-bis(4-Hydroxyphenyl)-propionitrile
E₁  estrone
E₂  17β-estradiol
E₃  estriol
ER  estrogen receptor
ERE  estrogen response element
ERK  extracellular-related kinase
FBS  fetal bovine serum
FITC  fluorescein isothiocyanate
GABA  γ-aminobutyric acid
GnRH  gonadotropin-releasing hormone
GPCR  G-protein coupled receptor
h  hours
HEK  human embryonic kidney
HFD  high fat diet
HPA  hypothalamic-pituitary adrenal axis
HPG  hypothalamic-pituitary gonadal axis
ICC  immunocytochemistry
ICV  intracerebroventricular
JNK  c-Jun NH2 terminal kinase
kb  kilobases
<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Definition</th>
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<tbody>
<tr>
<td>kDa</td>
<td>kilodaltons</td>
</tr>
<tr>
<td>LH</td>
<td>luteinizing hormone</td>
</tr>
<tr>
<td>LHa</td>
<td>lateral hypothalamus</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>ME</td>
<td>median eminence</td>
</tr>
<tr>
<td>MCH</td>
<td>melanin-concentrating hormone receptor 1</td>
</tr>
<tr>
<td>MNAR</td>
<td>modulator of non-genomic activity of estrogen receptor</td>
</tr>
<tr>
<td>mRNA</td>
<td>messenger ribonucleic acid</td>
</tr>
<tr>
<td>α-MSH</td>
<td>melanocyte-stimulating hormone</td>
</tr>
<tr>
<td>MPP</td>
<td>methyl-piperidinopyrazole</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>ODN</td>
<td>oligonucleotide</td>
</tr>
<tr>
<td>OVX</td>
<td>ovariectomized</td>
</tr>
<tr>
<td>PBS</td>
<td>phosphate buffered saline</td>
</tr>
<tr>
<td>PCR</td>
<td>polymerase chain reaction</td>
</tr>
<tr>
<td>PI3K</td>
<td>phosphatidylinositol 3-kinase</td>
</tr>
<tr>
<td>PKC</td>
<td>protein kinase C</td>
</tr>
<tr>
<td>POA</td>
<td>preoptic area</td>
</tr>
<tr>
<td>PP</td>
<td>pancreatic peptide</td>
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xvi
<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Definition</th>
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<tbody>
<tr>
<td>PPT</td>
<td>4,4′,4″-(4-Propyl-[1H]-pyrazole-1,3,5-triyl)trisphenol</td>
</tr>
<tr>
<td>PVN</td>
<td>paraventricular nucleus</td>
</tr>
<tr>
<td>PYY</td>
<td>peptide YY</td>
</tr>
<tr>
<td>RNA</td>
<td>ribonucleic acid</td>
</tr>
<tr>
<td>(R,R)-THC</td>
<td>(R,R)-5,11-Diethyl-5,6,11,12-tetrahydro-2,8-chrysenediol</td>
</tr>
<tr>
<td>RT-PCR</td>
<td>reverse transcriptase polymerase chain reaction</td>
</tr>
<tr>
<td>siRNA</td>
<td>small interfering ribonucleic acid</td>
</tr>
<tr>
<td>STAT</td>
<td>signal transducer activator of transcription</td>
</tr>
<tr>
<td>SV40</td>
<td>simian virus 40</td>
</tr>
<tr>
<td>T-Ag</td>
<td>T-antigen</td>
</tr>
<tr>
<td>VMN</td>
<td>ventromedial nucleus</td>
</tr>
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</table>
Chapter 1

Introduction
1.1 Introduction

Appetite is regulated by an interplay of neuropeptides, neurotransmitters and hormones produced from both central and peripheral sites. Centrally, appetite and energy homeostasis is chiefly regulated in the hypothalamus by a complex neural circuitry comprised of over 100 putative orexigenic (appetite stimulating) and anorexigenic (appetite inhibiting) neuropeptides – including neuropeptide Y (NPY), melanin-concentrating hormone (MCH), galanin, orexin, α-melanocyte-stimulating hormone (α-MSH), neurotensin (NT) and corticotropin-releasing hormone (CRH) (1-7). These appetite-stimulatory and appetite-inhibitory circuits of the hypothalamus are, in turn, controlled by peripheral endocrine signals. Two key hormones, estrogens and leptin, are thought to be involved in regulating these peptidergic feeding circuits by altering secretion and gene expression of the feeding-related neuropeptides (8, 9). Additionally, reduced nutritional status or body mass results in infertility and delayed reproductive maturation while disturbing the course of the ovarian cycle (10). Perturbed gonadotropin-releasing hormone (GnRH) secretion, the central regulator of the hypothalamic-pituitary-gonadal (HPG) axis, is postulated as the most important etiological factor for nutritionally-induced reproductive disorders (9). Although the relationship between nutrition and reproductive success has been extensively studied, the exact mechanism linking the two physiological processes is yet to be determined. NPY neuromodulators of the hypothalamus have emerged as key factors involved in regulating both feeding behaviour and reproductive homeostasis (11-13). Although several research groups have documented the importance of NPY in the regulation of the reproductive axis and energy homeostasis, the peripheral hormonal regulators of NPY neurons have not been fully
investigated. Recently, estrogen and leptin have emerged as key modulators of the NPY neuron.

Estrogen and leptin receptors are expressed in overlapping NPY neuronal populations in the arcuate nucleus (ARC) and ventromedial nucleus (VMN) (14-18). Previous studies have demonstrated deficiencies in either leptin or estrogen levels can result in an upregulation of hypothalamic NPY mRNA in the ARC (19-21). The hypothesis that 17β-estradiol (E2) and leptin can positively or negatively regulate specific NPY subpopulations to control feeding and reproductive physiology is yet to be verified. The use of hypothalamic cell models provides a novel tool to study the direct regulation of these neuropeptides by E2 and leptin. In this thesis, the first and second studies characterize the E2- and leptin-mediated regulation of NPY secretion in embryonic- and adult-derived mouse hypothalamic cell lines. The molecular mechanisms involved in the differential regulation of NPY secretion and receptors responsible were investigated. In addition, as GnRH is one of the most important peptides required for normal reproductive function, the third study in this thesis characterizes the direct effect of NPY on GnRH gene expression. GnRH-synthesizing GT1-7 neurons were used to study the receptors, signaling pathways and transcriptional events of NPY and conditioned medium treatments from NPY-synthesizing cell lines in a GnRH neuronal cell model.

The purpose of this thesis was to evaluate the ability of NPY to regulate GnRH neuronal populations and describe the regulation of NPY-expressing neuronal cell lines by peripheral endocrine hormones - E2 and leptin - that are required to maintain normal feeding and reproductive homeostasis. Using clonal immortalized hypothalamic neuronal cell lines, I provide detailed mechanics of this circuit and describe the differential regulation of the NPY neuron. In addition, these studies provide further and more
comprehensive evidence of the significance of NPY in the regulation of the GnRH neuron and overall reproductive function. These studies contribute to our understanding of both leptin and estrogen physiology, and provide evidence that NPY neurons are heterogeneous in nature with intrinsically different responses to these hormones.

1.2 Reproductive function

1.2.1 The hypothalamic-pituitary-gonadal axis

The key to species survival depends on the ability to reproduce. In mammals, reproduction is maintained by a delicate feedback loop involving the hypothalamus, pituitary gland and gonads, which form the HPG axis (Figure 1.1) (22, 23). At the pinnacle of the HPG axis are GnRH neurons. GnRH is secreted into the median eminence (ME) of the hypothalamus in pulses, and through the hypophyseal portal vasculature, signals the anterior pituitary to secrete gonadotropin hormones, luteinizing hormone (LH) and follicle stimulating hormone (FSH) (24). These hormones are released into the systemic blood system and act at the gonads to regulate steroid hormone production and gametogenesis. Specifically, FSH allows for the maturation of ovarian follicles in females and spermatogenesis in males, and LH induces sex steroid hormone synthesis and release, gametogenesis and regulates ovulation (25). The sex steroids produced by the gonads include estrogens, progestins and androgens, which are produced in sexually dimorphic quantities (26). Gonadal steroids indirectly control their own secretion through positive and negative feedback loops, which maintain hormonal levels within a narrow range and enable normal reproductive function (27, 28). In particular, gonadal steroids modulate both the hypothalamus and pituitary to control the release of GnRH and gonadotropin hormones. The regulation of normal secretory mechanisms, transcription
The hypothalamus produces gonadotropin-releasing hormone (GnRH), which is released in a pulsatile fashion to control the synthesis and secretion of pituitary hormones, luteinizing hormone (LH) and follicle stimulating hormone (FSH). LH and FSH in turn are secreted into systemic circulation to act on the ovaries and testes to regulate steroidogenesis. The gonadal steroids estrogen and testosterone are then secreted into the systemic circulation to regulate production and secretion of GnRH through positive and negative feedback loops, which occurs both directly at the GnRH neuron and indirectly through afferent neuronal circuitry upstream of GnRH neurons.

Figure 1.1. Schematic illustration of the hypothalamic-pituitary-gonadal axis. Adapted from Kim 2010.
levels, receptor activation and cellular signaling cascades in the HPG axis are essential to maintain normal reproductive function.

1.2.2 Gonadotropin-releasing hormone (GnRH) in the hypothalamus

There are two GnRH genes in mammals. GnRH-1 (GnRH) was originally discovered by McCann and colleagues in the 1960’s, when extracts from rat hypothalamic cultures were able to stimulate the release of LH (24). Additional studies identified the active factor in the hypothalamic extracts to be a decapeptide that was capable of stimulating both LH and FSH release from the anterior pituitary (29). This decapeptide, now known as GnRH, is highly conserved in mammals (30). The gene encoding GnRH spans 4.5 kb of DNA on chromosome 8 in humans and consists of three introns and 4 exons encoding a 92 amino acid propeptide (31, 32). Post-translational processing of the 92 amino acid propeptide is completed by endopeptidase, carboxypeptidase E and peptidyl-glycine α-amidating monooxygenase to generate a 23 amino acid signal peptide, a 59 amino acid peptide product called GnRH-associated peptide and the decapeptide GnRH (33-37). A second form of GnRH, termed chicken GnRH-2, is expressed in mammals and found in the brainstem and medial hypothalamus (38). This highly conserved peptide is present on chromosome 20 in humans, but its function appears to be silenced in mice, humans, cattle and rat (39, 40). As a result reproductive cycles studied by endocrinologists have focused almost exclusively on GnRH.

In the rodent, GnRH is synthesized by a small population of GnRH-expressing neurons (400-1000) mainly localized in the anterior hypothalamus, specifically in the medial preoptic nucleus (POA) (36). GnRH neurons originate from the olfactory placode
during fetal life and migrate rostrally along the cribiform plate, through the nasal septum towards the anterior hypothalamus in the developing brain (30). GnRH is secreted in a pulsatile manner into the hypophyseal portal vasculature to reach the anterior pituitary gonadotrophs where it stimulates the secretion of LH and FSH (41).

1.2.3 Regulation of GnRH neurons

GnRH neurons are under the control of a number of regulatory neuromodulators including γ-aminobutyric acid (GABA) (42), kisspeptins (43), NPY (44, 45), neurotensin (NT) (46), dopamine (47), norepinephrine (NE) (48), nitric oxide (49), activin (50), histamine (51), androgen (52), estrogen (53) and melatonin (54), among many others (55). Some of the latest and likely most relevant regulators of the GnRH neuron are discussed in greater detail below.

GABA-expressing neurons have been shown to be a prominent inhibitory regulator of GnRH neuronal function (56). GnRH neurons express the GABA receptor and are directly innervated by GABA-expressing neurons (57). Both the down-regulation of GABA neurotransmitter levels in pre-synaptic terminals innervating GnRH cell bodies and decreases in GABA receptor expression in GnRH-expressing neurons are thought to be key events in enabling the pre-ovulatory surge (58). Together, GABA-expressing neurons are thought to have a prominent role in regulating GnRH neuronal function and the reproductive axis.

Kisspeptin-expressing neurons of the hypothalamus represent another set of neurons that are critical to reproductive function (59). Kisspeptin is the natural ligand of the previously orphan receptor, GPR54 (60, 61). In 2003, two groups independently identified an absence of puberty onset and hypogonadotropic hypogonadism in patients with a loss of function mutation in the GPR54 gene (62, 63). Additional studies
demonstrated that kisspeptin is a key regulator of the GnRH neuron and thereby the HPG axis (59, 61, 64). Kisspeptin has been demonstrated to directly depolarize and increase the firing rate of GnRH neurons, which have been shown to express GPR54 (43). Furthermore, Kisspeptin treatment results in an increase in GnRH release in a number of species (59, 60, 65, 66). Although the cellular mechanisms of kisspeptin hormonal regulation are incomplete, kisspeptin has emerged as an important regulator of reproductive function.

NT-expressing neurons can also stimulate GnRH mRNA and release in vivo (46). NT neurons from the anteroventral periventricular nucleus (AVPV) innervate and act on GnRH neurons in a similar manner to NPY (67). NT has also been shown to regulate the amplitude of GnRH secretion, which is seen in rats where centrally infused NT resulted in increased LH release (46, 68). The precise role of NT-expressing neurons in the regulation of the HPG axis remains to be determined; although it is likely that NT neurons play a synergistic role to other neuronal populations such as kisspeptin, GABA and NPY to regulate GnRH neuronal function.

NPY has been acknowledged for many years as a major afferent regulator of reproductive function (69). NPY neurons in the ARC project to GnRH cell bodies in the POA and to GnRH pre-synaptic terminals in the ME (44, 70). Additionally, immunohistochemical evidence indicates co-localization of NPY receptors in GnRH neurons (71). This neuroanatomical evidence for connections between NPY and GnRH neurons establishes a possible mechanism in which NPY can directly influence the reproductive axis. Several studies, both in vivo and in vitro, have shown NPY stimulates GnRH secretion (45, 72, 73). In ewes, NPY infusion into the third ventricle substantially increases GnRH secretion in the ME (74). However, depending upon the steroidal
environment and species, NPY can also down-regulate the reproductive axis through GnRH (75). NPY injections into the third ventricle in ovariectomized rats led to a reduction in plasma LH (76). Chronic NPY administration inhibited gonadotropin secretion in intact female rats (76). In ovariectomized rabbits, NPY perfusion significantly decreased mean levels of GnRH, while the same NPY perfusion stimulated mean levels of GnRH in intact rabbits (77, 78). Overall, the role of NPY on the reproductive axis has produced conflicting evidence, with both stimulatory and inhibitory effects published. Although a number of studies have investigated the role of NPY on GnRH secretion, the effect of NPY on GnRH neurons at the transcriptional level has not been elucidated.

GnRH neurons act as the final channel for a number of neuroendocrine signals that regulate reproductive function. However, classical in vivo approaches cannot firmly establish the direct action of key neuromodulators on the GnRH neuron, primarily because the GnRH system receives input from numerous sources (79). As a result, the mechanism underlying the regulation of GnRH neurons by these neuromodulators are still being investigated.

1.3 Energy homeostasis and reproductive function

1.3.1 Hypothalamic nuclei associated with regulation of food intake

The energy balance equation states that body mass remains constant when caloric intake equals caloric expenditure. Chronic deviations from this balance results in a change in body mass (80). Although the energy balance equation may appear simplistic in nature, in reality, energy homeostasis involves the interaction of an array of neuropeptides, neurotransmitters and hormones produced at both central and peripheral sites (9, 81). The hypothalamus is the central regulator of feeding behaviour and energy
homeostasis, integrating neuronal, metabolic and endocrine signals. Several distinct hypothalamic nuclei are involved in this process including, the lateral hypothalamic area (LHA), paraventricular nucleus (PVN), dorsomedial nucleus (DMN), VMN and ARC (82, 83). It is now well known that these feeding nuclei are controlled by a neural circuitry comprised of orexigenic and anorexigenic neuropeptides including NPY, MCH, galanin, orexin, galanin-like peptide (GALP), α-MSH, NT and CRH (8, 84). Table 1.1 provides an overview of the feeding neuropeptides and overall effect on feeding behaviour. Energy homeostasis is carefully maintained through the regulation of these neuropeptides by both peripheral and central signals.

1.3.2 Energy homeostasis and reproductive function

Reproduction is an energy intensive process that is metabolically gated. In humans, under-nutrition or excessive exercise results in reproductive dysregulation (81). Conversely, obesity and diabetes can also impair reproductive function. This relationship has been demonstrated in mammals where a deficiency or excess of nutrients results in impaired gonadial function, delayed reproductive maturation, and a disturbance in the course of the ovarian cycle (85, 86). The hypothalamus plays a crucial role in integrating these two physiological processes, where metabolic sensory stimuli including hormonal neuromodulators and hypothalamic neuropeptides that control energy homeostasis can also influence the HPG axis. A decrease in GnRH and/or LH secretion is postulated to be the most important etiological factor for nutritionally induced reproductive disorders (9, 86). Although the relationship between nutrition and reproductive success has been described, the exact mechanisms and neuropeptides linking these two physiological processes has yet to be determined. NPY neurons of the hypothalamus have emerged as a
key neuromodulator involved in regulating both feeding behaviour and reproductive homeostasis (13, 78, 87, 88).

<table>
<thead>
<tr>
<th>Anorexigenic peptides</th>
<th>Orexigenic peptides</th>
</tr>
</thead>
<tbody>
<tr>
<td>Adrenomedullin</td>
<td>Neuropeptide Y (NPY)</td>
</tr>
<tr>
<td>Bombesin</td>
<td>Agouti-related peptide (AgRP)</td>
</tr>
<tr>
<td>Cholecystokinin (CCK)</td>
<td>Galanin</td>
</tr>
<tr>
<td>Calcitonin</td>
<td>Galanin-like peptide (GALP)</td>
</tr>
<tr>
<td>Cocaine-and amphetamine regulated transcript (CART)</td>
<td>Growth hormone-releasing hormone (GHRH)</td>
</tr>
<tr>
<td>Corticotropin releasing hormone (CRH)</td>
<td>Growth hormone (GH)</td>
</tr>
<tr>
<td>Ciliary neurotropic factor (CNTF)</td>
<td>Ghrelin</td>
</tr>
<tr>
<td>Gastrin-releasing peptide (GRP)</td>
<td>Melanin-concentrating hormone (MCH)</td>
</tr>
<tr>
<td>Galanin-like peptide (GALP)</td>
<td>Opioids</td>
</tr>
<tr>
<td>Glucagon-like peptide-1 (GLP-1)</td>
<td>Orexin-A/B</td>
</tr>
<tr>
<td>Glucagon</td>
<td>Prolactin</td>
</tr>
<tr>
<td>Melanocortin</td>
<td>Peptide YY (PYY)</td>
</tr>
<tr>
<td>Melanocyte-stimulating hormone (MSH)</td>
<td></td>
</tr>
<tr>
<td>Neuromedin U</td>
<td></td>
</tr>
<tr>
<td>Neurotensin</td>
<td></td>
</tr>
<tr>
<td>Oxytocin</td>
<td></td>
</tr>
<tr>
<td>Pituitary adenylate cyclase-activating polypeptide (PACAP)</td>
<td></td>
</tr>
<tr>
<td>Pro-opiomelanocortin (POMC)</td>
<td></td>
</tr>
<tr>
<td>Somatostatin</td>
<td></td>
</tr>
<tr>
<td>Thyrotropin-releasing hormone (TRH)</td>
<td></td>
</tr>
<tr>
<td>Uroctin</td>
<td></td>
</tr>
<tr>
<td>Vasactive intestinal polypeptide (VIP)</td>
<td></td>
</tr>
</tbody>
</table>
1.4 Neuropeptide Y

1.4.1 Synthesis

NPY is a 36 amino acid neuropeptide first discovered in the porcine brain by Tatemoto et al. in 1982 (89). NPY is highly conserved in many species and forms the pancreatic peptide family with Peptide YY (PYY) and pancreatic polypeptide (PP) (69, 89, 90). The NPY gene is located on human chromosome 7 at the locus 7p15.1 (69). The NPY gene contains 3 introns that are separated by 4 exons (69). The coding region of the prepro-NPY gene is 551 bp, which encodes a 97 amino acid prepropeptide. This prepro-NPY peptide includes a signal peptide, the 36 amino acid NPY peptide product, and the C-terminal peptide of NPY (CPON) (69). After translation, prepro-NPY is directed to the endoplasmic reticulum where the signal peptide is cleaved to form pro-NPY. Prohormone convertase then cleaves Pro-NPY at a dibasic site to generate NPY$_{1-39}$ and CPON. Two further truncations at the C-terminal end of Pro-NPY by a carboxypeptidase and the peptidylglycine α-amidating monooxygenase lead to the biologically active 36 amino acid peptide product. The mature NPY product can be further processed by two enzymes, dipeptidyl peptidase 4 and aminopeptidase P, resulting in NPY$_{3-36}$ and NPY$_{2-36}$, respectively (90). NPY is widely expressed in various regions of the brain, including the hypothalamus, amygdala, hippocampus, nucleus of the solitary tract (NTS), locus coeruleus, nucleus accumbens and cerebral cortex (69). Additionally, NPY fibres have been shown to innervate a number of brain regions including the PVN, supraoptic nucleus (SON), POA, DMH and ME (91-93). Peripheral NPY expression is seen in endothelial cells, spleen, heart, adrenal medulla and liver (69). In accordance with its wide distribution, NPY participates in the control of several physiological functions,
including feeding behaviour, water consumption, learning and memory, locomotion, cardiovascular homeostasis, hormone secretion, emotional behaviour, reproduction and circadian rhythms (94).

1.4.2 NPY receptors

NPY exerts its biological effects through G-protein coupled receptors, which have been characterized based on their downstream physiological effects. The NPY receptor family includes: 1) the Y1 receptor, a post-synaptic receptor involved in feeding, vasoconstriction and reproduction (95), 2) the Y2 receptor, a pre-synaptic PYY preferring-receptor expressed abundantly in the periphery and implicated with metabolic syndrome (96), 3) the Y3 receptor, a controversial putative NPY-preferring receptor (97), 4) the Y4 receptor, a PP preferring-receptor involved in food intake (98), 5) the Y5 receptor, which is also involved in feeding, neuroprotection and reproduction, 6) the putative Y6 receptor, which has been cloned but the physiological function remains to be discovered (90, 94). Much of NPY receptor functionality has been studied using pharmacological truncated and substituted NPY analogs that are receptor specific (94, 99). The pharmacological characteristics, binding preferences and functional significance of each NPY receptor subtype are described in Table 1.2.

1.4.3 Signaling pathways activated by NPY

NPY receptors are, in most cases, coupled to pertussis toxin-sensitive G-proteins (i.e. Gi and Go proteins). Therefore, the most common response to NPY is the inactivation of adenylyl cyclase, which in turn, results in the inhibition of 3’,5’-cyclic monophosphate (cAMP) synthesis (100). However, NPY has also been demonstrated to induce cAMP production by inhibiting cAMP degradation by inactivating phosphodiesterases in neuroblastoma cell lines (101).
In many cell types, NPY has also been linked to the induction of calcium mobilization and calcium signaling pathways. NPY raises intracellular calcium concentrations, which occurs through inositol 1,4,5-phosphate-dependent (IP3) or -independent pathways and activating or blocking membrane bound calcium channels (102). In addition, NPY has been demonstrated to activate the mitogen-activated protein kinases (MAPK), as shown in erythroleukemia cells (92, 102). In particular, NPY has been confirmed to induce the phosphorylation of p42/p44 (ERK1/2). This activation of the MAPK pathway by NPY is suspected to occur through a phosphoinositide 3-kinase (PI3K)-dependent or pertussis toxin sensitive mechanism (Figure 1.2). Finally, NPY has also been demonstrated to induce vasodilation of human subcutaneous arteries via intracellular events involving nitric oxide (103, 104). Notably, NPY signaling systems activated are not helpful in providing the NPY-receptor subtype activated, since each receptor subtype has been demonstrated to be capable of activating the same intracellular pathways in transfected cells and tissue-specific studies.

The study of NPY second messenger systems has been essential to the understanding of anxiety, memory, hypertension and drug addiction (94). However, the NPY signaling events activated in the GnRH neuron, ultimately controlling the reproductive axis, have not yet been fully described.

1.4.4 NPY effects on energy homeostasis and reproduction

NPY neurons are a prime candidate linking energy balance and reproduction in the hypothalamus (10, 13, 81). The ability of NPY to regulate feeding behaviour was reported when intracerebroventricular (ICV) injection of NPY elicited a strong feeding
Table 1.2. NPY receptor characteristics. Adapted from Gehlert. Neuropeptides, 38; 135-140 (2004)

<table>
<thead>
<tr>
<th>Receptor</th>
<th>Agonist affinity</th>
<th>Distribution</th>
<th>Physiological role</th>
</tr>
</thead>
<tbody>
<tr>
<td>Y1</td>
<td>NPY &gt; [Leu31, Pro34] &gt;&gt; NPY (13-36) &gt;&gt; PP</td>
<td>Vascular smooth muscle, adipocytes, cerebral cortex, colon, hypothalamus</td>
<td>Food intake, blood pressure, seizure regulation, anxiety, pain, depression, ethanol consumption, reproduction?</td>
</tr>
<tr>
<td>Y2</td>
<td>PYY &gt; NPY &gt; NPY (13-36)</td>
<td>Hippocampus, adipocytes, renal proximal tubular cells, hypothalamus, amygdala</td>
<td>Food intake, blood pressure, seizure regulation, anxiety, bone formation, pain, GI motility, angiogenesis</td>
</tr>
<tr>
<td>Y4</td>
<td>PP &gt; [Leu31, Pro34] &gt; PYY &gt; NPY</td>
<td>Brain, heart, coronary artery, ileum, testis, lungs</td>
<td>Food intake, GI motility, reproduction?</td>
</tr>
<tr>
<td>Y5</td>
<td>NPY &gt; NPY (3-36) = [D-Trp32]</td>
<td>Dentate gyrus, hypothalamus, thalamus, cortex</td>
<td>Food intake, seizure regulation, anxiety, reproduction?</td>
</tr>
</tbody>
</table>
Figure 1.2. Schematic illustration of the signal transduction mechanisms activated by NPY.

NPY can modulate a variety of pathways through the activation of G-protein coupled receptors, resulting in: adenylyl cyclase inhibition and thus inhibition of PKA. Conversely, NPY can also increase cAMP levels and stimulate PKA activity by inhibiting PDE3B. NPY can also activate PLC and PI3K activity in a tissue specific manner. Mitogenic signals are stimulated through redundant mechanisms including the nitric oxide pathway (not depicted here).
response, even in satiated animals eventually leading to obesity (105-107). Subsequent studies supported NPY as a natural orexigenic neuropeptide. NPY expression in the hypothalamus dramatically increased in rats under poor metabolic conditions (108). Additional studies demonstrated that peripheral administration of NPY stimulates feeding and chronically administered doses results in obesity (109). Similarly, leptin-deficient mice have strongly elevated hypothalamic NPY levels and are morbidly obese (110, 111). Finally, NPY mRNA expression is increased during times of food restriction, further indicating that the activation of the NPY system is involved in the stimulation of feeding (106, 109). Moreover, increased NPY levels return to baseline levels after re-feeding, indicating hypothalamic NPY is a key physiological signal for energy homeostasis.

Shortly after NPY was identified as an orexigenic neuropeptide, additional studies implicated NPY in reproductive physiology. NPY neurons in the ARC project to GnRH cell bodies in the POA and to GnRH pre-synaptic terminals in the ME (70, 93). Further studies demonstrated NPY knock-out (NPY-KO) mice are not capable of generating a normal LH surge, necessary stage for ovulation (13). Importantly, NPY mRNA has been shown to accumulate in the ARC just prior to the LH surge, signifying that NPY may regulate the LH surge (88, 112). Although the importance of NPY in the reproductive axis has been demonstrated, the direct effect of NPY on GnRH neurons at the transcriptional level has not been elucidated.

1.5 Estrogen

1.5.1 Synthesis and metabolism

Estrogens are steroid hormones that are important endocrine effectors of reproduction, cardiovascular physiology, neuronal growth and differentiation, neuroprotection, cognition, sexual differentiation and regulation of mood (113-115). The
most common forms of estrogen found in the body are: estrone, E₂, 17α-estradiol and estriol (113, 116, 117). Estrone, estriol and 17α-estradiol are considered short acting estrogens with a much lower binding affinity to ERs than E₂ (118). However, both 17α-estradiol and E₂ have high ER affinity in the brain, and have been implicated in the control of hippocampal synaptic plasticity and neuroprotective effects in the brain (119). Although 17α-estradiol is emerging as a key regulator of neural development, it is not found in the circulation and is unaffected by ovariectomy, castration and/or adrenalectomy, suggesting 17α-estradiol is produced in the brain (120, 121). As a result, E₂ is considered to be the most potent and dominant form of estrogen in the body (117, 122). Estrogens are synthesized from a microsomal member of the p450 superfamily, aromatase cytochrome p450 (aromatase) (123). Through a series of reactions, aromatase in granulosa cells catalyzes the conversion of C19 androgenic steroid substrates produced from thecal cells to form a phenolic A ring structure, which is characteristic of estrogenic compounds (113). Estrogens can be produced in a wide range of tissues (124). In premenopausal women, the ovaries are the principal source of estrogens that circulates to act on distal tissues. However, in men and women, estrogenic compounds are also produced from extragonadal sites that operate primarily via paracrine mechanisms (115). These sites include the mesenchymal cells of adipose tissue, osteoblasts and chondrocytes of bone, vasculature endothelium, aortic smooth muscle and in the brain (115).

1.5.2 Estrogen receptors

The biological actions of E₂ are primarily mediated through two specific nuclear estrogen receptors (ER), ER-α and ER-β, which are part of the nuclear receptor superfamily (19, 120, 125, 126). There are two distinct genes that encode ERs, which may encode isoforms generated by alternative splicing (127, 128). In particular, data
supports that ER-β has multiple splice variants at the protein level (128, 129). Thus far, studies have largely focused on ER-β1 (ER-β), the originally cloned sequence (130). Additional isoforms ER-β2 and ER-β5 are derived from alternative splicing of the last coding exon (exon 8) (131). Studies examining the putative functions of ER-β2 and ER-β5 isoforms have found that only ER-β can bind to ligand and induce conformational changes as determined by protease digestion assays (132). As a result, current studies are examining the biological role of ER-β2 and ER-β5 isoforms. Presently, ER-β2 and ER-β5 isoforms are hypothesized to exist in an activated state, as these isoforms cannot bind to E2 (133).

ERs are multidomain proteins that are comprised of: 1) A-B domain containing activating function 1 (AF-1), 2) C region that contains a highly conserved DNA-binding domain (DBD) and two zinc fingers critical for DNA-binding, 3) D region that acts as a hinge, and 4) E domain, which contains the AF-2 region and is responsible for ligand recognition and binding (134). ER-α and ER-β contain evolutionary conserved DBDs, which are critical for receptor-DNA recognition and specificity, and C-terminal ligand binding domains (LBD) that recognize specific estrogenic compounds to exert the appropriate biological response (135). Although ER-α and ER-β show considerable homology in their DBD (90%), the receptor subtypes share only 53% amino acid identity at the carboxyl-terminal LBD (136). Additionally, the ER subtypes are products of different genes and studies have indicated that each ER can have unique and overlapping biological functions in a tissue- and cell context-dependent manner (136, 137). This is exemplified in studies that compared the phenotypes observed in the individual lines of ER knockout mice (ERKO), the αERKO and βERKO, which exhibit phenotypes that generally mirror the respective ER expression patterns (19, 114). The most notable
phenotypes in the female αERKO mice include an underdeveloped reproductive tract, hypergonadotropic hypergonadism, lack of pubertal onset, and excess adipose tissue, whereas in the male, testicular degeneration and epididymal dysfunction are major factors (114). These phenotypes combined with deficits in sexual behavior result in infertility in both sexes of the αERKO mice. In contrast, βERKO males are fertile but demonstrate neuronal deficits and an abundance of astroglial cells; however, βERKO females exhibit inefficient ovarian function and subfertility (114, 138).

ERs are widely distributed throughout the body and display overlapping expression in a number of tissues (120, 136). ER-α is expressed in the uterus, liver, kidney and heart, whereas ER-β is expressed in the ovary, prostate, lung, gastrointestinal tract, bladder and hematopoietic cells. ER-α and β are co-expressed in the mammary glands, epididymis, thyroid, adrenal glands, bones, and in regions of the brain (120, 136). The binding of E₂ to ER induces conformational changes in the receptor that leads to dimerization, protein-DNA interaction, recruitment of co-regulators/transcription factors and ultimately the formation of a pre-initiation complex, as described in greater detail in the next section (116, 136).

Evidence is accumulating that non-genomic E₂ signaling may also be mediated by the seven-transmembrane domain G-protein coupled receptor, GPR30 (139). Filardo and colleagues identified E₂ as a natural ligand for GPR30 as E₂ activated the MAPK pathway in MCF-7 cells in the absence of the classical ERs, ER-α or -β (139, 140). Subsequent studies further implicated the novel GPR30 receptor to be directly involved in mediating cellular responses of E₂ (141-144). Transfection of GPR30 into MDA-MB-231 cells induced E₂ responsiveness in the ER-deficient cell line (140). In 2005, Thomas et al. described the specific binding of E₂ to GPR30 in transfected HEK293 with a Kₐ of
3 nM (145). This binding was eliminated by GPR30 silencing in HEK293 cells that were treated with small interfering RNA (siRNA) specific to GPR30 (145). GPR30 is now suspected to act as a scaffold, recruiting kinases for other signaling molecules that could regulate the expression of conventional ERs (140, 142, 144).

Using green fluorescent protein (GFP) chimeric construct fusing GFP to the carboxy-terminus of GPR30, GPR30 has been localized to the endoplasmic reticulum and the plasma membrane (142). GPR30 transcripts are reported to be widely expressed in humans in various tissues including heart, lung, ovary, liver and brain (140, 142, 143). In the brain, immunohistochemical (IHC) studies have revealed GPR30 is expressed throughout neurons in the hypothalamus, pituitary, hippocampus and brainstem. These studies indicate that in addition to ERs, GPR30 is an endogenous receptor of E2 and is involved in the activation and regulation of cellular physiology.

1.5.3 Signaling pathways activated by estrogen

1.5.3.1 Genomic mode of estrogen action

The genomic mechanism of E2 signaling involves E2 binding to cytosolic or nuclear ERs producing a conformational change in their AF-2 domains, which allows ER homodimerization and subsequent nuclear translocation (116, 146). In the nucleus, ER acts as a ligand-dependent transcription factor, binding with high affinity to E2 responsive elements, which are cis-acting enhancers/repressors located within the regulatory regions of target genes (136, 147) (Figure 1.3). However, subsequent studies demonstrated ERs inhibit progesterone receptor (PR) and glucocorticoid receptor (GR) activation on promoters lacking EREs (148, 149). These observations suggested ERs could form ER
Figure 1.3. Schematic illustration of estrogen signaling mechanisms.

The classical pathway includes estrogen directly binding to cytosolic ER, resulting in ER dimerization and protein-protein interactions with other transcription factors to regulate transcription. Nonclassical estrogen signaling results in the rapid activation of intracellular signaling kinases of the MAPK, PI3K, AMPK, PKA and PLC pathways. Rapid signaling cascades can occur through estrogen binding to cytosolic and membrane-bound ERs or through the GPR30 receptor localized at the endoplasmic reticulum or cellular membrane. Estrogen is freely permeable, gaining access to intracellular ERs and GPR30 receptors.
multiprotein complexes with coregulatory proteins that can bind to non-ERE promoter sites to directly regulate gene expression (116). Additional studies led to the identification of a host of coregulatory proteins that interacted with the LBD, AF-1 and AF-2 domains of ERs. One of the first ER-interacting proteins was identified after the cloning and characterization of steroid receptor coactivator-1 (SRC-1) gene (122, 150). SRC-1 was initially demonstrated to directly interact with ERs using yeast two-hybrid screening and has been shown to enhance transcriptional activities in the presence of E2 using luciferase assays. Sheppard et al. identified a conserved motif within SRC-1 called the nuclear receptor box (LXXLL; L = leucine, X = any amino acid), which is necessary and sufficient for coactivator binding to activated ERs (151). The LXXLL structural motif is also found in other ER coactivators, including TRAP220, CREB-binding protein (CBP), p300, and the activating signal cointegrators (ASC-1 and ASC-2), which can also modulate ER activity (150). Coregulatory proteins of ERs contain intrinsic histone acetylase activity (HAT), which is known to facilitate chromatin remodeling at target promoters and ER activity (122, 150). A number of ER coregulatory proteins have been identified since the original identification of SRC-1, of which several have been identified, including GRIP1, AIB1, CBP/p300, TRAP220, PGC-1, p68 RNA helicase, and SRA (152). Table 1.3 lists some of these coregulatory proteins, including their function and interaction with ERs.

The classical actions of E2 ultimately result in the regulation of a number of target genes, including matrix and structural proteins, regulatory enzymes, surface receptors, ion channels, transcription factors and peptides. Depending on the cell, promoter context, co-regulators expressed and ratio of ERα:ERβ, the DNA-bound
ERs utilize a network of coactivators and corepressors that provide a balanced and precise control of ER-mediated regulation of gene expression.

### Table 1.3. Co-activators in estrogen receptor physiology

Adapted from Hall et al. Molecular Interventions, 5; 343-357 (2005)

<table>
<thead>
<tr>
<th>AF-2 Coactivators</th>
<th>Steroid receptor coactivator-1 (SRC-1)</th>
<th>HAT</th>
<th>Binds ERs AF-2 through LXXLL motifs</th>
</tr>
</thead>
<tbody>
<tr>
<td>CREB-binding protein (CBP/p300)</td>
<td>HAT</td>
<td></td>
<td>Binds ERs AF-2 through LXXLL motifs</td>
</tr>
<tr>
<td>Thyroid hormone receptor activating protein of 220 kDa (TRAP220, TRAP/DRIP)</td>
<td>HAT</td>
<td></td>
<td>Binds ERs AF-2 through LXXLL motifs</td>
</tr>
<tr>
<td>Activating signal cointegrator-1 (ASC-1)</td>
<td>Bind HATs and NRs</td>
<td></td>
<td>Binds ERs AF-2 through</td>
</tr>
<tr>
<td>AF-1 Coactivators</td>
<td>Steroid receptor activator (SRA)</td>
<td>Splicing</td>
<td>Binds ERs AF-1</td>
</tr>
<tr>
<td>p68 RNA helicase (P68)</td>
<td>RNA helicase</td>
<td></td>
<td>Binds ERs AF-1</td>
</tr>
<tr>
<td>Protein methyltransferase 1 (CARM1)</td>
<td>Arginine histone methyltransferase</td>
<td></td>
<td>Binds ERs AF-2 indirectly through association with p160s</td>
</tr>
<tr>
<td>PPAR coactivator-1 (PGC1)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Coiled-coil coactivator (CoCoA)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Secondary Coactivators</td>
<td>E6-associated protein (E6-AP)</td>
<td>Ubiquitin ligase</td>
<td>Binds ERs AF-2</td>
</tr>
<tr>
<td></td>
<td>Receptor potentiating factor-1 (RPF-1)</td>
<td>Ubiquitin ligase</td>
<td>Binds ERs AF-2</td>
</tr>
<tr>
<td></td>
<td>Tethering surface for other cofactors; splicing</td>
<td></td>
<td>Binds the hinge region of the ERs</td>
</tr>
</tbody>
</table>
receptor exerts either a positive or negative effect on expression of the downstream target gene.

1.5.3.2 Non-genomic mode of estrogen action

E$_2$ also acts through non-genomic, rapid signaling mechanisms by binding to plasma membrane-bound or cytoplasmic ERs or GPR30, which activate signal cascades that can directly lead to cell-specific biological effects (120, 143, 146). The concept of non-classical E$_2$ signaling was originally suggested because E$_2$ could induce cellular changes that were far too rapid to be accounted for by classical E$_2$ signaling (153). This was first observed in 1967 by Szego and Davis, where E$_2$ exposure increased cAMP in the uterus of OVX mice in 15 seconds, which is considered too rapid for genomic responses of E$_2$, which often take hours for final changes in protein expression to take place (154). Additional studies found E$_2$ could bind to receptors located at the cell membrane and initiate rapid cAMP accumulation in endometrial cells (155). Since then, investigators have demonstrated that non-genomic E$_2$ signaling involves the mobilization of intracellular calcium, stimulation of adenylate cyclase activity and activation of the MAPK and PI3K signaling pathways (156, 157). The non-genomic actions of E$_2$ are mediated through functional domains of the receptor that likely interact with scaffold proteins such as the modulator of non-genomic action estrogen receptor (MNAR), caveolin-1 and proximal signaling molecules including: G proteins, striatin, p130Cas, ras, p85$\alpha$ and Shc (158-161). These ER-interacting proteins have been shown to couple ER to kinases such as Src and PI3K to mediate rapid E$_2$ activation of AKT and ERK. The activation of signal transduction pathways may then enhance the activation of downstream signaling components to ultimately elicit genomic responses. For instance,
transcription factors Elk-1, C/EBPβ and CREB are all targets for phosphorylation by the MAPK signaling pathway (151, 158-161).

Although there is an abundance of research conducted on E₂ signaling, the relative contribution of genomic and non-genomic actions to certain gene responses remains undetermined. These signaling mechanisms may occur concurrently or in series, but subsequently converge at the level of transcription. The molecular responses to E₂ are likely to vary depending upon a number of conditions, such as the differential expression of ERs, interaction of coregulatory/scaffold proteins, cell membrane or nuclear ER content, steroidal/hormonal milieu and duration of E₂ exposure.

1.5.4 Effects of estrogen on reproduction and feeding behaviour

Estrogens have been demonstrated to regulate both energy homeostasis and reproductive function in the hypothalamus (162, 163). E₂ is critical for the synthesis and secretion of GnRH, paradoxically exerting both a stimulatory and inhibitory effect on GnRH release (53, 79, 164). Estrogens act in the central nervous system to directly inhibit GnRH transcription and secretion, in both male and female mice (27). However, positive estrogen action is also required for the generation of the preovulatory LH surge in females (165). Despite the importance of estrogen action on reproductive function, the mechanisms regulating the hormonal response to estrogen are poorly understood. This is primarily due to the complex circuitry of the hypothalamus and scattered distribution of GnRH neurons in the brain (32, 166). However, carefully designed in vivo studies have shed light on the importance of estrogen in the regulation of GnRH release. In OVX rhesus monkeys and mice, exogenous E₂ administration that reflect physiological levels were sufficient to induce a GnRH surge (167). However, E₂ directly reduces GnRH expression and release in the homogenous GT1-7 cell lines, and thus, the repressive
effects of E₂ on GnRH neurons are thought to be overcome by afferent neuronal fibres stimulated by estrogen (53). Estrogens could potentially stimulate a number of afferent fibres that are intimately associated with dendrites, cell bodies or nerve terminals of GnRH neurons. Excitatory neuropeptides and neurotransmitters that have been implicated in GnRH regulation are NPY, NT, norepinephrine, glutamate and aspartate (79). Continuous and increasing estrogen exposure could enhance the synthesis and release of these stimulatory factors and may overcome the direct inhibitory effect of estrogen on GnRH neurons. As a result, estrogen is hypothesized to regulate the HPG axis through a complex cellular network that is not yet fully characterized.

Estrogens are also well-recognized negative regulators of energy homeostasis and feeding behaviour. Donohoe et al. found that subcutaneous injections of E₂ and 17α-estradiol reduced food intake in OVX rats (168). However, E₂ treatments reduced food intake significantly more compared to 17α-estradiol treatments (168). In addition, 17α-estradiol is synthesized in the brain and not the ovaries, which suggests that the OVX mice that have increased weight gain and food intake occurs through 17α-estradiol independent mechanisms (121). In fact, in OVX mice brain 17α-estradiol levels are significantly higher compared to wild type controls, suggesting E₂ may negatively regulate 17α-estradiol syntheses in the brain (121). Together, these studies have led to a larger focus on E₂ in the regulation of body weight and food intake.

Postmenopausal women display increased weight gain, visceral obesity and have an increased risk of diabetes (169, 170). E₂ replacement therapy normalizes these abnormalities. This negative effect on energy homeostasis by E₂ has also been demonstrated in rodent studies. During the estrous cycle, peak levels of E₂ observed during the afternoon of proestrous results in significantly reduced food intake (171).
Ovariectomized (OVX) rats display increases in food intake and increases in adipose tissue deposition, which can be readily reversed with the re-introduction of estrogen (20, 172). E₂ is thought to regulate energy homeostasis through two major pathways: an anorectic action through the central nervous system and a direct action on tissue metabolism (162, 173). The central action of E₂ that regulates feeding behaviour appears to be mediated through the regulation of multiple hypothalamic orexigenic and anorexigenic neuropeptides. One study found that castration of female mice resulted in a decrease in anorexigenic pro-opiomelanocortin (POMC) and CRH mRNA expression, which was normalized after E₂ injections (162). Another study found that E₂ was required for normal action of the gut derived peptides cholecystokinin (CCK) and ghrelin, the adipocyte derived hormone leptin, and the hypothalamic neuropeptide melanin-concentrating hormone (MCH) on satiety signals in the hypothalamus (174-176). Overall, E₂ has a well-characterized anorectic effect in mammals, but the mechanisms and hypothalamic targets have yet to be fully elucidated. Recent studies, however, have implicated NPY neurons as a key central target of E₂ in the hypothalamus.

1.5.5 Estrogen-mediated regulation of NPY neurons

E₂ acts as a homeostatic feedback molecule between the periphery and the brain, regulating energy balance and reproduction. The feedback mechanisms employed by E₂ could occur through the modulation of several neuropeptidergic circuits. NPY neurons of the hypothalamus have emerged as a key target of estrogen, as NPY has a potent role in regulating both reproductive function and energy homeostasis. NPY neurons express both ER-α and ER-β in vivo and in vitro (164, 177-179). Studies suggest E₂ can modulate both reproduction and feeding by regulating NPY mRNA expression in clonal, immortalized hypothalamic cell models (164, 178). Here, E₂ differentially regulated NPY mRNA
expression in two distinct NPY-expressing hypothalamic cell lines. In the mHypoE-42 NPY cell line, \( E_2 \) tonically downregulated NPY mRNA throughout a 72 h time course (164). In the mHypoE-38 NPY cell line, 8 h \( E_2 \) treatment resulted in a decrease in NPY mRNA expression, whereas a 24 h \( E_2 \) treatment in the same cell line resulted in a 4-fold increase in NPY mRNA that corresponded with increased ER-\( \beta \) mRNA levels (164). Together, this study suggests \( E_2 \) may differentially modulate NPY-expressing neurons, which may result in an anorectic and reproductive effect. Although the transcriptional regulation of \( E_2 \) on NPY-expressing cell lines has shed light on the dual role of \( E_2 \), a number of questions remain unanswered including the \( E_2 \)-mediated control of NPY secretion.

1.6 Leptin

1.6.1 Synthesis and metabolism

In 1994, Friedman and colleagues discovered and characterized an obese gene (\( ob \)) mutated in the mouse strain \( ob/ob \) (180). The \( ob \) gene was later found to encode a 4.5 kb mRNA sequence that was predominantly expressed in adipose tissue (111, 181). This 4.5 kb sequence is comprised of three exons that span 15 kb of genomic DNA. Analysis of cloned sequences upstream of the transcriptional start site revealed that the 217 bp sequence upstream of the 5’ region is required for basal leptin gene expression in adipocytes (181). The evolutionary conserved \( ob \) gene encodes for a 167 amino acid 16 kDa protein called leptin (181). Leptin plasma concentrations are directly proportional to one’s body fat or body mass index (BMI), although this can vary with gender, nutritional status and time of day (15, 182). Physiological leptin concentrations in males and females range from 4 ng/ml to 100 ng/ml, observed in lean and obese subjects, respectively (182).
1.6.2 Leptin receptors and signaling events

The gene for the leptin receptor (Ob-R) is encoded by the *db* gene and is alternatively spliced into several different receptor isoforms; one full length (isoform Ob-Rb), and several shorter isoforms spliced at the C-terminal coding exon (Ob-Ra, Ob-Rc, Ob-Rd, Ob-Re, Ob-Rf) (173, 174). These receptor proteins have identical sequences in their extracellular and transmembrane domains, and also share the first 29 amino acids of the cytoplasmic domain. However, the Ob-Rb isoform has a 302 amino acid intracytoplasmic domain that includes several motifs for protein-protein interactions (175, 176). The remaining shorter isoforms have truncated intracytoplasmic regions (173, 176). Ob-Rs are a member of the class 1 cytokine receptor family. This receptor family uses an assortment of cytoplasmic-signaling transducers to mediate changes in gene transcription and cellular events (183). Ob-Rb is predominately expressed in the hypothalamus (21, 176); however, the truncated isoforms are expressed in a wide range of tissues, including hypothalamus, thymus, heart, lung, liver, spleen, kidney, stomach and adipose tissue (184). The activation of cellular signaling cascades is thought to occur primarily through the Ob-Rb isoform, as the short leptin receptor isoform does not contain the post-receptor signaling machinery (185). The full-length Ob-Rb is known to activate the JAK-STAT cascade as a major pathway in leptin signaling. Recent studies have demonstrated continuous leptin injections result in the activation of STAT3 in hypothalamic nuclei (179). Studies in peripheral tissues have also suggested alternate leptin signaling mechanisms. Leptin has been shown to induce activation of the PI3K-phosphodiesterase type 3B-(PDE3B)-cAMP pathway, resulting in a decrease in cAMP levels in the hypothalamus (186). The MAPK family has also been proposed in leptin receptor signaling, as leptin treatments induce MAPK/ERK activity in the hypothalamus.
Most recently, AMPK, a fuel-sensing enzyme responsible for maintaining metabolic homeostasis, has been shown to play a critical role in leptin action in the hypothalamus (183, 184) (Figure 1.4). To add to the complexity of leptin signaling, several groups have identified negative regulators of leptin signaling. Specifically, suppressor of cytokine signaling (SOCS)-3, a leptin induced signaling protein, negatively regulates leptin signaling by binding to SH2 binding domains on the Ob-R (185). In addition, protein tyrosine phosphatase (PTP1B), originally identified as a negative regulator of insulin signaling, was found to also reduce leptin signaling by impairing leptin-induced JAK/STAT signaling as demonstrated in cells overexpressing PTP1B (186). The discovery of the negative regulators of leptin signaling has led scientists to hypothesize that overreactivity of the negative regulators of leptin signaling is a potential causal mechanism of leptin-resistant obesity.

The role of the short form of leptin receptors remains to be defined; however, studies have suggested the short isoforms may act as a free cytosolic binding protein for leptin, thus regulating free leptin concentrations. Despite the functional uncertainty of the short leptin receptor, the distinct tissue distribution and abundance of short leptin receptor isoforms suggests that these receptors play an important role in the biological action of leptin.
Figure 1.4. Schematic illustration of leptin receptor signaling. Adapted from Harvard and Ashford. Neuropharmacology, 44, 947-849 (2003)

Leptin binds to the Ob-R, which leads to increased activity of intracellular JAK2 kinases associated with membrane-proximal regions of the Ob-R. JAK2 phosphorylates Ob-R tyrosine residues that lead to the activation of the STAT3 and MAPK signaling pathways. PI3K is also involved in the regulation of rapid signaling kinases and membrane polarity. Through unknown mechanisms, leptin can directly regulate the activity of AMPK signaling. SOCS3 and PTP1B negatively regulate leptin signaling by binding to Ob-R tyrosine phosphorylated sites to prevent STAT3 activity and by dephosphorylating tyrosine residues, respectively.
1.6.3 Effects of leptin on feeding behaviour and reproduction

The idea of a blood-borne factor produced from fat mass that relays information to the central nervous system was originally conceptualized by Kennedy and colleagues as early as 1953 (187). This factor, leptin, was finally identified by Friedman’s group in 1994 and has since been extensively studied and characterized (180). Leptin is a blood borne signal released in proportion to fat stores that informs the hypothalamus about peripheral energy stores. Specifically, leptin has an appetite suppressing effect (181). Leptin administration centrally or peripherally decreases food intake and increases energy expenditure (182, 188). Lack of functional leptin receptors or signaling in mice results in severe obesity (111, 184). Alternatively, the administration of leptin to obese mice with defects in the ob gene (resulting in leptin insufficiency) can correct this abnormal phenotype (189, 190). Leptin also regulates energy homeostasis in the periphery by regulating muscle metabolism (191). Specifically, leptin stimulates beta-oxidation in skeletal muscle (191). In addition, ob/ob mice depict a large increase in their triglyceride content resulting in impaired beta cell function. Together, leptin regulates energy homeostasis centrally by regulating food intake and peripherally by regulating metabolic expenditure.

Adequate leptin levels are also required for normal reproductive function. The link between leptin and reproduction became apparent when ob/ob mice were reported to have a number of reproductive abnormalities (192). These mice are infertile, and similar to the obese phenotype, abnormal reproductive function can be normalized by peripheral injections of recombinant leptin (193-195). The reproductive malfunction in ob/ob mice is thought to be due to reduced gonadal steroids and reduced HPG activity (196). Prolonged and continuous leptin treatment increases uterine and ovarian weight in
females, and increases seminal vesicle and testicular weight in males (196). Leptin has also been implicated in triggering the onset of puberty. Exogenous leptin treatments result in precocious vaginal opening, an indicator for pubertal status (194). Together, the evidence available thus far suggests leptin is a key peripheral signal that is required for normal reproductive function and energetic status, as inert or absent leptin results in reproductively deficient and obese animals. Although leptin is thought to control these physiological processes through specific nuclei located in the hypothalamus, the intermediary signals between leptin, feeding and reproductive homeostasis remains unclear.

1.6.4 Leptin-mediated regulation of NPY neurons

Evidence clearly indicates that the central nervous system, particularly the hypothalamus, is a major site of leptin action (197, 198). Ob-Rb is critical for intracellular leptin signaling and has been localized to a number of hypothalamic nuclei responsible for reproductive and metabolic function, including the ARC, DMH, LHA and VMH (21, 197, 198). Two important neuropeptides, POMC and NPY, which are involved in both feeding and reproductive homeostasis, have emerged as key hypothalamic targets of leptin.

Neurons containing anorexigenic peptide-products of the POMC gene express the Ob-R and make direct synaptic connections on GnRH-expressing neurons (199). Interestingly, POMC gene expression is modified with varying leptin levels, where untreated ob/ob mice show a 50-70% decrease in POMC gene expression, which can be restored to wild-type levels with the treatment of leptin (200, 201). The most promising peptide product of POMC that may be implicated in the leptin-mediated control of feeding and reproduction is α-MSH (202). The leptin-mediated inhibitory action on food
intake was shown to be at least partially mediated through the α-MSH receptor, melanocortin type 4 (MC4) (203). However, studies using MC4 specific agonist/antagonist studies have failed to demonstrate that MC4 receptor plays a crucial role on reproductive parameters despite the apparent synaptic connection between POMC- and GnRH-expressing neurons (204). As a result, MC4 activity is suspected to regulate GnRH synthesis and release indirectly by modulating receptors and responsiveness to other key GnRH stimulators. Thus, although POMC/α-MSH plays an important role in the control of energy homeostasis, studies have yet to conclusively link MSH to reproductive function.

The orexigen NPY is a neuropeptide that also plays a dual role in regulating both feeding and reproduction in the hypothalamus. NPY can directly stimulate GnRH secretion, as demonstrated in push-pull cannulae studies in vivo and GT1-7 neuronal cell lines in vitro (45, 78, 88). NPY is also a potent orexigenic compound, as peripheral and central injections cause increased food intake (205). NPY-expressing neurons in the ARC also contain Ob-R, suggesting NPY is directly responsive to circulating leptin (198). Additionally, NPY mRNA is elevated in both fasted and ob/ob mice (206). Previous work in our lab has demonstrated that leptin can directly down-regulate NPY mRNA in hypothalamic cell lines; however, ensuing studies on whether leptin can directly regulate NPY secretion to control energy balance and reproduction have yet to be completed (164). The hypothesis that leptin can positively or negatively regulate neuropeptide synthesis of afferent neuronal populations to feeding and reproductive nuclei have yet to be verified. The use of hypothalamic cell lines will provide new models that allow the study of the regulation of these neuropeptides by critical steroids and peripheral hormones.
1.6.5 Leptin Resistance

A deficit in leptin does not underlie most cases of obesity in humans. In fact, obese individuals exhibit elevated circulating leptin levels due to an increase in adipose tissue mass (207, 208). Paradoxically, the elevated leptin level observed does not result in a decrease in feeding. As a result, this failure of high leptin levels to suppress feeding and decrease body weight suggests the presence of resistance to the anorexigenic effect of leptin (209). Although leptin has a profound effect on regulating appetite and body weight, the mechanisms of leptin resistance are not understood. To date, scientists have proposed two mechanisms of leptin resistance and are actively investigating these hypotheses (208). In the first hypothesis, impaired transport of leptin across the blood brain barrier (BBB) is suspected to result in central leptin resistance (207, 210). In support of this hypothesis is the finding that the concentration of leptin in the cerebrospinal fluid (CSF) from obese humans is not increased in proportion to their elevated serum leptin levels (207). The second and more likely hypothesis points to downstream signaling defects in hypothalamic neurons as a primary cause of leptin resistance. Munzberg et al. showed that in diet induced obese (DIO) mice (a classical mouse model prone to leptin resistance and obesity when provided an experimental high-fat diet) recombinant leptin completely failed to activate STAT3 in hypothalamic extracts, indicating leptin resistance in the hypothalamus of DIO mice (211, 212). Because Munzberg et al. used whole hypothalamic extracts, the precise neuronal populations that develop leptin resistance remain unknown. Further studies localized leptin resistance specifically to the ARC. After 16 weeks of high-fat-diet feeding, leptin-activated phospho-STAT3 staining within the ARC was dramatically decreased. This decrease in phospho-STAT3 was correlated with significantly higher SOCS3 levels in the
ARC (211). This study suggests that the ARC is selectively leptin resistant in DIO mice (compared to wild-type control) and this may be caused by elevated SOCS3 in this hypothalamic nucleus (211, 212).

The characteristics of specific neuronal populations that become leptin-resistant in the ARC are unknown. However, the specific neurons that exist in the ARC that express leptin receptors include, NPY, POMC and GALP neurons (213, 214). Based on this knowledge, additional studies should examine the individual neuronal subpopulations to determine the precise cell type and mechanisms involved in leptin resistance.

1.7 Cell models

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 presents a highly complex environment to study the direct regulation of neuropeptides and cellular events by neuromodulators (215). Additionally, the limited number of specific neuronal cell populations that are responsible for key physiological effects such as feeding and reproductive function calls for new complementary 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 these reasons, researchers have turned to immortalized, clonal, hypothalamic cell lines to generate a comprehensive picture of the molecular biology involved with the regulation of specific neuroendocrine hypothalamic neurons. Cell lines provide a homogeneous population of cells that allow for the study of the direct regulation of signaling pathways and molecular events (Figure 1.5) (216).
However, it is important to note there are limitations to the use of cell lines. The lack of complexity, afferent innervations and endogenous stimuli prevents researchers from concluding whether cell lines will respond identically to cells in vivo. Despite these limitations, cell lines can be used to further develop theories of biological function prior to completing studies in vivo. Studies reported using cell lines to date have been instrumental in several novel findings that have been replicated in vivo (53, 217). In particular, cell models created by both Belsham et al. and Mellon et al. have been instrumental in the study of neuroendocrine regulation of key hypothalamic neuropeptides (218-220).

1.7.1 GnRH-expressing GT1-7 neurons

GnRH neurons are a scarce population of neurons that are widely distributed throughout the hypothalamus (79). Because of this, there are limited studies that examine the cellular and molecular mechanisms that regulate GnRH neurons. There are now four GnRH-expressing cell models used to study these cellular events: the GT1 (218), GN (221), GnV (222) and GRT (223) cells. Each cell model was created using well-characterized oncogenes targeting tumorigenesis in GnRH-expressing neurons. Of the cell lines listed, the GT1 cells have proven to be the most similar to endogenous GnRH-expressing neurons. GT1 neurons were immortalized by directing tumorigenesis in GnRH-expressing neurons in transgenic mice through the expression of the simian virus 40 (SV40) T-antigen oncogene at the 5’ regulatory region of the GnRH gene (218). Tumors from two offspring were cultured and cloned into GT1 cells, which were further subcloned into three homogeneous cell populations labeled as GT1-1, GT1-3 and GT1-7. Most studies to date have been performed using the GT1-7 cell lines because of the high level of GnRH mRNA, expression of mature neuronal markers and classic neuronal
Figure 1.5. 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 geneticin to select for retrovirus-infected cells. 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 (219,220).
morphology (218, 224, 225). Additionally, GnRH is secreted in a pulsatile manner and responds appropriately to neurotransmitters, and intrahypothalamic injections of GT1-7 cells can rescue fertility in hypogonadal mice (hpg mouse) (226). Together, studies completed to date suggest the GT1-7 neurons are representative of GnRH neurons in vivo, and thus provide the most valid and well-characterized model to study the cellular and molecular regulation of GnRH neurons. In the third study of this thesis, the GT1-7 cell model was analyzed for the transcriptional regulation of GnRH mRNA levels in response to NPY stimulation.

1.7.2 Embryonic hypothalamic cell lines – mHypoE-xx

The GnRH-expressing cell lines described above represent only one cell type from the enormous range of cell types in the hypothalamus. Thus a number of laboratories have set out to develop hypothalamic cell lines to compensate for this lack of representation. Cell lines representative of the central nervous system have been created by subcloning tumorigenic cell populations. CNS cell lines, Neuro2A and PC12 were isolated from naturally occurring neuroblastoma and pheochromocytomas, respectively (227). However, these cell models are not truly representative of fully differentiated neurons. Other groups have utilized retroviral gene transfer of the SV40 T-antigen oncogene to mass immortalize hypothalamic cells. In 1990, Rasmussen and colleagues developed the RCF-8, RCF-12 and RCA-6 cell lines from rat embryonic hypothalamic cultures (228). However, few studies have employed these cell lines, as they have not been fully characterized. Kasckow et al. in 2003 also retrovirally transferred embryonic hypothalamic cultures with the SV40 T-antigen to produce one cell line, IVB, which was found to be a parvocellular CRH-expressing cell line (229). The lack of a representative collection of hypothalamic cells that have been thoroughly characterized for the study of
hypothalamic physiology prompted the Belsham group to generate an array of immortalized cell models from the hypothalamus (216). Using the retroviral transfer of the SV40 T-antigen to primary hypothalamic cell cultures from fetal mice on days 15, 17 and 18, Belsham et al. were able to subclone over 60 embryonic cell lines labeled as mHypoE-‘clone number’ (219). These cell lines express neuronal cell markers, neurosecretory machinery, have clearly defined perikarya and neurites and have been thoroughly characterized with over 100 neuroendocrine markers. Importantly, a number of these cell lines generated express neuropeptides linked to energy and reproductive homeostasis, providing new models to study the molecular biology of hypothalamic neurons (216, 230). In the studies presented in this thesis, two embryonic, NPY-expressing cell lines were used: mHypoE-38 and mHypoE-42. The cell lines express a complement of neuropeptides listed in Figure 3.2 and Figure 4.1.

1.7.3 Adult hypothalamic cell lines – mHypoA-xx

Because the embryonic hypothalamic cell lines generated contributed to an immense wealth of knowledge in the neuroregulation of hypothalamic neurons, the Belsham group immortalized adult hypothalamic cell models to understand key mechanisms involved in adult neuroendocrine cell types. In order to immortalize adult neurons, cells were treated with ciliary neurotrophic factor (CNTF) to induce proliferation, thus predisposing cell cultures to the retroviral transfer of the SV40 T-antigen oncogene (220). Over 50 adult mouse cell lines were established labeled as mHypoA-‘clone number’. Similar to the embryonic neuronal cells, the adult cell lines express mature neuronal markers, exhibit neuronal morphology and have been characterized for the expression of various neuropeptides and receptors. These cell lines will be key to understanding hypothalamic physiology and can be used for the direct
comparison to embryonic neuronal cell lines. Overall, the hypothalamic cell lines now made available by our lab and others, allows the study of mechanisms by which peripheral hormones and neuromodulators can regulate hypothalamic neuroactivity. In particular, I have taken advantage of these novel hypothalamic cell lines to piece together the complex circuitry and cell-mediated responses that regulate energy and reproductive homeostasis. In the studies outlined in this thesis, two adult hypothalamic cell models were used: mHypoA-2/12 and mHypoA-59. Studies were completed in the mHypoA-2/12 and mHypoA-59 as they express robust levels of NPY and hormone receptors of interest. The phenotypic characterization of these cell lines were completed and listed in Figure 3.2 and Figure 4.1.

1.7.4 NPY-GFP cell line

The Belsham group has recently devised a novel method of immortalizing NPY-expressing neurons from the adult hypothalamus. NPY-GFP transgenic mouse hypothalamii were dissected individually and immortalized as described above in section 1.7.3 (Belsham, unpublished data). Hypothalamii are from the adult transgenic mice NPY-GFP mouse (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). NPY neurons were than selected using flow cytometry. NPY-GFP cell lines have been thoroughly characterized using RT-PCR, ICC and NPY secretion assays. These cell lines will be important to understand the control mechanisms utilized by mature NPY neurons in terms of basic physiological functions and stimulus control. In this thesis, the NPY-GFP cell model is used in conjunction with other hypothalamic cell models to describe the leptin-mediated regulation of NPY secretion. The phenotypic characterization of these cell lines is described in Figure 4.2.
1.8 Hypothesis and aims

Neural networks of the hypothalamus and brainstem structures have been identified as the homeostatic control systems of food intake. This homeostatic control system is comprised of neuropeptides, neurotransmitters and hormones and is a crucial determinant of feeding behaviour. Another extensive cortico-limbic neural system, the hypothalamus-ventral tegmental-accumbens pathway, processes food intake in response to cues from the environment. This non-homeostatic mechanism of food intake is undoubtedly a powerful regulator of appetite that contributes to the obesity epidemic observed today. Importantly, in this thesis I have focused on the homeostatic mechanisms of food intake in relation to the control of reproductive function with a specific interest in neuropeptides involved in this regulation.

Severe metabolic challenges or hormonal deficits disrupt reproductive function at all three levels of the HPG axis, with reduced GnRH neuronal activity as a primary cause of reproductive impairment. To effectively synchronize reproductive effects with energy homeostasis, neuronal populations must be receptive to peripheral hormonal cues regulating these processes. Two key endocrine hormones, E\textsubscript{2} and leptin, can reduce food intake by regulating the expression and secretion of hypothalamic neuropeptides involved with feeding, and also positively regulate the reproductive system through afferent neuronal systems that synapse on GnRH neurons. NPY neuronal populations have emerged as key central targets of these hormones and may integrate both their feeding and reproductive effect. Studies completed to date demonstrate that leptin and E\textsubscript{2} treatments decrease NPY mRNA in the whole hypothalamus \textit{in vivo}. Although, these studies do not indicate whether these NPY neuronal populations directly respond to leptin and E\textsubscript{2} treatments, the NPY secretory responses to hormonal stimulation, nor do they fully elucidate the mechanisms by which this hormonal regulation occurs. Importantly, NPY released from neurons upstream of GnRH
neurons have been demonstrated to stimulate GnRH secretion *in vitro*, a suspected link between the feeding and reproductive axis. However, the transcriptional regulation of GnRH mRNA and NPY receptor(s) and signaling kinases involved remain to be determined.

It was therefore hypothesized that NPY neurons serve as integration centres to modulate the effects of estrogen and leptin on reproduction and food intake. This integration at the level of the NPY neuron is likely reflected by differential responses to these hormones with regard to NPY secretion and signaling mechanisms. These differential signaling and secretory responses should reflect the ability of these NPY neuronal populations to decrease food intake and stimulate GnRH neuronal populations.

Using well-characterized hypothalamic cell lines that endogenously express key receptors of interest and secrete basal levels of NPY and GnRH, the hypothesis was tested through 3 specific aims (Figure 1.6):

**Aim 1**: Determine the receptors, signaling mechanisms and regulation of NPY secretion by E2 in individual hypothalamic NPY-synthesizing cell lines. These results are presented in Chapter 3.

**Aim 2**: Determine the NPY secretory responses to leptin in individual NPY-secreting cell lines and elucidate potential signaling mechanisms involved. In addition, this aim will analyze the effect of prolonged leptin exposure on NPY secretory responses and intracellular signaling mechanisms activated. These results are discussed in Chapter 4.

**Aim 3**: Determine the effects of NPY on GnRH mRNA levels in the GT1-7 GnRH-expressing neurons and identify potential signaling mechanisms and receptors involved. In addition, this aim will examine the effect of conditioned media treatments from NPY-synthesizing cell lines on GnRH mRNA transcript levels. These results are described in Chapter 5.
We hypothesize that the hypothalamus is comprised of a heterogeneous population of NPY neurons, a feeding-responsive NPY neuron and reproductive-responsive NPY neuron, which are differentially regulated by estrogen and leptin to equilibrate feeding and reproductive homeostasis. Taking an in vitro approach, we examined the direct regulation of NPY neuronal cell lines by estrogen and leptin treatments. In addition, we also examined the direct regulation of GnRH mRNA transcription in response to NPY treatment in GnRH-expressing GT1-7 cell lines, indicating a potential indirect mechanism of leptin and estrogen on the reproductive axis. Together, using cell models derived from the hypothalamus, we will begin to describe the complex architecture of NPY hypothalamic circuits involved in energy homeostasis and reproductive function.

GnRH, gonadotropin-releasing hormone; NPY, neuropeptide Y.
Chapter 2

Materials and methods
2.1 Cell culture and reagents

Immortalized cells were grown in monolayer in Dulbecco’s modified Eagle medium (DMEM) (Sigma-Aldrich Inc, Oakville, ON) supplemented with 5% fetal bovine serum (FBS) (Hyclone Laboratories Inc., Logan, UT), 4.5mg/ml glucose and 1% penicillin/streptomycin and maintained at 37°C in an atmosphere of 5% CO2 21% O2 and 74% N2. GT1-7 neurons were grown in the same conditions above, except in 10% FBS. NPY was obtained from American Peptide (Sunnyvale, CA), while NPY receptor specific agonists Peptide YY, D-[Trp32]-Neuropeptide Y (human, rat), [Leu31, Pro34]-Neuropeptide Y, Neuropeptide Y (13-36), and Pancreatic Polypeptide (rat) were obtained from Bachem (Torrance, CA). 17β-Estradiol was obtained from Sigma-Aldrich. Additional pharmacological agents ER-α selective agonist propylpyrazole triol (PPT), ER-β-selective agonist diarylpropionitrile (DPN), ER-α selective antagonist methylpiperidino-pyrazole (MPP) and ER-β antagonist R,R-tetrahydrochrysene (R,R)-THC were obtained from Tocris Bioscience. β-Estradiol 6-(O-carboxy-methyl)oxime: BSA (estrogen-BSA) was obtained from Sigma-Aldrich. Leptin was obtained from the National Hormone and Peptide Program (Torrence, California). The MAPK family MEK1/2 inhibitor U0126 [1,4-Diamino-2,3-dicyano-1,4-bis(o-aminophenylmercapto) butadiene ethanolate] and PI3-K inhibitor LY294002 [2-(4-morpholinyl)-8-phenyl-1(4H)-1-benzopyran-4-one-hydrochloride] were obtained from Cell Signaling Technologies Inc. (Danvers, MA) and dissolved in dimethylsulfoxide (DMSO) (Sigma-Aldrich). The selective PKA inhibitor H89 [N-[2-(p-Bromocinnamylamino)ethyl]-5-isoquinolinesulfonamide dihydrochloride] was obtained from Sigma Aldrich and dissolved in H2O. LY294002 and U0126 were applied to neurons one hour before NPY
treatment and had final concentrations of 25 µM (optimal concentrations were determined in previous studies) (164, 178, 231). H89 was applied in the same manner as above at a final concentration of 30 µM (232). The NPY Y1 subtype receptor was blocked with 1 h, 1 µM pretreatments of the NPY Y1 antagonist, Diphenylacetyl-D-Arg-4-hydroxybenzylamide (BIBP-3226) (Bachem) (233). The AMPK inhibitor Compound C 6-[4-[2-(1-Piperidinyl)ethoxy]phenyl]-3-(4-pyridinyl)-pyrazolo[1,5-a]pyrimidine dihydrochloride was obtained from Tocris Bioscience (Ellisville, MS) and treated at a concentration of 20 µM (234).

2.2 Semi-quantitative RT-PCR

2.2.1 One step RT-PCR

Total cellular RNA was isolated using the guanidinium isothiocyantate phenol chloroform extraction method. RNA was treated with Turbo DNase (Ambion, Austin, TX) and one step RT-PCR was performed using the OneStep RT-PCR kit (Qiagen, Mississauga, ON) according to the manufacturers protocol. The primer sequences are listed in Table 2.1 along with the utilized annealing temperature.

2.2.2 Two step RT-PCR

Total RNA was isolated using the guanidinium isothiocyantate phenol chloroform extraction method. cDNA was made using the Applied Biosystems High Capacity cDNA Reverse Transcriptase Kit (Foster City, CA). For RT-PCR, Mango Taq polymerase was used according to the manufacturer’s protocol (Bioline, Taunton, MA). PCR products were electrophoresed in 2% agarose gels and stained in an ethidium bromide solution [10mg/ml ethidium bromide in 300 ml 1X TAE (Tris-acetic acid-EDTA)]. Gels were visualized under UV light and quantified by densitometry. Primer sequences used are listed in Table 2.1 along with the utilized annealing temperature.
Table 2.1. Primer sequences.

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2.3 Real-Time RT-PCR

RNA from time-course and co-culture experiments was isolated by the guanidium isothiocyanate phenol choloroform extraction method. Analysis of GnRH mRNA levels was completed using real-time RT-PCR. cDNA was made using the Applied Biosystems High Capacity cDNA Reverse Transcriptase Kit (Foster City, CA). Real-time RT-PCR reactions were performed with 100 ng of cDNA template using SYBR green PCR master mix and run on the Applied Biosystems Prism 7000 real-time PCR machine. GnRH primer sequences were as follows: GnRH SYBR sense - 5’ CGT TCA CCC CTC AGG GAT CT -3’; and SYBR anti-sense - 5’ CTC TTC AAT CAG ACT TTC CAG AGC -3’; Amplicon size – 51 bp. Gamma-actin sequences are as follows: actin SYBR sense, 5’-CTT CCC CAC GCC ATC TTG -3’ and SYBR antisense, 5’- CCC GTT CAG TCA GAT CTT CAT -3’; Amplicon size – 79 bp. Real-time RT-PCR values were calculated using the relative standard curve method and normalized to gamma-actin at the corresponding time points.

2.4 Enzyme Immunoassay

In Chapter 3, mHypoE-42 and mHypoA-2/12 neurons were grown to 90% confluency and serum starved in phenol-red free DMEM for 4 hours prior to incubation with 10 nM E$_2$, PPT, DPN, E$_2$-BSA or vehicle alone for 1 hour at 37°C. 60 mM KCl were completed for 15 minutes. KCl treatments were completed in all secretion studies as a positive control. KCl treatment results in membrane depolarization and a large influx of Ca(2+) for tens of minutes to induce exocytosis (235). In the pharmacological inhibitor and ER antagonist studies, mHypoE-42 and mHypoA-2/12 neurons were treated with vehicle or with E$_2$ (10 nM) in the presence or absence of pharmacological inhibitors directed against the PI3K, MAPK and AMPK pathways or ER specific antagonists MPP
and (R,R)-THC. Inhibitors and antagonists were applied for 1 hour prior to E$_2$ treatments. Cell suspensions were collected (in triplicate) and NPY-like immunoreactivity was measured by an enzyme immunoassay (Phoenix Pharmaceuticals, CA) according to the manufacturer’s protocol (assay sensitivity 0.09 ng/ml).

In Chapter 4, mHypoE-38, mHypoA-59 and NPY-GFP neurons were grown to 90% confluency and then serum starved in DMEM for 4 hours prior to incubation with 10 nM leptin or vehicle for 1 hour at 37°C. 60 mM KCl were completed for 15 minutes. Pharmacological inhibitors used in these experiments were applied as described above. For the leptin-resistance studies, the cells were pretreated with 10 nM leptin or vehicle for 8 or 24 hours. At time zero, the cells were washed with 1xPBS and placed in fresh serum-free medium for 2 hours. Cells were then re-challenged with leptin and media was collected 8 or 24 hours later. Cell suspensions were collected (in triplicate) and NPY-like immunoreactivity was measured by an EIA (Phoenix Pharmaceuticals, CA) according to the manufacturer’s protocol (assay sensitivity 0.09 ng/ml).

2.5 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 Hank’s balanced salt solution supplemented with 0.5 mM EDTA and 1% bovine serum albumin (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\textregistered 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 by GFP fluorescence after gating to remove cell aggregates. All FACS was completed by the Faculty of Medicine Flow Cytometry Facility, University of Toronto.

2.6 Radioactive Immunoassay

GT1-7 cells were grown to 90% confluence and cell medium was replaced with serum-free opti-MEM containing 100 nM IBMX (Sigma-Aldrich) for 4 hours prior to incubation with 100 nM NPY, 100 nM NPY receptor specific agonist, 30 μM forskolin or vehicle alone for 15 min at 37\textdegree C. To extract cAMP from the cells, 1 ml of ice-cold ethanol was added to each plate and left for 10 min. Cell suspensions were collected and centrifuged at 20,800g and 4\textdegree C for 5 min. The supernatant was divided into 100 μL aliquots, and were dried down using Labconco Centrivap DNA concentrator for 3-5 hours. cAMP levels were determined by radioimmunoassay (RIA) (Biomedical Technologies Inc, MA) according to the manufacturers protocol.

2.7 Western Blot Analysis

In Chapter 5, GT1-7 were treated with 100 nM NPY, 30 μM forskolin or vehicle alone for a 1 h time course. Cells were washed with ice-cold PBS and lysed in a high-salt buffer (20 mM Tris hCl, 150 mM NaCl, 1 mM Na\textsubscript{2}EDTA, 1 mM EGTA, 1% Triton, 2.5 mM sodium pyrophosphate, 1 mM β-glycerophosphate ,1 mM Na\textsubscript{3}VO\textsubscript{4}, 1 mM leupeptin) (Cell Signaling Technology, MA) supplemented with 1% protease cocktail inhibitor (Cell Signaling Technology) and 1 mM PMSF. Cell lysates were centrifuged at 20,800g for 10 min at 4\textdegree C and the collected supernatant was stored at -80\textdegree C. Protein concentration was determined using the bicinchonicnic acid (BCA) protein assay kit (Pierce Biotechnology,
IL). Total protein (50 µg) was resolved on a 8% SDS-PAGE and blotted onto immuno-Blot PVDF membrane (Bio-Rad Laboratories, Hercules, CA). Membranes were blocked with 5% BSA (Sigma) in Tris-buffered saline containing 0.2% Tween 20 (TBS-T), supplemented with a phosphatase inhibitor cocktail (Sigma) for 30 to 60 min and then washed 3 times with TBS-T. Blots were then incubated with primary antibodies against phospho-AKT (Ser473, 1:1000 Cell Signaling technology), phospho-ERK (Thr202/Tyr204, 1:1000: Cell Signaling Technology) phospho-CREB (Ser133, 1:1000: Cell Signaling Technology), phospho-PKA (Thr197, 1:1000: Cell Signaling Technology) or G, (1:5000: Santa Cruz Biotechnology Inc, Santa Cruz, CA) overnight at 4 °C followed by 3 washes in TBS-T and then incubated with secondary horseradish peroxidase-labeled secondary goat anti-rabbit at 1:5000 dilution for 1-2 hours at room temperature. Membranes were visualized with enhanced chemiluminescence (ECL kit, GE Healthcare, UK) on the Kodak Imager 2000R.

In Chapter 4, mHypoE-38, mHypoA-59 and NPY-GFP neurons were treated with leptin (10 nM) or vehicle alone over a 30-minute time course and protein was harvested as described above and blotted onto PVDF membrane as described above. For leptin resistance experiments, cells were pretreated with 10 nM leptin for 8 and 24 hours before leptin treatment media was changed to serum-free DMEM for 2 hours. Cells were re-challenged with 10 nM leptin and protein was isolated after 15 and 30 minutes. Blots were then incubated with primary antibodies phospho-JAK2 (Tyr1007/1008, 1:1000, Cell Signaling technology), phospho-AMPK (Thr172, 1:1000, Cell Signaling Technology), phospho-ACC (Ser79, 1:1000, Cell Signaling Technology), phospho-STAT3 (Tyr705, 1:1000, Cell Signaling Technology), phospho-ERK1/2 (Thr202/Tyr204) 1:1000, Cell Signaling Technology), phospho-JNK (Thr183/Tyr185, 1:1000, Cell Signaling
Technology), phospho-CREB (Ser133, 1:1000, Cell Signaling Technology), phospho-AKT (Ser473, 1:1000, Cell Signaling Technology), G (1:5000, Santa Cruz Biotechnology Inc, Santa Cruz, CA) or leptin receptor (SC-1834, 1:1000, Santa Cruz Biotechnology Inc) overnight at 4 °C followed by 3 washes in TBS-T. Blots were then treated as illustrated above.

For Western blot studies, phospho-proteins were normalized to the loading control G-beta. Although total protein comparisons are ideal, our group has found in previous studies that Gbeta is a reliable indicator of loading status (164, 178, 236-241).

2.8 Immunocytochemistry

In Chapter 3, mHypoE-42 and mHypoA-2/12 cells were plated on eight-well chamber slides (BD Biosciences) in DMEM overnight. Cells were fixed in 2% paraformaldehyde, blocked with 1% BSA–PBS and incubated with primary antibody overnight at 4°C. No antibody wells served as controls. The primary ER-α antibody was used at a 1:50 1% BSA–PBS dilution of mouse monoclonal anti-human for ER-α (DakoCytomation). For colocalization, primary antibody for rabbit polyclonal caveolin-1 (Santa Cruz Biotechnology) was used at a 1:50 dilution. Cells were washed with PBS and incubated with a FITC-conjugated AffiniPure goat anti-mouse secondary antibody (Jackson ImmunoResearch) in a 1:100 dilution at room temperature for 90 min. Cells were then washed with PBS and incubated with rhodamine-labeled (Texas Red) biotinylated goat anti-rabbit IgG secondary antibody (Vector Laboratories) in a 1:100 dilution at room temperature for 30 min. After washing cells with PBS, gaskets were removed from the chamber slides and mounted with DakoCytomation fluorescent mounting media. Fixed cells were then visualized with a confocal laser scanning microscope at a magnification of 400x (LSM 510; Carl Zeiss). FITC fluorescence was
excited by the 488 nm argon laser line, whereas rhodamine was excited by the 543 nm helium–neon laser line.

2.9 Co-culture

In Chapter 5, NPY-containing media from mHypoE-38 neurons grown to 80-90% confluence was harvested from 60 mM KCl or vehicle treated cells. Media were collected after treatment and run through a Zeba de-salting spin column (Thermo Scientific, IL). As a control, GT1-7 neurons were treated with KCl- or vehicle-treated medium from mHypoE-38 neurons and RNA from GT1-7 neurons was harvested after 4 hours and subjected to real-time RT-PCR, as described above.

2.10 Statistics

Data are presented as the mean ± the standard error of the mean (SEM). Data were analyzed by one- or two-way analysis of variance (ANOVA) followed by a Bonferroni multiple comparisons test or by a Student’s t-test using Graphpad Prism (Graphpad Software Inc., CA), as indicated in the figure legends, with the exception of the three-way ANOVA. Three-way ANOVAs were performed on SigmaStat version 2.01 for Windows (Jandel Scientific, San Rafael, CA). Experiments were performed on three to twelve separate occasions. Comparisons were considered statistically significant at p <0.05.
Chapter 3

17β-estradiol inhibits NPY secretion through membrane-associated estrogen receptor (ER)-α in clonal, immortalized hypothalamic neurons

Manuscript in press within the International Journal of Obesity.

Citation:
Estrogen inhibits NPY secretion through membrane-associated estrogen receptor (ER)-α in clonal, immortalized hypothalamic neurons
Dhillon SS, Belsham DD.
Int J Obes. Accepted May 12 2010
Manuscript # 2010IJO00022RR

Contributions:

• SSD completed experiments and wrote the manuscript
• DDB edited the manuscript and provided scientific input, direction and funding
3.1 Abstract

17β-estradiol (E$_2$) has an inhibitory effect on food intake by acting centrally in the hypothalamus; although it is not clear which neuronal cell types are functionally required for this effect. Previous studies from our lab and others have implicated NPY neurons as an important central anorectic target of E$_2$. The present study was designed to investigate whether E$_2$ can directly regulate NPY secretion and examine the cellular mechanisms and receptors responsible for this anorexigenic action of E$_2$. Clonal, murine, hypothalamic neuronal cell models, mHypoE-42 and mHypoA-2/12, were investigated for NPY secretory responses to E$_2$ in the presence or absence of pharmacological inhibitors directed against the PI3K, MAPK and AMPK pathways or to ER specific agonists/antagonists. E$_2$ significantly decreased NPY secretion in both the mHypoE-42 and mHypoA-2/12 neurons. The E$_2$-mediated repression of NPY secretion in the mHypoE-42 and mHypoA-2/12 neurons required ER-α, but not ER-β, as demonstrated by studies using ER-specific agonist/antagonists. Additionally, using immunocytochemistry (ICC) I detected colocalization of ER-α and the cell membrane-associated scaffold protein caveolin-1. Importantly, using E$_2$-conjugated BSA (E$_2$-BSA) and ER antagonists, I was able to demonstrate that the E$_2$-mediated decrease in NPY secretion occurred through cell membrane-bound ER-α. Finally, using a combination of pharmacological inhibitors, I found that inhibition of the PI3K or AMPK pathway blocked the E$_2$-mediated decrease in NPY secretion. These findings indicate that the central anorectic action of E$_2$ occurs at least partially through hypothalamic NPY-synthesizing neurons. This regulation of NPY secretion occurs through non-genomic signaling mechanisms and possibly through cell membrane-bound ER-α.
3.2 Introduction

E\textsubscript{2} is thought to negatively regulate energy homeostasis through two pathways: an anorectic action through the central nervous system and a direct action on tissue metabolism \((242)\). The central action of E\textsubscript{2} is hypothesized to occur through the regulation of multiple orexigenic and anorexigenic neuropeptides implicated in feeding behaviour \((243-245)\). A large body of evidence suggests NPY is involved with the E\textsubscript{2}-mediated decrease in feeding behaviour \((245)\). NPY is a potent orexigenic peptide, as the central administration of NPY stimulates feeding and repeated doses results in an increase in body weight \((107, 246)\). Interestingly, the withdrawal of E\textsubscript{2} by OVX in mice results in significantly greater levels of NPY mRNA expression in the ARC \((20, 245)\). This effect was reversed by E\textsubscript{2} administration. Additional studies found E\textsubscript{2} significantly decreased the sensitivity to the orexigenic effect of NPY \((247)\). Finally, E\textsubscript{2} treatment resulted in a decrease in NPY release from microdissected hypothalamic sites and PVN cultures \((244)\). Although E\textsubscript{2} appears to regulate NPY mRNA and release, whether E\textsubscript{2} acts directly on NPY neurons or solely through afferent neuronal connectivity to influence NPY levels remains to be determined.

The actions of E\textsubscript{2} are thought to be primarily mediated through two specific nuclear receptor isoforms, ER-\(\alpha\) and ER-\(\beta\) \((117, 125)\). Studies undertaken to delineate the ER subtype responsible for the central anorexigenic action of E\textsubscript{2} have produced conflicting data, implicating both ER-\(\alpha\) and ER-\(\beta\) as the main receptor subtype. A study of food intake and body weight demonstrated systemic injections of E\textsubscript{2} or the ER-\(\alpha\) agonist 4,4',4"-(4-propyl-[1H]-pyrazole-1,3,5-triyl)trisphenol (PPT) significantly reduced food intake, but not the ER-\(\beta\) agonist 2,3-bis(4-hydroxyphenyl)-propionitrile (DPN) \((248)\). In contrast, ICV injection of E\textsubscript{2} and anti-sense oligonucleotides (ODN) directed
against ER-β attenuated the E₂-mediated decrease in food intake, whereas ER-α ODN had no effect (249). Thus, it remains unclear whether E₂ acts through ER-α or ER-β in the hypothalamus to influence food intake. To complicate matters, recent studies have suggested NPY neurons do not express ER-α in the hypothalamus (171). In this study, I confirmed the presence of ER-α in NPY-GFP FAC-sorted cells.

The aim of the current study is to determine the secretory events, rapid signal transduction cascades and the E₂ receptor subtype responsible for the anorectic action of E₂ on NPY-expressing neurons. To examine the mechanisms by which E₂ regulates NPY within an individual NPY-expressing neuron, I used hypothalamic neuronal cell models, mHypoE-42 (219) and mHypoA-2/12 (220), recently generated by our lab. These cell lines have been thoroughly characterized, demonstrate neurosecretory properties, express neuron specific markers and have classical neuronal morphology (216, 230). In the present study, I demonstrate that 17β-estradiol (E₂) directly decreases NPY secretion in both the mHypoE-42 and mHypoA-2/12 neurons. Using pharmacological ER-α and ER-β receptor specific agonists/antagonists, I was able to determine the E₂-mediated decrease occurred via ER-α in both hypothalamic cell lines. Additionally, I detected the colocalization of ER-α and the membrane-associated signaling protein caveolin-1. I found this cell membrane-bound ER-α to be directly involved in the E₂-mediated decrease in NPY secretion using a combination of E₂-BSA and ER antagonists. Finally, I provide evidence that the PI3K and AMPK signaling pathways play an important role in the E₂ regulation of NPY secretion.
3.3 Results

3.3.1 Expression of ER-α in FAC-sorted NPY-GFP neurons

A recent study by Olofsson et al. demonstrated ER-α is not expressed by NPY neurons of the hypothalamus in vivo (171). This result is in contrast to previous studies that colocalized ER-α in NPY neurons. As a result, it is currently unclear whether NPY neurons express ER-α and are a direct target of E₂ in vivo. Thus I sought to determine whether NPY neurons express ER-α in vivo using transgenic mice that express humanized Renilla-GFP (hrGFP) driven by the NPY promoter. NPY-GFP neurons were FAC-sorted (96% accuracy in sorting for GFP fluorescence) from the hypothalamus and ER-α expression was measured/detected using RT-PCR (Figure 3.1). I confirmed the expression of ER-α in NPY neurons, which suggests E₂ may directly act on NPY neurons in vivo.

3.3.2 Expression of the ER subtypes and other hypothalamic markers in mHypoE-42 and mHypoA-2/12 neurons

The mHypoE-42 and mHypoA-2/12 neurons display neuronal morphology in culture. A partial list of markers expressed in the cell lines is presented (Figure 3.2A), including neuropeptides, receptors, and signaling molecules. RNA isolated from the mouse hypothalamus was used as a positive control. The presence of these receptors and the expression and secretion of NPY (Figure 3.2B) at appreciable levels indicates that the mHypoE-42 (NPY concentration: 0.55±0.034 ng/mL) and mHypoA-2/12 (NPY concentration: 0.77±0.027 ng/mL) neurons have the appropriate machinery to respond to E₂ and are suitable models to study the E₂ mediated regulation of NPY secretion. Media from GT1-7 neurons was used as a negative control as they do not synthesize NPY.
Figure 3.1. Expression of ER-α mRNA in NPY-GFP neurons using RT-PCR

NPY-GFP neurons were isolated from the hypothalamus of the NPY-GFP transgenic mouse using fluorescence-activated cell sorting (FACS). Hypothalamus, Hypo; no-template control, NTC. ER-α amplicon size: 344 bp.
3.3.3 Regulation of NPY secretion by E\textsubscript{2} in mHypoE-42 and mHypoA-2/12 neurons

Although evidence indicates that E\textsubscript{2} can regulate NPY mRNA and release in vivo, it is not yet known whether this regulation can occur directly at the level of the NPY neuron (164, 178). mHypoE-42 and mHypoA-2/12 neurons were exposed to E\textsubscript{2} (10 nM) for 1 h. This dose was found to be the optimal treatment concentration in our cell models in previous studies (164). As a control to ensure accurate NPY measurements, cells were treated with the well-characterized depolarizing agent, KCl. 60 mM KCl treatments resulted in a ~1.5-fold increase in NPY secretion in both mHypoE-42 and mHypoA-2/12 neurons. Analysis of the results in the embryonic mHypoE-42 neurons indicates that NPY secretion was significantly reduced by E\textsubscript{2} (vehicle, 1.02±0.085; E\textsubscript{2}, 0.83±0.10) (Figure 3.3A). Similarly, in the adult hypothalamic cells, mHypoA-2/12, NPY secretion was significantly inhibited by E\textsubscript{2} exposure (vehicle, 1.09±0.0381; E\textsubscript{2}, 0.78±0.052) (Figure 3.3B).

3.3.4 E\textsubscript{2}-mediated regulation of NPY secretion is dependent on ER-\(\alpha\) in the mHypoE-42 and mHypoA-2/12 neurons

I next examined the role of ER-\(\alpha\) and ER-\(\beta\) in the E\textsubscript{2}-mediated down-regulation of NPY secretion. The effects of vehicle, E\textsubscript{2}, ER-\(\alpha\) selective agonist PPT, ER-\(\beta\)-selective agonist DPN, ER-\(\alpha\) selective antagonist MPP and ER-\(\beta\) antagonist (R,R)-THC on NPY secretion are shown in Figure 3.4. In the mHypoE-42 neurons, 1 h 10 nM PPT treatment (0.72±0.033) reduced NPY secretion compared to vehicle control (1.0±0.034) (Figure 3.4A). In agreement with the embryonic cell lines, the mHypoA-2/12 PPT treatment group (0.61±0.11) had a significant reduction in NPY secretion when compared to the
Figure 3.3. Estrogen directly decreases NPY secretion in mHypoE-42 and mHypo-A2/12 neurons.

mHypoE-42 and mHypoA-2/12 neurons were serum-starved for 4 h before treatment with 10 nM E2 for 1 h, vehicle for 1 h or 60 mM KCl for 15 min. Cell culture medium was then collected and assayed for NPY-like immunoreactivity by an EIA. Absolute secretion values for the mHypoE-42 and mHypoA-2/12 cells are 0.55 ng/ml and 0.77 ng/ml, respectively. Results shown are relative to control (set to 1.0) and expressed as mean +/- SEM (n = 9 independent experiments, each in triplicate); *, p<0.05 versus control, as per one-way ANOVA with Bonferroni’s post-hoc test.
vehicle control group (1.0±0.059) (Figure 3.4B). In contrast to PPT treatments, the ER-β agonist DPN failed to elicite any changes in NPY secretion in both cell lines. Additionally, ER-α antagonist MPP (1 µM) blocked the E2-mediated decrease in NPY secretion in both the mHypoE-42 (E2, 0.83±0.10; MPP+E2, 1.02±0.074) (Figure 3.4C) and mHypoA-2/12 (E2, 0.78±0.052; MPP+ E2, 0.98±0.042) (Figure 3.4D) neurons. Conversely, in the presence of the ER-β antagonist (R,R)-THC (1 µM), E2 still reduced NPY secretion in both the mHypoE-42 ((R,R)-THC, 0.97±0.0179; (R,R)-THC+E2, 0.81±0.11) and the mHypoA-2/12 ((R,R)-THC, 1.03±0.19; (R,R)-THC+E2, 0.72±0.084) (Figure 3.4E and 3.4F). Importantly, ER antagonist treatments alone did not significantly change NPY release levels compared to controls. ER agonist/antagonist concentrations used in this study are optimal treatment concentrations established in previous studies (136, 248, 250, 251). These results indicate that activation of ER-α mediates the effects of E2 on the repression of NPY secretion.

3.3.5 E2 decreases NPY secretion via membrane-bound ER-α

There is some evidence that classical nuclear ER-α can localize to the membrane and lead to the activation of intracellular signaling cascades (non-genomic E2 signaling) (116, 117, 158). I attempted to detect ER-α at the level of the cell membrane by colocalizing ER-α with the cell membrane protein caveolin-1. Using ICC with ER-α-and caveolin-1-specific antibodies, I found ER-α could be colocalized at the cell membrane (Figure 3.5). In order to implicate the membrane bound ER-α in the E2-mediated decrease in NPY secretion, I used cell membrane impermeable E2-BSA in the presence of ER antagonists MPP and (R,R)-THC. Interestingly, I observed that E2-BSA failed to decrease NPY secretion in the presence of the ER-α antagonist, MPP (1 µM), in both embryonic (MPP+E2-BSA, 1.01±0.032) (Figure 3.6A) and adult (MPP+E2-BSA,
Figure 3.4. Estrogen attenuates NPY secretion via ER-α in mHypoE-42 and mHypoA-2/12 neurons.

(A) mHypoE-42 and (B) mHypoA-2/12 cells were serum-starved for 4 h before treatment with 10 nM estrogen (E2), 10 nM ER-α agonist PPT, 10 nM ER-β agonist DPN or vehicle alone for 1 h. (n=6 independent experiments for agonist treatments). (C) mHypoE-42 and (D) mHypoA-2/12 neurons were serum-starved for 4 h before treatment with 10 nM E2 or vehicle alone for 1 h. Cells were pretreated with the ER-α antagonist MPP for 1 hour at a concentration of 1 μM. (n=3 independent experiments for antagonist treatments). (E) mHypoE-42 and (F) mHypoA-2/12 neurons were serum-starved for 4 h before treatment with 10 nM E2 or vehicle alone for 1 h. Cells were pretreated with the ER-β antagonist, (R,R)-THC for 1 h at a concentration of 1 μM (n=3 independent experiments for antagonist treatments). For all experiments above, media was collected in triplicate and assayed for NPY-like immunoreactivity by EIA. Results are shown relative to corresponding control NPY levels (set to 1.0) and expressed as mean +/- SEM. (n=9 independent experiments for E2 and vehicle treatments). *p<0.05 versus control, as per one-way ANOVA with Bonferroni’s post-hoc test.
0.98±0.042) (Figure 3.6B) hypothalamic cell lines. However, E2-BSA decreased NPY secretion in the presence of the ER-β antagonist (R,R)-THC (1 µM) in the mHypoE-42 (((R,R)-THC, 0.97±0.0179; (R,R)-THC+E2-BSA, 0.82±0.0808) (Figure 3.6C) and the mHypoA-2/12 (((R,R)-THC, 1.03±0.019; (R,R)-THC+E2-BSA, 0.88±0.082) (Figure 3.6D) cell lines. Therefore, it is evident that membrane-bound ER-α mediates the effect of E2 on NPY secretion.

3.3.6 Inhibition of the PI3K and AMPK pathways affect the E2-mediated regulation of NPY secretion

I next determined whether the regulation of NPY by E2 is dependent on non-genomic signal transduction pathways, MAPK, PI3K and AMPK, using pharmacological inhibitors specific to these pathways. Neurons were treated with or without E2 (10 nM) in the presence or absence of pharmacological inhibitors LY294002 (25 µM), UO126 (25 µM) or Compound C (20 µM). As shown in Figure 3.7A and 3.7B, the E2-mediated decrease in NPY was blocked in the presence of the AMPK inhibitor, Compound C dihydrochloride, in both the mHypoE-42 (E2, 0.83±0.10; Compound C+E2, 1.05±0.075) and the mHypoA-2/12 (E2, 0.78±0.052; Compound C+E2, 1.03±0.030) neurons. Next, I found E2 co-treatment with the PI3K inhibitor, LY294002, also prevented the E2-mediated decrease in NPY secretion in both the embryonic (E2, 0.83±0.10; LY294002+E2, 0.98±0.059) and adult (E2, 0.78±0.052; LY294002+E2, 1.09±0.018) cell lines. In addition, I found that U0126 treatment, a MEK1/2 inhibitor, attenuated the E2-mediated decrease in NPY secretion, but this effect was not found to be statistically significant. Importantly, the pharmacological inhibitor treatments alone had no affect on basal NPY secretory levels. Together, our data indicates that activation of the AMPK and
Figure 3.5. ER-α can be localized at the cell membrane with caveolin-1 protein in the mHypoE-42 and mHypoA-2/12 neurons.

(A) Confocal laser scanning microscopy of cells labeled with ER-α–FITC or caveolin-1–rhodamine (Cav-Rhod). Colocalization of the ER-α–FITC (green) and caveolin–rhodamine (red) fluorescence was observed in the merge (yellow) of the two images. Negative control cells were in the absence of primary antibody (Ab) and resulted in minimal staining. Cells were visualized with a confocal laser-scanning microscope at a magnification of 400x. FITC fluorescence was excited by the 488 nm argon laser line, whereas rhodamine was excited by the 543 nm helium–neon laser line.
Figure 3.6. ER-α localized at the cell membrane is required for the estrogen-mediated decrease in the mHypoE-42 and mHypoA-2/12 neurons.

(A) mHypoE-42 and (B) mHypoA-2/12 neurons were serum starved for 4 h before treatment with 10 nM E2-BSA or vehicle alone for 1 h. Cells were pretreated with the ER-α antagonist MPP for 1 h at a concentration of 1 μM. (C) mHypoE-42 and (D) mHypoA-2/12 neurons were serum starved for 4 h before treatment with 10 nM E2-BSA or vehicle alone for 1 h. Cells were pretreated with the ER-β antagonist, (R,R)-THC for 1 h at a concentration of 1 μM. For all secretion experiments above, media was collected in triplicate and assayed for NPY-like immunoreactivity by EIA. Results are shown relative to corresponding control NPY levels (set to 1.0) and expressed as mean +/- SEM. (n=3 independent experiments for antagonist treatments and n=9 independent experiments for E2 and vehicle treatments). *p<0.05, **p<0.01 versus control, as per two-way ANOVA with Bonferroni’s post-hoc test.
Figure 3.7. PI3K inhibitor LY294002 and AMPK inhibitor Compound C attenuates NPY-mediated regulation of NPY secretion in mHypoE-42 and mHypoA-2/12 neurons.

mHypoE-42 and mHypoA-2/12 neurons were serum starved for 4 h before treatment with 10 nM E2 or vehicle alone for 1 h. Cells were pretreated with the 25 μM PI3K LY294002 (LY), 25 μM MAPK MEK1/2 inhibitor U0126 or 20 μM AMPK inhibitor compound C (C) for 45-60 min. NPY secretion was analyzed using an NPY-specific EIA. Results are shown relative to corresponding control NPY levels (set to 1.0) and expressed as mean +/- SEM. (n=3 independent experiments for inhibitor experiments and n=9 for E2 and vehicle treatments). *P<0.05 versus control, as per two-way ANOVA with Bonferroni’s post-hoc test.
PI3K pathways are critical for E$_2$ to decrease NPY secretion in mHypoE-42 and mHypoA-2/12 neurons.

3.4 Discussion

E$_2$ is a well-known negative regulator of energy balance, acting peripherally to increase metabolic activity and centrally to reduce food intake. The experiments completed in this study were inspired by previous work from our lab that demonstrated E$_2$ decreased NPY mRNA expression in the mHypoE-38 and mHypoE-42 neurons (164, 178). Interestingly, the NPY mRNA response to E$_2$ treatment observed in the neuronal cell lines depended on the ratio of ER-$\alpha$ and ER-$\beta$. Where ER-$\beta$ was linked to the induction of NPY mRNA expression by E$_2$ and ER-$\alpha$ was responsible for the reduction in NPY mRNA (164). Additional studies using pharmacological inhibitors found the E$_2$-mediated decrease in NPY mRNA observed at 8 hours required both the PI3K and MAPK pathway (178). Our findings extend these studies by providing the first evidence that E$_2$ exposure can rapidly regulate NPY secretion. I confirmed the expression of ER-$\alpha$ in NPY neurons using FAC-sorted NPY-GFP neurons. Next, using clonal, immortalized, hypothalamic, NPY-expressing cell lines I investigated the direct regulation of the orexigenic neuropeptide NPY as a central target for E$_2$. One hour E$_2$ exposure in both the embryonic mHypoE-42 and adult mHypoA-2/12 hypothalamic cell lines resulted in a ~20% decrease in NPY secretion. I propose the E$_2$-mediated decrease in NPY occurs through ER-$\alpha$, and not ER-$\beta$. Further, I found that ER-$\alpha$ can be localized to the cell membrane in our hypothalamic cell lines, and that this membrane-bound receptor was responsible for the reduction in NPY secretion. Finally, through the use of pharmacological inhibitors, I show that the PI3K and AMPK pathways are necessary for
the E₂-mediated decrease in NPY secretion. This study adds to the growing list of evidence that E₂ can regulate NPY levels to modulate feeding behaviour.

E₂ acts a homeostatic feedback molecule between the periphery and the brain regulating reproduction and energy homeostasis (162, 217). E₂ has a verified central anorectic role to suppress hyperphagia, as ICV E₂ treatment reduces food intake in OVX mice (172, 252, 253). This anorexigenic effect could occur through the modulation of several orexigenic and anorexigenic signals. One study found that the castration of female mice resulted in a decrease in gene expression of the anorexigenic neuropeptides POMC and CRH, which normalized after 12 and 24-hour E₂ injections (245, 254). Another study found E₂ was required for normal action of the gut-derived peptides CCK and ghrelin, the adipocyte-derived hormone leptin, and hypothalamic neuropeptide MCH on satiety signaling in the hypothalamus (175, 176, 247). NPY can be added to the growing list of neuropeptides and hormones that are regulated by E₂. OVX mice have increased NPY mRNA levels in the ARC and E₂ treatment in the PVN results in a decrease in NPY release (20, 244). Finally, using hypothalamic neuronal cell lines, our lab has previously shown E₂ can directly decrease NPY mRNA as a potential anorexigenic mechanism of E₂ in the hypothalamus (164). Our study corroborates these gene expression studies and provides evidence that E₂ exposure to hypothalamic embryonic and adult cell lines can rapidly decrease NPY secretion. Our findings present the first demonstration that the anorexigenic action of E₂ can act directly through hypothalamic NPY-synthesizing neurons by reducing NPY release.

ER-α and ER-β are widely distributed throughout the hypothalamus, with both receptor subtypes present in the ARC and POA (14, 16). Although ER-α and ER-β show considerable homology in their DBD and carboxyl-terminal LBD domains, they are
products of different genes and studies have indicated that each ER may have a unique physiological role in a tissue- and cell context-dependent manner (122). Studies to date have produced conflicting evidence in identifying the ER that is largely responsible for the central anorectic role of E$_2$. One report demonstrated the inhibitory effect of E$_2$ on feeding was blocked with the ICV administration of ODN directed against ER-β, but not ODN directed against ER-α (249). However, the ER-α knockout mice display an obese phenotype with increased food intake and decreased energy expenditure, implicating the ER-α subtype in the regulation of feeding (173, 176). Conversely, ER-β knockout mice display normal energy intake and expenditure (114). Additional studies found the ER-α agonist, PPT, but not ER-β agonist, DPN, reduced total food intake in OVX rats (255). Previous studies in our lab found ER-α to be critical in the decrease in NPY mRNA; however, changes in mRNA expression may not translate into changes in protein content (164). To examine which ER subtype is responsible for the E$_2$-mediated reduction in NPY secretion, I treated our neuronal cell lines with ER agonists PPT and DPN, and ER antagonists MPP and (R,R)-THC. I found the ER-α agonist PPT significantly decreased NPY secretion in both the mHypoE-42 and mHypoA-2/12 neurons, whereas DPN failed to invoke a change in NPY secretion. These results were further corroborated by the results of the ER antagonist studies. The ER-α antagonist MPP blocked the E$_2$-mediated decrease in NPY secretion, whereas the ER-β antagonist (R,R)-THC, did not. Although I do not implicate ER-β in the regulation of NPY secretion, it does not rule out the possibility that ER-β can regulate other key feeding-related hypothalamic neuropeptides. Accordingly, these studies propose that E$_2$ invokes an anorectic response by decreasing NPY directly via ER-α in the NPY cell models.
Recent studies have shown E\textsubscript{2} can mediate non-genomic signaling cascades through a subpopulation of classical ERs located at the plasma membrane (256, 257). Caveolae are thought to facilitate E\textsubscript{2} signal transduction by providing a location for numerous signaling proteins (258). In this study, I was able to co-localize ER-\(\alpha\) to the cell membrane invagination scaffold protein caveolin-1, suggesting E\textsubscript{2} can activate non-genomic membrane bound signaling kinases. In order to investigate whether membrane bound ER-\(\alpha\) plays a role in the regulation of NPY secretion, I examined the effect of the cell membrane-impermeable E\textsubscript{2}-BSA in the presence and absence of ER antagonists on NPY release. Here, I demonstrated a similar ER-\(\alpha\)-dependent decrease in NPY secretion, indicating E\textsubscript{2} attenuates NPY release through membrane-bound ER-\(\alpha\). These studies parallel previous work that supports ER-\(\alpha\) as the anorexigenic target of E\textsubscript{2} in the hypothalamus and present the first line of evidence of the necessary role of membrane bound ER-\(\alpha\) in directly regulating NPY levels.

To date, E\textsubscript{2} has been reported to activate a number of intracellular signaling cascades through the interaction of various scaffold proteins in a tissue- and cell-specific manner, including: 1) the mobilization of intracellular calcium, 2) the activation of adenylate cyclase and cAMP synthesis, 3) the MAPK pathway and finally 4) the PI3K pathway (116, 146, 256, 259). Interestingly, previous gene expression studies completed in our lab directly linked the PI3K and MAPK pathways to the E\textsubscript{2}-mediated decrease in NPY gene expression (164). I therefore used pharmacological inhibitors against these pathways to determine their role in the E\textsubscript{2}-mediated regulation of NPY secretion. I found that the 1 hour E\textsubscript{2}-mediated decrease in NPY secretion was significantly attenuated with the PI3K inhibitor, but not the MAPK inhibitor. However, co-treatment of E\textsubscript{2} and the MAPK inhibitor failed to reduce NPY secretion to levels comparable to E\textsubscript{2} treatment
alone, suggesting the MAPK pathway may play some role in this effect. I next assessed the role of a novel fuel-sensing enzyme responsible for maintaining metabolic homeostasis, AMPK. AMPK is involved in regulating a number of feeding-related neuropeptides and is a well-characterized anorectic signaling target of leptin in the hypothalamus \((260, 261)\). I therefore hypothesized that \(E_2\) may also exert its anorectic decrease in NPY secretion via the AMPK signaling pathway. To assess the potential role of AMPK in mediating the decrease in NPY secretion by \(E_2\), I co-treated the mHypoE-42 and mHypoA-2/12 neurons with \(E_2\) and the pharmacological inhibitor Compound C. I found inhibition of the AMPK pathway was sufficient to block the \(E_2\)-mediated repression of NPY release. These results support the hypothesis that AMPK is required for the anorectic action of \(E_2\) on the NPY neuron. Taken together, these data outline the potential signaling mechanisms that contribute to the reduction in NPY secretion by \(E_2\).

Considering that the mHypoE-42 and mHypoA-2/12 cell lines are neurons taken from the entire hypothalamus, I can only speculate on the precise nuclei of the hypothalamus from which each clonal cell line originated. To date, a number of feeding-related hypothalamic nuclei have been implicated in the \(E_2\)-mediated decrease in food intake. Kalra and colleagues \((7)\) have shown the anorectic action of \(E_2\) may be mediated through NPY neurons in the PVN of the hypothalamus, although other areas of the hypothalamus, especially the ARC where the vast majority of NPY-synthesizing neurons are located, can not be ruled out from these studies. Other rodent studies have found \(E_2\) decreases food intake via the VMN \((252)\). Interestingly, the POA has been shown to be important in the anorectic action of \(E_2\) in the rat, although the POA is mainly comprised of GnRH, NT and CRH-expressing neurons \((254)\). In order to speculate where the mHypoE-42 and mHypoA-2/12 neurons originated from \textit{in vivo}, I reviewed published
data that reported the expression of specific neuropeptides and receptors in these feeding-related hypothalamic nuclei. I found that: 1) in situ hybridization studies implicate ER-β to be the predominant receptor of the PVN and ER-α to be the predominant receptor in the VMH, suggesting the mHypoE-42 and mHypoA-2/12 neurons did not originate from the either of these nuclei (14, 16); 2) 95% of neurons that co-express AgRP/NPY are localized to the ARC (246); 3) The POA does not express NPY (254); and 4) ER-α, ER-β and the leptin receptor (Ob-R) are expressed abundantly in the ARC in vivo. Taken together, the ARC is the only hypothalamic nuclei known to express ER-α, ER-β, NPY, AgRP and Ob-R. Overall, these observations suggest that the hypothalamic anorectic target of E₂ may be NPY-synthesizing neurons that reside in the ARC nucleus and additional studies in vivo could confirm this (Table 3.1).

Intact E₂ signaling is essential for the maintenance of energy homeostasis. Delineating the hypothalamic targets and signal transduction cascades of E₂ is therefore critical for our understanding of E₂-related feeding disorders. Our studies demonstrate novel non-genomic mechanisms by which E₂ can directly regulate the release of the most potent orexigenic feeding-related peptide, NPY, in embryonic and adult hypothalamic cell lines. I have found that E₂ can rapidly reduce NPY secretion within 1 hour of exposure through neurons likely located in the ARC nucleus. Furthermore, I demonstrate that this decrease in NPY secretion is mediated through non-genomic E₂ signaling via ER-α localized at the cell membrane. Specifically, I found that E₂ directly reduces NPY secretion through a PI3K and AMPK dependent mechanism. These findings provide new information towards our understanding of the central role of E₂ that may be involved in the anorexigenic activity of this steroid hormone (see Figure 3.8).
Table 3.1. Expression of ER, NPY, Ob-R and AgRP in hypothalamic nuclei.

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<th>PVN</th>
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Legend: mRNA expression of neuropeptides and receptors in hypothalamic nuclei. PVN, paraventricular nucleus; VMN, ventromedial nucleus; mPOA, medial preoptic nucleus; ARC, arcuate nucleus. Listed in the table is the presence (+) or absence (-) of specific genes. ER, estrogen receptor; NPY, neuropeptide Y; Ob-R, leptin receptor; AgRP, agouti-related peptide.
Figure 3.8. Model of the potential cellular signaling pathways involved in estrogen regulation of NPY secretion.

Previous experiments completed by the Belsham group using siRNA directed against ER-α and ER-ß found that estrogen-mediated repression of NPY mRNA levels required both ER-α and ER-ß or ER-α alone. We have extended these studies and directly linked NPY secretory responses to estrogen treatments. Using receptor specific agonists/antagonists and E2-BSA, we found membrane-bound ER-α was responsible for the repressive effect of estrogen on NPY secretion. Additionally, pharmacological inhibitor studies found this effect to occur through the PI3K and AMPK signaling pathways.
Chapter 4

Leptin differentially regulates NPY secretion in NPY-expressing hypothalamic cell lines through distinct intracellular signal transduction pathways

Manuscript is in preparation.

Citation:
Leptin directly decreases NPY secretion via AMPK- and PI3K-dependent mechanisms that is blocked after induction of leptin resistance in hypothalamic NPY neuronal cell models.
Dhillon SS, Centeno ML, Kim GL, Belsham DD.
To be submitted June 2010.

Contributions:

• SSD completed experiments and wrote the manuscript
• MLC immortalized and characterized NPY-GFP cell line
• GLK characterized the NPY-GFP cell line (RT-PCR)
• DDB edited the manuscript and provided scientific input, direction and funding
4.1 Abstract

Leptin acts as a key peripheral hormone in the hypothalamus regulating food intake and metabolism. Leptin also conveys metabolic information to the reproductive axis through unknown mechanisms. Accumulating evidence shows that NPY neurons are involved in mediating the anorexigenic and reproductive effects of leptin via hypothalamic neuronal circuits. To determine the molecular and cellular basis for this dual role of leptin, I determined the ability of leptin to differentially regulate NPY neurons in vitro. mHypoE-38, mHypoE-42, mHypoA-59 and NPY-GFP cell lines were characterized for leptin receptors, neuronal markers and NPY secretion using RT-PCR and EIA. In the mHypoE-38 neurons, 1 h leptin treatment directly increased NPY secretion, and this effect was directly linked to the MAPK and PI3K signaling pathways using pharmacological inhibitors. Treatment with leptin increased the phosphorylation status of Jak2, JNK and CREB as shown by Western blot analysis. In the mHypoE-42 neurons, leptin failed to change NPY secretion. However, in the mHypoA-59 and NPY-GFP neurons, leptin decreased NPY secretion, which was linked to the AMPK and PI3K pathways. Western blot analysis further demonstrated that leptin reduced the phosphorylation status of AMPK in these two cell models. Additionally, AMPK activation via (aminomimidazole carboxamide ribonucleotide) AICAR treatment directly increased NPY secretion, highlighting the importance of AMPK activity in NPY neuronal function. Prolonged leptin exposure in the NPY-GFP cells prevented leptin-induced changes in AMPK phosphorylation and reductions in NPY secretion, indicating NPY neurons are susceptible to leptin resistance. This is the first demonstration that leptin can regulate individual NPY neuronal cell models through distinct intracellular signaling
pathways and secretory responses, which may serve as a metabolic signal for food intake and a stimulatory signal for the reproductive axis.

4.2 Introduction

Leptin, an adipocyte-derived hormone and product of the *ob* gene, acts on its receptor in the hypothalamus to reduce appetite and body weight (188, 189). Several studies have demonstrated this anorexigenic role of leptin, as mutations in the *ob* gene (*ob/ob* mice) or leptin receptor (*db/db* mice) are associated with morbid obesity, and both central and peripheral administration of leptin decreases food intake in a number of species (184). Although this leptin-induced reduction in appetite has been found to occur through hypothalamic circuitry comprised of both orexigenic (i.e. NPY) and anorexigenic (i.e. POMC) peptides, the mechanisms involved have yet to be defined (6, 7, 262). In addition to the role of leptin in energy homeostasis, leptin has recently been recognized as a key regulator of the reproductive axis (196, 204). This reproductive effect of leptin was first observed in *ob/ob* mice, which have lower levels of LH and are infertile, a phenotype that can be reversed with leptin administration (195). Conversely, both transgenic mice overexpressing leptin and the peripheral administration of leptin results in accelerated puberty (193). This action of leptin on the reproductive axis is believed to occur through the GnRH neuron, as leptin administration elevates GnRH levels *in vivo* (263). However, immunohistochemistry (IHC) studies have demonstrated that Ob-R are not present in GnRH-expressing neurons in the rat hypothalamus, indicating that leptin may indirectly stimulate GnRH activity through interneurons synapsing on GnRH-expressing neurons (264). Although the anorexigenic and reproductive effects of leptin have been well described, the identity of leptin-responsive target cells that reduce appetite and stimulate the HPG axis remains unclear.
NPY neurons have emerged as a key target of leptin in the hypothalamus, as NPY neurons within the ARC express Ob-R mRNA and protein (6, 265). NPY is a potent orexigenic peptide that stimulates feeding upon central administration and increases body weight after repeated doses (107). NPY has also been acknowledged for many years as a major regulator of the reproductive axis. Morphological evidence indicates co-localization of NPY receptors on GnRH neurons and studies in vivo and in vitro have shown that NPY stimulates GnRH secretion (45, 70, 266). Given the dual role of NPY as an orexigenic and reproductive signal, NPY neurons may act as an intermediary target of leptin to regulate both the metabolic and reproductive axis. Although ICV leptin administration results in a decrease in NPY mRNA levels in the whole hypothalamus, the direct effect of leptin on individual NPY neurons and the signaling mechanisms through which leptin acts have yet to be documented.

To directly study the effects of leptin on NPY neuronal function, I used hypothalamic NPY-expressing neuronal cell models; mHypoE-38, mHypoE-42, mHypoA-59 and NPY-GFP, which were immortalized using the retroviral transfer of primary hypothalamic cell culture with SV40 T-antigen (219, 220). These cell lines have been thoroughly characterized, demonstrate neurosecretory properties, express neuron specific markers and have classical neuronal morphology (216). In the present study, I hypothesized that NPY neuronal cell lines can be differentially regulated by leptin to regulate the pleiotropic neuroendocrine responses of leptin. Based on this evidence, I propose that leptin stimulates reproductive NPY neuronal cell lines that would impinge on GnRH-expressing neurons to stimulate the reproductive axis, and inhibits feeding-related NPY neuronal cell lines to reduce food intake. I have found that leptin exposure to the mHypoE-38 cell lines stimulated NPY secretion through a MAPK- and PI3K-
dependent mechanism. However, in the mHypoA-59 and NPY-GFP cell lines, leptin directly decreased NPY secretion via AMPK- and PI3K-dependent mechanisms. AMPK phosphorylation was inhibited with leptin exposure in these two cell lines. Additionally, prolonged leptin exposure prevented the leptin-mediated decrease in AMPK phosphorylation and NPY secretion in the NPY-GFP cell line, suggesting NPY neuronal populations are susceptible to leptin resistance. These data indicate that leptin can differentially regulate NPY neuronal cell lines through the activation of intrinsically different pathways. This differential leptin regulation of NPY may act to stimulate the reproductive axis and reduce food intake, thereby providing a mechanism by which leptin controls both of these important physiological processes.

4.3 Results

4.3.1 Expression of the Ob-R and other markers in mHypoE-38, mHypoE-42, mHypoA-59 and NPY-GFP neurons

mHypoE-38, mHypoE-42 and mHypoA-59 cells were immortalized as previously described. NPY-GFP cells were immortalized and then FAC-sorted by GFP fluorescence. Dividing cells were retrovirally infected with the SV40 T-antigen cDNA sequence. NPY-GFP cell lines were probed for NPY and GFP protein using specific antibodies and visualized using ICC to confirm cell phenotype (Figure 4.1A). Cell lines were further characterized for neuronal markers, receptors and neuropeptides (Figure 4.1B). The cells secrete NPY as detected by EIA (Figure 4.2A-D).
Figure 4.1. Characterization of NPY-expressing hypothalamic cell models.

(A) The immortalized hypothalamic cultures obtained from the NPY-GFP mouse express GFP and NPY, as determined by ICC, confirming that the mixed cultures contain immortalized NPY-GFP neurons. (B) RNA was isolated from hypothalamus, mHypoE-38, mHypoE-42, mHypoA-59 and NPY-GFP cell lines and analyzed using semi-quantitative RT-PCR. Listed in the table is the presence (+) or absence (-) of specific genes. T-Ag, T-antigen; NSE, neuron-specific enolase; GFAP, glial fibrillary acidic protein; NPY, neuropeptide Y; AgRP, agouti-related peptide; POMC, proopiomelanocortin; ER, estrogen receptor; Ob-Rb, leptin receptor long form.

mHypoE-38 and mHypoE-42 RT-PCR data has been previously published by Titolo et al. Mol Endocrinol. 2006
4.3.2 Regulation of NPY secretion by leptin in mHypoE-38, mHypoE-42, mHypoA-59 and NPY-GFP neurons

Although evidence indicates that leptin can regulate NPY mRNA \textit{in vivo}, it is not known whether leptin can directly regulate NPY secretion (265). mHypoE-38, mHypoE-42, mHypoA-59 and NPY-GFP neurons were exposed to leptin (10 nM) for 1 h. KCl treatments resulted in a ~1.4-fold increase in NPY secretion in all cell lines. Analysis of the results indicates that in the mHypoA-59 and NPY-GFP neurons, NPY secretion is reduced by leptin (mHypoA-59: vehicle, 1.0±0.06: leptin, 0.78±0.03) (NPY-GFP: vehicle 1±0.14: leptin, 0.8±0.04) (Figure 4.2A,B). In the mHypoE-42 neurons, leptin treatments failed to produce a change in NPY secretion compared to vehicle control (Figure 4.2C). On the other hand, NPY secretion in mHypoE-38 neurons increased in response to leptin treatment (vehicle, 1.0±0.07: leptin, 1.21±0.06) (Figure 4.2D). When comparing the cell lines, it is evident that leptin can differentially regulate NPY secretion, indicating an inherent difference in phenotype between the cell lines.

4.3.3 Leptin increases NPY secretion in the mHypoE-38 neurons via PI3K and MAPK pathways

Leptin has been reported to act through the PI3K, AMPK, MAPK and JAK/STAT pathways in the whole hypothalamus (213). In an attempt to delineate the molecular mechanisms responsible for the differential regulation of NPY secretion by leptin, I utilized Western blot analysis to examine the activity of key signaling kinases in the mHypoE-38, mHypoA-59 and NPY-GFP neurons. The present and subsequent studies were not completed using the mHypoE-42 neurons due the failure of leptin to elicit a
Figure 4.2. Leptin directly regulates NPY secretion in NPY-expressing hypothalamic cell lines.

NPY-GFP, mHypoA-59, mHypoE-42 and mHypoE-38 neurons were serum-starved for 4 h before treatment with 10 nM leptin for 1 h, vehicle for 1 h or 60 mM KCl for 15 min. Cell culture medium was then collected and assayed for NPY-like immunoreactivity by an EIA. Results shown are relative to control (set to 1.0) and expressed as mean +/- SEM (n=6 independent experiments for the mHypoE-42 cells and n = 9 independent experiments for all other cell lines, each completed in triplicate). *, p<0.05 versus H2O control, as per one-way ANOVA with Bonferroni’s post-hoc test.
change in NPY secretion. In the mHypoE-38 neurons, leptin significantly induced the phosphorylation of Jak2 (Figure 4.3A), CREB (Figure 4.3B) and JNK (Figure 4.3C). Leptin failed to change the phosphorylation status of AMPK in the mHypoE-38 cells. To investigate the signaling kinases directly implicated in the leptin-mediated increase in NPY secretion, pharmacological inhibitors directed against PI3K (LY294002), MAPK (U0126) and AMPK (Compound C) pathways were applied for 1 h prior to 1 h leptin treatments. I observed that when mHypoE-38 neurons were co-treated with leptin and the PI3K or MAPK inhibitors, the leptin-mediated increase in NPY secretion was significantly attenuated (Figure 4.3D). The inhibitors alone had no effect on basal levels of NPY secretion. These results indicate that in mHypoE-38 neurons, leptin acts through the MAPK and PI3K pathways to directly increase NPY secretion.

4.3.4 Leptin decreases NPY secretion in the mHypoA-59 and NPY-GFP neurons via AMPK and PI3K pathways

Accumulating evidence shows that NPY neurons are involved in the anorexigenic action of leptin (6, 200, 267, 268). To determine the signaling mechanisms activated by leptin in the NPY-GFP (Figure 4.4) and mHypoA-59 (Figure 4.5) cell lines, I used Western blot analysis to analyze phosphorylation activity of key signaling kinases. Leptin transiently decreased phospho-AMPK, indicating AMPK inhibition. To determine whether the PI3K, MAPK or AMPK pathways are required for the leptin-mediated decrease in NPY secretion, mHypoA-59 and NPY-GFP neurons were pre-treated with inhibitors for 1 h, followed by a 1 h leptin co-treatment.
Figure 4.3. Leptin increases NPY secretion in the mHypoE-38 cell line via PI3K and MAPK pathways.

mHypoE-38 neurons were serum starved for 4 h before treatment with 10 nM leptin or vehicle alone over a 30 min time course. At the indicated time points, cell lysates were harvested and subjected to SDS-PAGE. Western blot analysis was performed with enhanced chemiluminescence using phospho-specific antibodies directed against (A) JAK2 (B) CREB (C) JNK. Results shown are relative to corresponding control protein levels (set to 1.0) and expressed as mean +/- SEM. Phospho-protein is normalized to G-beta. (D) mHypoE-38 neurons were serum starved for 4 h and then pretreated (1 h) with MEK inhibitor (25 μM U0126), PI3K inhibitor (25 μM LY294002), AMPK inhibitor (20 μM Dorsomorphin), or with vehicle alone prior to treatment with leptin (10 nM) (+) or vehicle (-) for 1 h. Cell culture medium was then collected and assayed for NPY-like immunoreactivity by EIA. Results shown are mean ± SEM (n ≥ 3 independent experiments each in triplicate) *, p < 0.05 versus control, as per two-way ANOVA with Bonferroni’s post-hoc test.
I found that inhibiting either AMPK with Compound C or the PI3K pathway with LY294002 prevented the leptin-mediated decrease in NPY secretion. These data provide evidence that shows that AMPK and PI3K pathways are important mediators of the anorexigenic action of leptin in the putative feeding-related NPY cell lines mHypoA-59 and NPY-GFP.

4.3.5 AICAR directly stimulates NPY secretion in mHypoA-59 and NPY-GFP neurons

Treatment with AICAR activates AMPK activity in numerous cell types, including neuronal cells (269, 270). I analyzed the effect of AICAR on NPY secretion in the presence and absence of leptin in the mHypoA-59 and NPY-GFP neurons (Figure 4.6). AICAR alone directly increased NPY secretion in the mHypoA-59 and NPY-GFP neurons. Together, these results indicate that AMPK activity is critical for NPY secretory release and provides a potential mechanism by which orexigenic compounds (i.e. ghrelin) can stimulate NPY secretion.

4.3.6 Leptin pre-treatment attenuates leptin-mediated phosphorylation of AMPK in NPY-GFP neurons

High fat diets (HFD) or hyperleptinemia often result in impaired leptin signaling, a state defined as leptin resistance. To determine the effects of prolonged leptin exposure on neuronal signaling, I pre-treated NPY-GFP neurons with leptin for 8 and 24 h. Following leptin pre-treatment, cells were washed with PBS, placed in fresh medium for 2 h and then re-challenged with leptin to determine neuronal responsiveness. Using Western blot analysis, I determined that a 24 h leptin pre-treatment prevented the leptin-induced
Figure 4.4. Leptin decreases NPY secretion in the NPY-GFP cell line via AMPK and PI3K pathways.

(A) NPY-GFP neurons were serum starved for 4 h before treatment with 10 nM leptin or vehicle alone over a 30 min time course. At the indicated time points, cell lysates were harvested and subjected to SDS-PAGE. Western blot analysis was performed with enhanced chemiluminescence using phospho-specific antibodies directed against (A) AMPK (n=4 independent experiments). Results shown are relative to corresponding control protein levels (set to 1.0). Phospho-protein is normalized to Gβ. (B,C) NPY-GFP neurons were serum starved for 4 h and then pretreated (1 h) with AMPK inhibitor (20 μM Compound C (C)), PI3K inhibitor (25 μM LY294002 (LY)) or with vehicle alone prior to treatment with leptin (10 nM) for 1 h. Cell culture medium was then collected in triplicate and assayed for NPY-like immunoreactivity by EIA. Results shown for all experiments are expressed as the mean ± SEM (n=3 independent experiments for inhibitor experiments and n=9 independent experiments for leptin and vehicle treatments); *, p < 0.05 versus control, as per two-way ANOVA with Bonferroni’s post-hoc test.
Figure 4.5. Leptin decreases NPY secretion in the mHypoA-59 cell line via AMPK and PI3K pathways.

(A) mHypoA-59 neurons were serum starved for 4 h before treatment with 10 nM leptin or vehicle alone over a 30 min time course. At the indicated time points, cell lysates were harvested and subjected to SDS-PAGE. Western blot analysis was performed with enhanced chemiluminescence using phospho-specific antibodies directed against (A) AMPK (n=4 independent experiments). Results shown are relative to corresponding control protein levels (set to 1.0). Phospho-protein is normalized to G-β. (B,C) mHypoA-59 neurons were serum starved for 4 h and then pretreated (1 h) with AMPK inhibitor (20 μM Compound C (C)), PI3K inhibitor (25 μM LY294002 (LY)) or with vehicle alone prior to treatment with leptin (10 nM) for 1 h. Cell culture medium was then collected in triplicate and assayed for NPY-like immunoreactivity by EIA. Results shown for all experiments are expressed as the mean ± SEM (n=3 independent experiments for inhibitor experiments and n=9 independent experiments for leptin and vehicle treatments); *, p < 0.05 versus control, as per two-way ANOVA with Bonferroni’s post-hoc test.
Figure 4.6. AICAR increases NPY secretion in the NPY-GFP and mHypoA-59 cell lines.

(A) NPY-GFP and (B) mHypoA-59 neurons were serum starved for 4 h and then treated with AMPK activator AICAR (0.1, 1, 10 mM) or with vehicle alone for 1 h. (C) NPY-GFP and (D) mHypoA-59 neurons were pre-treated with AICAR (1 mM) followed by a 1 h co-treatment of AICAR and leptin (L). Cell culture medium was then collected and assayed for NPY-like immunoreactivity by EIA. Results shown for all experiments are expressed as the mean ± SEM (n=3 independent experiments, each in triplicate); *, p < 0.05 versus control, as per one-way ANOVA with Bonferroni’s post-hoc test.
reduction in phospho-AMPK (Figure 4.7). However, 8 or 24 h leptin pre-treatment prevented leptin signaling. These data indicate that the endogenous leptin signaling in a NPY cell model is impaired with prolonged leptin exposure.

4.3.7 Leptin pre-treatment attenuates the leptin-mediated decrease in NPY secretion in NPY-GFP neurons

To address whether impaired leptin signaling induced from prolonged leptin exposure can prevent the leptin-mediated reduction in NPY secretion, NPY-GFP cells were pre-treated with leptin for 8 or 24 h. Following leptin pre-treatment, cells were washed with PBS, placed in fresh medium for 2 h and then re-challenged with leptin for 1 h. Media were collected and NPY immunoreactivity was measured using an enzyme immunoassay. I found that 8 and 24 h leptin pre-treatment prevented the leptin-mediated decrease in NPY (Figure 4.8). These results suggest that leptin responsiveness decreases in NPY neurons after experiencing hyperleptinemic conditions, and is consistent with the possibility that cellular leptin resistance occurs at least partially through hypothalamic NPY neuronal subtypes.

4.4 Discussion

The hypothalamus is a key site for the integration of both central and peripheral endocrine signals involved in circadian rhythms, thermogenesis, satiety and reproduction (215, 216). The diverse functions of the hypothalamus are controlled by a number of heterogeneous, specialized cell populations. Recent evidence strongly supports the notion that key peripheral hormones may differentially regulate specific hypothalamic cell populations. For instance, NPY and POMC neurons respond differently to leptin
Figure 4.7. Prolonged leptin exposure prevents the leptin-mediated decrease in phospho-AMPK in the NPY-GFP cell line.

NPY-GFP cells were pre-treated with 10 nM leptin or vehicle for 8 or 24 h, then washed and placed in fresh media. Two hours after treatment removal, the cells were rechallenged with 10 nM leptin (black bar) or vehicle (white bar). Relative phospho-AMPK was measured using Western blot analysis and normalized to Gβ as a loading control. Data are shown as mean +/- SEM (n=3-6 independent experiments).*p<0.05, as compared to the time-matched vehicle control and analyzed with three-way ANOVA with a Bonferroni’s post-hoc test.
Figure 4.8. Prolonged leptin exposure prevents the leptin-mediated decrease in NPY secretion in the NPY-GFP cell line.

NPY-GFP cells were pre-treated with 10 nM leptin or vehicle for 8 or 24 h, then washed and placed in fresh media. Two hours after treatment removal, the cells were rechallenged with 10 nM leptin (black bar) or vehicle (white bar) and NPY secretion was analyzed using an EIA. Data are shown as mean +/- SEM (n=3-9 independent experiments). *p<0.05, as per three-way ANOVA with Bonferroni’s post-hoc test.
treatments in the LHA: leptin induces SOCS-3 mRNA in NPY neurons, but induces both SOCS-3 and Fos expression in POMC neurons (6). Interestingly, individual NPY neurons may also respond differently to endocrine signals, depending on the function, location and efferent projections of the neuron. Titolo et al. demonstrated that under the same E2 treatment, two separate NPY neuronal cell lines show distinct responses in NPY mRNA expression (164, 178). This differential response to E2 was found to be dependent on the ratio of ER-α to ER-β and demonstrates that different subpopulations of NPY neurons respond uniquely to E2, and in doing so, may convey opposing messages to their respective feeding and reproductive projections (164, 178). I hypothesized that NPY neuronal cell lines may be differentially regulated by leptin, which in turn would regulate multiple neuroendocrine processes. Specifically, I propose that 1) the reproductive role of leptin may act through a specific subpopulation of Ob-Rb-containing NPY neurons to achieve the GnRH pulse generator/surge, and 2) the anorexigenic role of leptin may act by inhibiting NPY neuropetidergic circuitry that project to feeding-related hypothalamic nuclei. Studying this hypothesis in vivo would be exceptionally difficult; thus, I have taken an in vitro approach to demonstrate that individual NPY cell lines can be differentially regulated by leptin treatment. Leptin treatment resulted in an increase in NPY secretion in the mHypoE-38 neurons. Interestingly, NPY-containing conditioned media treatments from the mHypoE-38 neurons induced an increase in GnRH mRNA levels from the GT1-7 neurons as discussed in Chapter 5. Thus, the increase in NPY peptide induced by leptin in the mHypoE-38 neurons may act on GnRH neurons to stimulate the reproductive axis. Conversely, in the mHypoA-59 and NPY-GFP neurons, leptin decreased NPY secretion, which would contribute to an anorexigenic effect of leptin. These results suggest a novel mechanism in which circulating leptin can act on
different hypothalamic NPY subpopulations to regulate both metabolic and reproductive functions.

Cell-based studies and *in vivo* experiments have led to a relatively detailed understanding of the intracellular signaling pathways activated by leptin within the hypothalamus in relation to specific biological function (7, 15, 82, 241). Bates *et al.* determined that the hypothalamic control of reproduction by leptin occurs through STAT3 independent mechanisms (271). In addition, indirect evidence implicated a reproductive role of the MAPK pathway in leptin action, as tyrosine 985 mutations, a key amino acid phosphorylation site that leads to the activation of the MAPK pathway, leads to infertility (271, 272). Studies have yet to examine whether the PI3K pathway is involved in the reproductive effect of leptin. Moreover, the individual signaling pathways implicated in the role of leptin in reproductive function have yet to be attributed to a specific neuronal population(s). I have found that leptin directly increases NPY secretion in the mHypoE-38 neurons. This increase in NPY secretion may stimulate the reproductive axis through downstream efferent projections to GnRH neurons. Interestingly, the leptin-mediated increase in NPY secretion was dependent on the MAPK pathway, which was previously demonstrated to be directly linked to the reproductive role of leptin *in vivo* (272). For the first time, our studies also implicate the PI3K pathway in the leptin-mediated increase in NPY secretion, as demonstrated by inhibitor studies using LY294002. Together, these data implicate the MAPK and PI3K pathways in the leptin-mediated increase in NPY neuronal activity in NPY-expressing mHypoE-38 cell lines, which may in turn act to stimulate the reproductive axis through GnRH neurons.

Leptin also activates multiple non-genomic signaling pathways that are likely critical in the release of hypothalamic neuropeptides to regulate appetite. Leptin activates
STAT3 proteins that bind to the phosphorylated 1138 tyrosine residue sites on Ob-Rb, which are key signaling molecules in POMC neurons (201, 273). However, deletion of STAT3 from NPY neurons does not alter NPY mRNA expression responses to leptin in vivo (271, 273). PI3K stimulation by leptin has been observed in cultured hypothalamic cells, and inhibition of hypothalamic PI3K activity in mice prevents leptin-mediated decreases in appetite (274-276). Interestingly, in hypothalamic slices, leptin appears to elicit cell depolarization via PI3K activity in POMC neurons; in contrast, leptin withdrawal activates the PI3K pathway in NPY neurons, suggesting leptin hyperpolarizes NPY neurons via PI3K-dependent mechanisms (277, 278). In fact, leptin has previously been demonstrated to inhibit hypothalamic cell lines by hyperpolarization via K$_{ATP}$- and PI3K-dependent mechanisms (276, 279). Most recently, considerable leptin research has focused on AMPK, an energy sensor activated by an increasing ratio of AMP:ATP in multiple cell types (270, 280). AMPK phosphorylates and inactivates ACC, a key enzyme in fatty acid synthesis (281, 282). Leptin has been demonstrated to inhibit AMPK activity in multiple regions of the hypothalamus (283). In addition, inhibition of hypothalamic AMPK is sufficient to reduce appetite and weight gain (191, 281). Taken together, leptin is hypothesized to stimulate ACC activity by inhibiting AMPK, ultimately decreasing food intake (283). Although the anorectic signaling mechanisms of leptin in the whole hypothalamus or specific hypothalamic nuclei have been studied, it is unclear which cell types leptin is acting through and whether leptin directly or indirectly regulates these neuronal cell types. In our studies, leptin directly decreased the phosphorylation status of AMPK, suggesting an increase in ACC activity in our NPY neuronal cell models; mHypoA-59 and NPY-GFP. Through inhibitor analysis, I demonstrate that both the AMPK and PI3K pathways are required for the leptin-mediated
reduction in NPY secretion. Conversely, stimulating AMPK activity through AICAR treatment directly stimulated NPY secretion. Together, this is the first study indicating that leptin directly decreases NPY secretion in NPY neuronal cell lines via AMPK- and PI3K-dependent mechanisms, which would ultimately reduce food intake and appetite in vivo.

Studies document that obese individuals with high levels of leptin undergo a failure to suppress feeding and decrease body weight (208, 209, 284). This suggests that hyperleptinemia may cause leptin resistance. To date, four mechanisms of leptin resistance have been hypothesized: 1) impaired leptin transport across the blood brain barrier (BBB) (207), 2) alterations in leptin signaling (284), 3) perturbations in developmental programming (212) and 4) increased Ob-R degradation (183, 285). Although each of these mechanisms may contribute to the entirety of leptin resistance, I examined the effect of prolonged leptin exposure on leptin signaling and leptin regulation of NPY secretion in the NPY-GFP neuronal cell line. I found that impaired leptin signaling might contribute to the leptin resistant state, as prolonged leptin exposure (8 and 24 h) prevented the leptin-mediated decrease in AMPK phosphorylation. In addition to attenuated leptin signaling in the NPY-GFP cell line, sustained leptin treatments also prevented the leptin-mediated decrease in NPY secretion. These results support the notion of neuronal resistance to prolonged hyperleptinemia and indicate for the first time that defective AMPK signaling and impaired NPY secretion may contribute to the leptin resistant state.

I used three cell lines obtained from mice during different stages of development for the studies in Chapter 4. The cell lines contain both clonal and mixed neuronal cells. The use of cell lines from both embryonic and adult sources could conceivably produce a
differential response to hormonal stimulants in vitro, since hormones such as leptin impose different physiological roles during different times of development (286, 287). However, our characterization of these cell lines demonstrates that these cell models are functionally similar to intact adult hypothalamic neurons. Importantly, the characterization of these cell lines for specific receptors and neuropeptides has allowed us to speculate on the nuclei from which these NPY neuronal cell lines are derived from in vivo.

The mHypoE-38 cell line that I propose is upstream of GnRH neurons express markers indicative of ARC origins. The expression of ER-α, ER-β, NPY and AgRP all suggest that this cell line is from the ARC, as ICC studies have identified that these markers are co-expressed almost exclusively in the ARC (17, 69, 163, 288, 289). Importantly, these cell lines do not express tyrosine hydroxylase (TH), a marker of NPY neurons that reside outside the ARC (290). In addition, IHC and retrograde analysis have found that 49% of NPY fibres that innervate GnRH neurons originate from the ARC (88, 291). Together, these studies are consistent with the notion that the mHypoE-38 cell line is derived from an ARC NPY neuronal population.

The adult mHypoA-59 neuronal cell line also displays markers that suggest an ARC origin, including ER-α, ER-β, NPY, AgRP and Ob-R (17, 69, 163, 288, 289). Even though both the hypothalamic cell lines seem to originate from the ARC, NPY neurons of the ARC may be functionally heterogeneous. Anatomical evidence now exists for a population of NPY neurons that also express the inhibitory neurotransmitter GABA in the dorsomedial part of the ARC, whereas a subset of non-GABAergic NPY cells exists in the ventral ARC (292). After additional research characterizes these cell lines and identifies bona fide markers specific to each NPY neuronal cell line, future studies could
be completed \textit{in vivo}. Finally, NPY-GFP cell lines represent a mixed population of NPY neurons from the entire hypothalamus, and thus these cells represent multiple nuclei. The leptin-mediated decrease in NPY secretion corroborates \textit{in vivo} studies that demonstrate that leptin treatments result in a decrease in NPY mRNA in the intact hypothalamus \cite{265,293}. Together, our studies using a combination of embryonic, adult, clonal and mixed NPY neuronal populations has allowed for a detailed mechanistic understanding of the regulation of NPY secretory responses to leptin.

Based on our findings that leptin differentially regulates intracellular signaling cascades and NPY secretory responses in NPY-synthesizing cell lines, I postulate that leptin-mediated increases in NPY secretion is a possible indirect mechanism by which leptin can exert a positive effect on the reproductive axis. However, leptin can also down-regulate NPY secretion from NPY neuronal cell lines, which may achieve the anorexigenic effect of leptin in the hypothalamus. During our experiments, prolonged leptin exposure impaired AMPK signaling and was accompanied by an impaired NPY secretory response to leptin. This finding implicates NPY neurons in the development of central leptin resistance. Additionally, leptin failed to alter NPY secretion in the mHypoE-42 neurons, suggesting that leptin does not affect all NPY-producing neurons. These data support the hypothesis that hypothalamic NPY neurons add another level of heterogeneity to the hypothalamus. Because the four cell lines responded to leptin differentially, and have a distinctive markers expressed, it seems likely that they come from distinct NPY neuronal subpopulations in the hypothalamus. The studies therefore provide a novel mechanism of leptin action in specific cell populations responsible for reproductive function and energy homeostasis (see \textbf{Figure 4.9}).

Figure 4.9. Model of the potential cellular signaling pathways involved in leptin regulation of NPY secretion.

Previous studies have demonstrated leptin can decrease NPY mRNA and phospho-AMPK levels in the whole hypothalamus. We have found that leptin can directly decrease NPY secretion from NPY-expressing neuronal cell lines, NPY-GFP and mHypoA-59, via the PI3K- and AMPK-dependent pathways. Prolonged leptin exposure resulted in impaired leptin action in the NPY-GFP cell line, with altered NPY secretory and phospho-AMPK responses. In contrast, in the mHypoE-38 cell line, leptin directly increased NPY secretion through MAPK- and PI3K-dependent pathways. This is the first demonstration that leptin can differentially regulate intracellular signaling and secretory responses in NPY neuronal cell lines.
Chapter 5

Neuropeptide Y induces gonadotropin-releasing hormone gene expression directly and through conditioned medium from mHypoE-38 NPY neurons.

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Contributions:

- SSD completed experiments and wrote the manuscript
- SG completed experiments for figure 6 and edited the manuscript
- DDB edited the manuscript and provided scientific input, direction and funding
5.1 Abstract

NPY regulates reproductive function at the level of the hypothalamus through control of GnRH secretion. However, the direct control of GnRH gene expression by NPY has not yet been studied. GT1-7 neurons were treated with 100 nM of NPY over a 36 h time-course. GnRH mRNA levels were significantly increased by NPY up to 12 h. I determined that GT1-7 neurons expressed Y1, Y2, and Y4 NPY receptors, but not Y5. Functional analysis of NPY receptor activation indicated that the Y1/Y4/Y5 receptor agonist [Leu31, Pro34] significantly induced cAMP accumulation in the GT1-7 neurons. Western blot studies demonstrated changes in the phosphorylation status of AKT, ERK1/2, CREB and ATF-1 after NPY exposure. Pharmacological inhibitors of the MAPK and PKA signal transduction pathways attenuated the NPY-mediated increase in GnRH transcription. This NPY-mediated increase in GnRH mRNA was also inhibited with the Y1-receptor specific antagonist BIBP-3326. The mHypoE-38 neurons secrete detectable levels of NPY and can be used to study the effect of NPY in the presence of other hypothalamic neuropeptides secreted. Conditioned medium from mHypoE-38 neurons induced an increase in GnRH mRNA, which was inhibited by the Y1 receptor antagonist BIBP-3326. Our findings highlight the importance of NPY in the transcriptional regulation of the GnRH gene and identify the receptors and signaling pathways required for the stimulation of the reproductive axis.

5.2 Introduction

NPY has been acknowledged for many years as a major afferent regulator of reproductive function (12, 13, 45, 112, 294). GnRH is a key decapetide that sits at the pinnacle of the HPG, and is released from a small population of neurons. NPY neurons in the ARC project to GnRH cell bodies in the POA and to GnRH pre-synaptic terminals in
the ME (70, 295, 296). Additionally, morphological evidence indicates co-localization of NPY receptors and GnRH neurons (70). This neuroanatomical evidence for connections between NPY and GnRH neurons establishes a possible mechanism by which NPY influences the reproductive axis. Several studies both in vivo and in vitro have shown NPY stimulates GnRH secretion (44, 45, 72, 88, 106). In ewes, NPY infusion into the third ventricle increases GnRH secretion substantially in the ME (74). Additionally, GT1-7 neurons exposed to NPY significantly increased GnRH secretion (45). Further studies demonstrated NPY-KO mice are not capable of generating a normal LH surge, a necessary stage of the estrous cycle (13). However, depending upon the steroidal environment and species, NPY can also downregulate the reproductive axis through GnRH (75, 76, 297-299). NPY injections into the third ventricle in OVX rats led to a reduction in plasma leutinizing hormone (LH) (300). Chronic NPY administration inhibited gonadotropin secretion and sexual function in intact female rats (297, 301). In OVX rabbits, NPY perfusion significantly decreases mean levels of GnRH. However, similar NPY perfusion stimulated mean levels of GnRH in intact rabbits (77). Overall, the physiological role of NPY on the reproductive axis remains unclear, with both stimulatory and inhibitory effects published depending on the steroidal milieu. Although a number of studies have investigated the role of NPY on GnRH secretion, the effect of NPY on GnRH neurons at the transcriptional level in the absence of steroid hormones has not been elucidated.

In the present study, I performed a series of experiments aimed to determine the direct effects of NPY on GnRH expression and define the underlying signaling mechanisms in the absence of steroidal modulators. These studies are difficult to perform in the intact animal as GnRH neurons receive input from many afferent neuronal cell
types (302). To directly study the effects of NPY on GnRH expression, I used two cell models: the clonal, GT1-7 GnRH-expressing neurons immortalized through targeted tumorigenesis (218); and the clonal, hypothalamic NPY-expressing neurons, mHypoE-38, immortalized using the retroviral transfer of a primary hypothalamic cell culture with simian virus 40 T-antigen (219). Both cell lines have been thoroughly characterized, and demonstrate neurosecretory properties, express neuron specific markers and have classical neuronal morphology (164, 219). I demonstrate that both commercial NPY peptide and NPY from mHypoE-38 (formerly called N-38) neurons increase GnRH expression. I further examined the cellular mechanisms that are involved and second messenger pathways. Finally, I demonstrate NPY induces GnRH gene expression, which may be involved in the realization of the preovulatory surge.

5.3 Results

5.3.1 Expression of NPY receptor subtypes in GT1-7 neurons and hypothalamic markers in mHypoE-38 neurons

The exact NPY receptor subtypes expressed in GT1-7 neurons has not been confirmed. However there is evidence that GnRH neurons in vivo and in vitro express some of the known NPY receptor subtypes (264). The presence of NPY receptor subtypes Y1, Y2 and Y4 mRNA was detected in GT1-7 neurons using RT-PCR; however, the Y5 subtype was not found to be expressed (Figure 5.1). Mouse hypothalamic RNA was used as a positive control as it confirms that the primers are specific for all receptor subtypes. The presence of these receptors indicates that GT1-7 neurons are sensitive to NPY and are therefore an appropriate model to study NPY-mediated regulation of GnRH mRNA expression. mHypoE-38 neurons were found to express an extensive list of neuropeptides, receptors and enzymes characteristic of neuroendocrine NPY
hypothalamic neurons (164, 219). Notably, the mHypoE-38 neurons express and secrete NPY at appreciable levels and are therefore an appropriate model to study the effects of NPY in the presence of other hypothalamic neuropeptides secreted by these hypothalamic neurons on GnRH mRNA expression in GT1-7 neurons.

5.3.2 Regulation of GnRH mRNA expression by NPY in GT1-7 neurons

There is some evidence that NPY upregulates GnRH gene expression in vivo (303); however, the direct transcriptional regulation of GnRH mRNA by NPY has not yet been studied in detail. Time course studies revealed that 100 nM NPY exposure prompted an increase in GnRH mRNA levels throughout the 36 h time course. GnRH mRNA was significantly up-regulated at the 2, 4 and 12 h time points compared to time matched controls (Figure 5.2A). Although all time points reveal an increase in GnRH mRNA expression, the 4 h time point was subsequently used thereafter as the standard NPY treatment time. A dose-response experiment was subsequently carried out where GT1-7 neurons were treated with 0.1 nM, 1 nM, 10 nM or 100 nM NPY for 4 h (Figure 5.2B). All treatment concentrations resulted in an increase in GnRH mRNA levels compared to vehicle control. However, the 100 nM NPY treatment concentration resulted in the most robust response observed. These findings demonstrate that NPY substantially increases GnRH mRNA expression in the GT1-7 GnRH neuron.

5.3.3 Effect of NPY receptor agonists on cAMP activity

NPY potently binds with differing affinities to a family of G-protein coupled receptors; Y1-Y5, that belong to the rhodopsin-like superfamily of receptors. To distinguish the precise receptor subtype(s) activated by NPY in the GnRH GT1-7 neuron,
Figure 5.1. Expression of NPY Y1, Y2 and Y4 receptor mRNA transcripts in GT1-7 neurons.

RNA harvested from GT1-7 neurons was used as a template for RT-PCR with primers specifically designed to amplify NPY receptor subtypes. Fragment sizes were NPY Y1- 481 bp, NPY Y2- 236 bp, NPY Y4- 530 bp, and NPY Y5- 291 bp. NTC, No template control. Hypothalamus was used as a positive control for all receptor subtypes.
Figure 5.2. NPY-mediated regulation of GnRH gene expression in GT1-7 neurons.

(A) GT1-7 cells were serum-starved for 2 h before treatment with 100 nM NPY or with vehicle alone over a 36 h time course; and (B) GT1-7 cells were serum-starved for 2 h before treatment with 0.1 nM, 1 nM, 10 nM, 100 nM NPY or with vehicle alone over a 4 h time course. Cell lysates were harvested and subjected to real-time RT-PCR to analyze GnRH mRNA expression. Results shown are relative to corresponding control mRNA levels (set to 1.0) and expressed as mean +/- SEM (n=4-5 independent experiments). GnRH is normalized to internal control gamma actin. (a) and *p<0.05, (b) p<0.01, (c) p<0.001 versus control, as per two-way ANOVA with Bonferroni’s post-hoc test (A) and one-way ANOVA with Bonferroni’s post-hoc test (B).
specific NPY receptor subtype agonists were administered for 15 min and cAMP synthesis, an indicator of GPCR activation was measured. As a control for cAMP synthesis and dependability of RIA measurements, GT1-7 neurons were treated with the well-characterized adenylyl cyclase activator, forskolin. 30 uM forskolin treatment resulted in a 2.5-fold increase in cAMP immunoreactivity compared to vehicle control (Figure 5.3). NPY (100 nM) and the Y1/Y4/Y5 receptor agonist (100 nM) [Leu\textsuperscript{31}, Pro\textsuperscript{34}] potently increased cAMP activity in GT1-7 neurons. Conversely, cAMP activity was not significantly changed when compared to vehicle control upon treatment with 100 nM of the Y2 selective agonist NPY\textsubscript{13-36}, Y4 selective agonist rat Pancreatic Polypeptide (rPP) or the Y5 agonist D-[Trp\textsuperscript{32}]. Thus, the cAMP sensitivity to the selective Y1/Y4 agonist [Leu\textsuperscript{31}, Pro\textsuperscript{34}] highlights the importance of the Y1 and potentially Y4 receptors in NPY-induced cAMP synthesis.

5.3.4 NPY rapidly phosphorylates PKA, ATF-1 and CREB in GT1-7 neurons

Because I found the MAPK and PKA pathways to be involved in NPY-mediated stimulation in GnRH mRNA expression, I then assessed phosphorylation of signaling proteins PKA and ERK1/2, and transcription factors CREB and activating transcription factor 1 (ATF-1) downstream of the PKA and MAPK pathways. To elucidate whether NPY activates specific signaling cascades in the GT1-7 neuron, 100 nM NPY was used over a 1 h time-course. Western blot analysis using phospho-specific antibodies demonstrated that NPY exposure induces the phosphorylation of PKA (Figure 5.4A), ERK (Figure 5.4B), ATF-1 (Figure 5.4C) and CREB (Figure 5.4D) at 5 min and returned to basal levels by 15 min post-treatment. These data suggest that the PKA and MAPK signal transduction pathway, as well as transcription factors CREB and ATF-1
Figure 5.3. NPY Y1 or Y4 receptor-mediated cAMP activity in the GT1-7 neurons.

GT1-7 cells were serum-starved for 4 h before treatment with 100 nM NPY receptor-specific agonists [Leu31, Pro34] (NPY Y1/Y4/Y5 R), NPY 13-36 (NPY Y2 R), Pancreatic Polypeptide rat (rPP) (NPY Y4 R), [D-Trp32] (NPY Y5 R), 30 uM forskolin (FSK) or with vehicle alone for 15 min. cAMP was analyzed using a cAMP-specific radioactive immunoassay (RIA). Results shown are relative to corresponding control cAMP levels (set at 1.0) and are expressed as mean +/- SEM (n=4 independent experiments). *P<0.05, **P<0.01 versus DMSO, as per one-way ANOVA with Bonferroni’s post-hoc test.
Figure 5.4. NPY activates signal transduction second messengers in GT1-7 neurons.

GT1-7 neurons were serum starved for 4 h before treatment with 100 nM NPY, 30 μM forskolin (FSK) or vehicle alone over a 1 h time course. At the indicated time points, cell lysates were harvested and subjected to SDS-PAGE. Western blot analysis was performed with enhanced chemiluminescence using phospho-specific antibodies directed against (A) PKA, (B) ERK1/2, (C) CREB, and (D) ATF-1. Results shown are relative to corresponding control protein levels (set to 1.0) and expressed as mean +/- SEM (n=4 independent experiments). Phospho-protein is normalized to G-beta. *p<0.05 versus H2O control, as per two-way ANOVA with Bonferroni’s post-hoc test.
plays a key role in the regulation of GT1-7 GnRH neurons.

5.3.5 **Inhibition of MAPK and PKA-C signaling pathways affects NPY-mediated regulation of GnRH mRNA expression in GT1-7 neurons**

As reported in the time course study above, treatment with 100 nM NPY resulted in increased GnRH mRNA expression in GT1-7 neurons over a 36 h time course (Figure 5.2A). To determine whether or not the increase in GnRH mRNA by NPY is mediated through the NPY Y1 receptor subtype, I used a NPY Y1 receptor specific antagonist. I demonstrate that the NPY Y1 antagonist BIBP-3326 inhibited the NPY-mediated increase in GnRH mRNA, suggesting NPY acts through the NPY Y1 receptor to increase GnRH mRNA (Figure 5.5A). Because I have shown NPY activates the MAPK and PKA pathways, I assessed their role in the NPY-mediated increase in GnRH mRNA seen at 4 h using pharmacological inhibitors. The co-treatment of NPY with the MAPK inhibitor U0126 or the PKA inhibitor H89, resulted in attenuation of the NPY-mediated increase in GnRH mRNA expression in the GT1-7 neurons compared to NPY treatment alone (Figure 5.5B). H89 alone did not significantly change GnRH mRNA expression, although U0126 moderately repressed basal levels of GnRH mRNA, but this was not statistically significant. These results indicate that both the MAPK and PKA pathways are critical for the regulation of GnRH mRNA by NPY in GT1-7 neurons.

5.3.6 **Regulation of GnRH transcription by conditioned media from NPY-secreting mHypoE-38 neurons is mediated through the NPY Y1 receptor subtype**

Although NPY induces a significant increase in GnRH mRNA expression in GT1-7 neurons over a 36 h time course, it is unclear whether other secreted neuropeptides from hypothalamic NPY neurons contribute to the regulation of GnRH mRNA levels.
Figure 5.5 NPY Y1 antagonist BIBP-3226 and MEK and PKA inhibitors attenuate NPY-mediated regulation of GnRH mRNA levels in GT1-7 neurons.

(A) GT1-7 neurons were serum starved for 2 h before treatment with 100 nM NPY or vehicle alone over a 4 h time course. Cells were pretreated with the NPY Y1 antagonist BIBP-3226 for 1 h at a final concentration of 1 μM before NPY exposure. Cell lysates were harvested after 4 hours of NPY exposure and subjected to real-time RT-PCR to analyze GnRH expression (n≥3 independent experiment). GnRH is normalized to internal control 18S. *P<0.05, **P<0.001.

(B) GT1-7 neurons were serum starved for 2 h before treatment with 100 nM NPY or vehicle alone over a 4 h time course. Cells were treated the MAPK MEK inhibitor [U0126] for 1 h at a final concentration of 25 μM before NPY exposure. The PKA inhibitor H89 was used in the same manner as above with a final concentration of 30 μM. Cell lysates were harvested after 4 hours of NPY exposure and subjected to real-time RT-PCR to analyze GnRH expression (n≥4 independent experiment). GnRH is normalized to internal control 18S. Statistical analysis compared all treatments to control. *P<0.05 versus DMSO control, as per two-way ANOVA with Bonferroni’s post-hoc test.
To determine the effect of additional neuropeptides on GT1-7 neurons, conditioned medium was collected from the NPY-secreting mHypoE-38 neurons. mHypoE-38 neurons were treated with 60 mM potassium chloride (KCl) or vehicle alone for 15 min. KCl treatments resulted in a 1.5 fold increase in NPY secretion from the mHypoE-38 neurons compared to vehicle controls (Figure 5.6A). In order to assess whether conditioning medium may regulate GT1-7 neurons, conditioned media from the mHypoE-38 neurons treated with vehicle or NPY-contained media was directly administered to GT1-7 neurons for 4 h. The conditioned medium was desalted (to remove KCl) using a Zeba desalting spin column to avoid changes in GT1-7 neurons. Real-time RT-PCR demonstrated that NPY-containing conditioned media significantly increased GnRH mRNA expression in GT1-7 neurons (1.25 fold increase) compared to GT1-7 neurons treated with vehicle conditioned media (set to 1) (Figure 5.6B). According to the secretion data in the mHypoE-38 neurons (Figure 5.6A), I calculated the concentration of NPY in the conditioned medium to be approximately 0.27 nM. Attempts to concentrate the medium, even 2-fold, resulted in complete cell death due to contamination or excessive salt concentrations (concentration of media was completed by drying down media contents in a centrifugal concentrator). The level of induction of GnRH by the conditioned medium therefore should be relatively lower than that of 100 nM NPY. This is confirmed by our dose curve (Figure 5.2B). As a control for desalting efficiency, GT1-7 cells were treated directly with KCl and gene expression was assessed at 4 h. No significant change in GnRH gene expression was detected with KCl alone (Figure 5.6B). This indicates that NPY secreted by mHypoE-38 NPY neurons is sufficient to induce GnRH mRNA levels in GT1-7 neurons, in a similar manner to which
Figure 5.6. Conditioned media from NPY-expressing mHypoE-38 neurons increases GnRH mRNA expression in GT1-7 cells and can be blocked by the NPY Y1 antagonist BIBP-3226.

(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 (medium from unstimulated mHypoE-38 cells) or KCl (60 mM) for 15 min and conditioned media 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).

(C) mHypoE-38 neurons were treated with vehicle or KCl (60 mM) for 15 min and conditioned media was collected and desalted. GT1-7 neurons were serum starved for 2 h before treatment with conditioned media or vehicle alone over a 4 h time course. Cells were pretreated the NPY Y1 receptor antagonist for 1 h at a final concentration of 1 μM before CM exposure. 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 versus vehicle control, as per one-way ANOVA with Bonferroni’s post-hoc test (A,B) and two-way ANOVA with Bonferroni’s post-hoc test (C).
NPY peptide alone stimulates GnRH mRNA expression. To ensure NPY secreted from the mHypoE-38 neurons mediates the increase in GnRH transcription and not other potential neurosecretory factors from these neurons, I pretreated cells with the highly selective NPY Y1 antagonist BIBP-3326. The co-treatment of NPY with BIBP-3326, resulted in attenuation of the NPY-mediated increase in GnRH mRNA expression in the GT1-7 neurons compared to NPY treatment alone, again suggesting NPY mediates its increase in GnRH mRNA through the Y1 receptor subtype. Together, these studies demonstrate that NPY is the key peptide released from the mHypoE-38 cell line required to stimulate GnRH mRNA levels from GT1-7 cells.

5.4 Discussion

The most recognized functions of NPY include the regulation of endocrine function, circadian rhythms, and satiety (90). Central administration of NPY stimulates feeding and repeated doses results in an increase in body weight (107). NPY gene expression and accumulation increase immediately before the preovulatory GnRH surge (112, 294). Much insight into the exact role of NPY in reproductive physiology has been achieved through the use of antisense oligonucleotides directed against NPY mRNA, injected into the ARC of mice or primates (294, 304, 305). If NPY de novo synthesis is blocked before the steroid-induced preovulatory rise in GnRH, the surge release of LH causing ovulation does not occur (304). Although several groups have documented the importance of NPY regulating the reproductive axis, the transcriptional regulation and cellular mechanisms governing the effect of NPY on GnRH transcription are not yet described. Interestingly, a brief study of the in vivo effects of NPY on GnRH gene expression found that GnRH mRNA levels were increased at 4 h (303), similar to what I have found in the GT1-7 neurons. This study speculated that the Y1 receptor was
responsible for the increase in GnRH mRNA levels using the Y1/Y4/Y5 receptor agonist [Leu$^{31}$, Pro$^{34}$], however the involvement of Y4 was not considered. In our study, I reveal that NPY exposure increases GnRH over a 36 h time course possibly through the Y1 or Y4 receptor in GT1-7 neurons.

NPY binds to at least four different receptor subtypes (Y1, Y2, Y4, Y5) belonging to the seven-transmembrane domain G protein-coupled receptor (GPCR) superfamily (101). Studies have attempted to elucidate which receptor is responsible for the stimulatory effects of NPY on GnRH neurons (45, 70, 306). Previous studies using either the GT1 cell line or GnRH neurons in situ have indicated that NPY can directly regulate GnRH secretion through receptor subtypes NPY-Y1 (45, 73, 307), NPY-Y2 (45, 308), or NPY-Y5 (266, 306). RT-PCR of GT1-7 total RNA failed to detect NPY Y5 receptor mRNA transcript. Co-localization studies completed in vivo have demonstrated that 55% of GnRH neurons express the Y5 receptor, indicating the Y5 receptor is differentially expressed in GnRH neurons (309). A number of studies have supported the Y1 receptor as the main receptor responsible for mediating the reproductive effects of NPY (45, 70, 75, 306, 310), although none have definitively ruled out the Y4 receptor. Most recently, Klenke et al. found that NPY inhibits GnRH activity through the Y1 receptor subtype in an explant model (311). The evidence that NPY acts through the Y1 receptor to mediate its effects on GnRH neurons may not be decisive, as pharmacological profiles have indicated that the selective Y1 agonist [Leu$^{31}$, Pro$^{34}$] also displays a significant affinity for the Y4 and Y5 receptor subtypes (101). I do not detect the expression of Y5 in GT1-7 cells. Recent evidence has provided compelling reasons to include NPY Y4 receptors as potential mediators of NPY action on the GnRH neuron. The NPY Y4 receptor, also known as the primary receptor for pancreatic polypeptide (PP), is the least studied NPY
receptor subtype. Nonetheless, ICV injection of the Y1 antagonist and Y4 agonist 1229U91 rapidly and robustly increase GnRH secretion (312, 313). Since PP is not found in the brain, NPY may therefore act as a central, albeit lower affinity ligand for the Y4 receptor. Interestingly, the NPY Y4 receptor knockout restores fertility in the leptin-deficient ob/ob mouse. However, ICV injections of human PP decreased plasma levels of LH in ovariectomized rats (314). It appears that high basal levels of NPY in the ob/ob mouse downregulates the HPG axis, specifically through Y4 receptor signaling (315).

The study implicating Y1 action in GnRH release in GT1 cells did not analyze expression of the Y1 receptor nor the role of NPY Y4 (it was not yet cloned at the time) (45). To approach which receptor subtype was active in the GT1-7 neurons, I have used specific agonists of NPY receptor subtypes and assessed cAMP levels using an RIA. Previous studies have demonstrated NPY primarily inhibits cAMP synthesis through actions on Gi (102). The Y1/Y4/Y5 selective agonist [Leu³¹, Pro³⁴] resulted in a surprisingly significant accumulation of cAMP compared to vehicle control, an indication of receptor activation. Nevertheless, cAMP activity was unchanged with the Y2 agonist NPY₁₃₋₃₆, Y4 agonist rPP, or the Y5 agonist D-[Trp³²], suggesting that Leu³¹, Pro³⁴ likely acted through the Y1 receptor. However, I cannot rule out the functional activity of the Y4 receptor, as rPP may have decreased activity in the mouse; thus I could not detect a significant elevation of cAMP in the GT1-7 neurons at the concentration used. Further, it has been suggested that rPP does not have high affinity for the Y4 receptor (312, 313). Our results demonstrating NPY can increase cAMP levels, suggesting NPY regulates cAMP in a neuron-specific mechanism. Interestingly, pretreatments with the NPY Y1 antagonist attenuated the NPY-mediated increase in GnRH mRNA, suggesting the Y1 subtype is the main receptor for the NPY-mediated transcriptional regulation of GT1-7 neurons. Despite
this, additional studies will be performed once more specific agonists/antagonists are available.

GPCR activation results in the dissociation of G-protein subunits that can either stimulate or inhibit adenylyl cyclase activity (100). The stimulation of adenylyl cyclase results in the conversion of ATP to cAMP. PKA becomes catalytically active in the presence of cAMP and mediates most of cAMP’s actions. Interestingly, PKA activity has been implicated in the stimulation of GnRH biosynthesis and secretion (316-318) and also has been shown to either have no effect or to decrease GnRH mRNA in GT1-7 neurons (318, 319). Fittingly, in our study, NPY significantly up-regulates PKA phosphorylation, suggesting the immediate increase in GnRH release could be a result of rapid PKA phosphorylation. Our results demonstrate treatment with the PKA inhibitor H89 alone did not alter basal GnRH mRNA levels, suggesting the basal levels of GnRH mRNA operate independently of the PKA pathway in GT1-7 neurons. However, in the presence of H89, NPY-mediated increases in GnRH mRNA were abolished, signifying that the PKA pathway was required for the NPY-mediated increase in GnRH mRNA.

GPCRs also have the ability to activate the MAPK signaling cascade through the interaction of specialized sub-domains (Gi and Gq/11) that dissociate with GPCR activation (and also through PKC or activation sites such as Ras or Raf) (320-322). Previous studies have demonstrated the activation of the MAPK pathway can regulate GnRH mRNA expression (222, 319). Insulin treatment increases GnRH mRNA levels in the Gnv-3 GnRH expressing neuron through the MAPK signaling cascade (222). Pharmacological inhibitor studies further demonstrated the MAPK pathway is critical for the melatonin-mediated down-regulation of GnRH mRNA expression (319). I show that basal levels of GnRH mRNA expression are slightly repressed with the treatment of the
MEK1/2 inhibitor U0126. In contrast, in the presence of U0126, NPY-mediated increases in GnRH mRNA levels are attenuated; implicating the MAPK pathway in NPY-induced increases in GnRH mRNA.

Two regions within the GnRH gene, known as the promoter and enhancer region, control GnRH transcription (79, 166, 323) (Figure 5.7). The promoter region is found proximal to the transcriptional start site of the GnRH gene, and is particularly important for basal GnRH gene expression (324). This region in the mouse and human is AT rich, and therefore could be a critical region for a number of transcription factor binding motifs (324). The enhancer region, found distal to the promoter region, was demonstrated to mediate neuron-specific expression through deletion analysis studies (225). This 300-bp enhancer site is located at the 5’ flanking site and binds to numerous nuclear proteins (79, 166, 323). The nuclear protein and immediate early gene cFos has been implicated in controlling GnRH mRNA levels, as its protein product dimerizes with cJun to form the transcription factor complex referred to as activator protein-1 (AP1). In order to elucidate potential factors involved in the regulation of GnRH transcription, I analyzed downstream effectors of the PKA and MAPK pathway, such as the transcription factors ATF-1 and CREB. Both are phosphorylated upon NPY stimulation in GT1-7 neurons. Interestingly, CREB may directly activate cFos, a component of the AP-1 protein critical for GnRH transcriptional regulation. Further studies are required to determine whether or not NPY regulates GnRH mRNA expression indirectly through the AP-1 complex or through other elements within the promoter or GnRH enhancer region.

I describe the regulation of GnRH gene expression by NPY in GT1-7 neurons, and importantly that conditioned medium from NPY-expressing mHypoE-38 neurons mimic this response. I demonstrated that the transcriptional regulation of GnRH mRNA
Figure 5.7. The Rat GnRH Promoter Region.

The neuron-specific enhancer responsible for rat GnRH gene expression has been characterized by Whyte et al. 1995. The enhancer region is approximately 300 bp located between -1863 and -1571 of the 5' flanking region. DNase 1 footprinting has demonstrated that multiple nuclear proteins bind to the GnRH enhancer regions in GT1-7 neurons (Lawson et al. 1996). The proximal/core promoter is an AT rich region that is essential for GnRH transcription.
in GT1-7 neurons responds to NPY predominantly through either the Y1 or Y4 receptor, and that NPY exposure increased GnRH mRNA over a 36 h time course in the absence of steroidal modulators. Western blot analysis revealed that NPY activates the PKA and MAPK signal transduction pathways, as well as transcription factors CREB and ATF-1 that are potential transcriptional mediators of the NPY response. Through the use of pharmacological inhibitors, I determined that the NPY-mediated induction of GnRH transcription is dependent on the signal transduction cascades PKA and MAPK pathways. These studies provide the paradigm in which to study the potential downstream transcriptional mechanisms involved in this response. Importantly, I also demonstrate NPY secreted from the mHypoE-38 neurons stimulates GnRH mRNA expression in GT1-7 neurons. Finally, with receptor specific antagonist studies, I demonstrated that the effects of NPY are mediated through the NPY Y1 or Y4 receptor subtype. Finally, conditioned media treatment experiments indicate that NPY and not other hypothalamic factors secreted by the mHypoE-38 cell lines was required to stimulate GnRH transcription in the GT1-7 cell lines. These studies provide expanded evidence of the significance of NPY in the regulation of the GnRH neuron itself in the absence of steroidal modulators (see Figure 5.8).
NPY directly increases GnRH mRNA levels in the GnRH-expressing GT1-7 GnRH neurons over an extended time-course. Conditioned media treatments from KCl-stimulated mHypoE-38 neurons also directly stimulates GnRH mRNA levels. This effect was found to occur through the NPY Y1/Y4 receptor subtype. NPY was found to stimulate the cAMP/PKA and MAPK pathways through Western blot analysis. The NPY-mediated increase in GnRH mRNA levels was linked to the MAPK and PKA signaling pathways using pharmacological inhibitors against key signaling molecules.
Chapter 6

Overall Discussion and Future Directions
6.1 Overall Discussion

Nutritional status can have marked effects on all aspects of physiology, particularly on growth, metabolism and reproduction (85-87, 325). Although the consequential effects of nutritional deprivation are well documented, the mechanisms by which the body perceives changes in nutritional status and uses this information to regulate reproductive processes and neuroendocrine function are less understood. Leptin and E2 are endocrine cues that link energy status to hypothalamic pathways regulating appetite and reproduction (7, 204, 259). To date, the preponderance of literature describing the E2- and leptin-mediated regulation of hypothalamic neuropeptides is based on whole hypothalamic extracts/tissue. Characterization of the direct regulation by E2 and leptin on individual neuropeptidergic neurons, however, lags considerably behind. NPY, a hypothalamic orexigenic peptide involved in the regulation of appetite and reproduction, work in concert with leptin and E2 (81, 291). Although scientists have studied NPY for over 20 years, much remains to be determined about the peripheral hormonal stimulants and the cellular mechanisms through which NPY neurons are regulated to maintain energy homeostasis and reproductive function. In this thesis, I examined the direct regulation of NPY neurons by endocrine hormones, E2 and leptin to evaluate the cellular signaling mechanisms and the secretory responses of individual NPY neuronal cell models. Interestingly, food deprivation that is accompanied by abnormal neuropeptidergic function inhibits the HPG axis at all levels, including the secretion of GnRH from the hypothalamus, the secretion of LH and FSH from the anterior pituitary and the secretion of gonadal steroids (85, 86, 326, 327). In my final studies, I determined the influence of NPY neuropeptide content from NPY-synthesizing neurons on the direct regulation of the HPG axis at the level of the GnRH neuron. This dissertation provides
additional mechanistic evidence of the relationship between feeding and reproductive homeostasis in individual hypothalamic neuropeptidergic cell lines.

Menopause, the age-related loss of estrogen and progestins, is associated with an increased risk of obesity and adiposity (169, 170, 328). E2 replacement therapy decreases body weight and reduces appetite in menopausal women, suggesting that E2 plays an important role in appetite regulation (329). Studies examining the suppressive effect of E2 on food intake have focused on the two main ER subtypes, ER-α and ER-β. Results from studies using ER specific agonists, ER-α knockdown and gene knockout mice studies provide evidence that ER-α mediates the anorectic action of E2 (19, 120, 248, 330). However, studies using an ER-β ODN knockdown approach also inhibited the anorexigenic effect of E2 (249). In addition to the uncertainty regarding which ERs are responsible for the anorectic action of E2, there have been no reports on the direct action of E2 on hypothalamic neuropeptide secretion. A recent study published by Olofsson et al. found that E2 inhibits NPY mRNA expression in hypothalamic explants through an ER-α-dependent mechanism (171). The study failed to co-localize ER-α in NPY neurons, suggesting that E2 works through an indirect mechanism.

Taking an alternative approach to confirm ER-α expression in NPY neurons, I used FACS to isolate NPY-GFP cells from the hypothalamus of the NPY-GFP transgenic mouse, and RT-PCR to detect ER-α. The result concurs with previous reports that demonstrate that ER-α and NPY are likely co-expressed in the same neurons in vivo (163, 164). Using a combination of ER specific agonists/antagonists and E2-conjugated BSA, I discerned that membrane-bound ER-α is directly responsible for the E2-mediated decrease in NPY secretion in two NPY neuronal cell lines, mHypoE-42 and mHypoA-2/12. Although feeding is regulated by a number of orexigenic and anorexigenic feeding
circuits, NPY is the most potent orexigenic compound (331). Therefore this E2-mediated decrease in NPY secretion observed in our neuronal cell model may indicate that NPY plays a large role in the anorectic action of E2.

Recent studies have demonstrated that AMPK and PI3K signaling are essential in the maintenance of food intake at the level of the hypothalamus (283). AMPK is a cellular energy sensor activated by peripheral endocrine signals during low energy status periods (280). Minokoshi et al. have demonstrated that exogenous leptin administration inhibits AMPK activity in the ARC (191). Inhibitor studies have further demonstrated that hypothalamic AMPK activity is essential for the anorectic action of leptin (281). In addition, studies on peripheral tissue have linked estrogen to increases in AMPK activity that translates into enhanced beta-oxidation and metabolism (332). However, such studies have not examined whether or not AMPK signaling is involved in the anorectic action of estrogen at the level of the hypothalamus. Studies have also strongly implicated the PI3K pathway in the hypothalamic neurons that influence food intake and body weight (209, 268, 275). The PI3K pathway has been considered a major signaling cascade in mediating the action of metabolic hormones insulin and leptin as demonstrated both in vitro and in vivo (276). E2 is also a major regulator of the PI3K pathway as demonstrated in vitro (178, 333). However, studies have yet to conclusively link estrogen and the PI3K pathway to energy homeostasis. In this thesis, our neuronal cell models have demonstrated for the first time that leptin directly decreases NPY secretion through an AMPK- and PI3K-dependent mechanism. In addition, I have linked the anorexigenic action of E2 to AMPK and PI3K activity in NPY cell models. A noteworthy observation here is that both leptin and E2 act through identical signaling mechanisms in order to alter NPY neuronal activity. The altered NPY neuronal activity, in turn, could reduce food
intake. These identical signaling mechanisms could prove to be a pharmacological target for menopausal women and/or leptin deficient individuals.

The failure of leptin to reduce appetite and weight loss in obese individuals gives rise to the notion of leptin resistance, which is impaired leptin action in obese states (208-211, 213, 284, 334). Leptin resistance is a complex subject that appears to occur at multiple levels of physiology. At the hypothalamic level, the mechanisms underlying leptin resistance have included impaired STAT3, PI3K, SOCS3 and PTP1B function in the ARC (214). High concentrations of leptin have effectively limited the activity of these signaling molecules in vivo (213, 335-337). Studies have yet to confirm the specific neuronal populations in the ARC that are susceptible to leptin resistance and whether or not the energy sensing kinase, AMPK, is altered in leptin resistant states. In this study, I have demonstrated that the leptin-mediated decrease in AMPK phosphorylation is greatly attenuated in NPY neuronal cell lines with prolonged exposure to leptin. This impaired AMPK signaling was accompanied by the inability of the NPY cell lines to respond appropriately to the leptin-mediated decrease in NPY secretion. This is the first demonstration of impaired AMPK signaling and NPY secretory response to prolonged leptin exposure. It provides essential information for potential neuroanatomical determinants of leptin resistance and of the underlying impaired signaling mechanisms that contribute to obese states.

In an unexpected finding, leptin was found to increase NPY secretion from the mHypoE-38 neuronal cell line after a 1 h treatment. This leptin-mediated increase in NPY secretion occurred through a MAPK-dependent mechanism. Previous studies have implicated MAPK signaling with the stimulatory role of leptin in reproduction and growth in mice (206, 272). Additional studies have also implicated the mHypoE-38 cell
line as a putative reproductive NPY neuronal cell line. Using this model, Titolo et al. demonstrated that $E_2$ induced a surge of NPY mRNA transcript levels after 24 h estrogen exposure that was proposed to be involved in the GnRH preovulatory surge (163, 164). Based on this evidence, I suspected that the leptin-mediated MAPK-dependent increase in NPY secretion could stimulate GnRH neuronal activity. I used GT1-7 GnRH-expressing cell lines and treated these cells with conditioned media from stimulated mHypoE-38 neurons. Interestingly, media taken from the stimulated mHypoE-38 NPY cell lines induced a significant induction in GnRH mRNA levels that occurred exclusively through the Y1 receptor subtype. As a result, I propose that leptin may stimulate a subset of NPY neuronal cells that project to GnRH cell bodies in order to stimulate the reproductive axis. Future in vivo studies will be instrumental in verifying and substantiating this hypothesis.

NPY has a paradoxical effect on the reproductive axis. Depending on the mode of its administration, the hormonal status of the experimental animal and the time course of the treatment, NPY can exert both suppressive and excitatory effects on the HPG axis (45, 75, 78, 88, 294). Besecke et al. have previously investigated the secretory responses of GnRH to NPY treatment in the GT1-7 immortalized cell line using a flow-through superfusion system (45). In this study, NPY directly stimulated the release of GnRH in a dose-dependent manner primarily through Ca(2+) dependent mechanisms. I have extended this research by showing that NPY can directly stimulate GnRH mRNA levels in the GT1-7 cell models. I am the first to show through inhibitor analysis that this occurs through a MAPK- and PKA-dependent mechanism. NPY is an essential neurotransmitter that serves as a communication bridge between the neural processes that regulate reproduction and those that maintain energy homeostasis. The studies presented herein
provide additional mechanistic descriptions of the control of GnRH-expressing cell lines by NPY in the absence of steroidal modulators.

The functional heterogeneity revealed in the NPY secretory responses to hormonal treatments in our NPY-expressing cell lines provides a cellular basis for the role of NPY neuronal subpopulations in regulating diverse neuroendocrine processes. This observation coincides with studies in vivo that support the notion of functionally diverse NPY neuronal populations. One of the first studies to identify biochemically distinct NPY neuronal populations used fluorescent double-immunolabeling for NPY and glutamic acid decarboxylase (292). This study found that only one third of ARC NPY neuronal perikarya co-expressed the glutamic acid decarboxylase. Ensuing studies found two distinct NPY subpopulations: 1) a subset of NPY and glutamic acid decarboxylase co-producing cells located in the dorsomedial ARC, and 2) a subset of NPY cells in the ventral ARC (292). Additional studies found heterogeneous electrophysiological properties of NPY neurons in vivo. Using patch-clamp recordings, Fioramonti et al. demonstrated that only 40% of NPY neurons are glucose-inhibited, indicating that a distinct population of NPY neurons that are glucose-sensing (338). Most recently, Padilla et al. demonstrated using a combination of fluorescence in situ hybridization (FISH) and lineage tracing studies that approximately 17% of NPY neurons are derived from POMC progenitor cells (202). This study indicated that a subset of NPY neurons in the hypothalamus is derived from a specific population of progenitor cells that remain distinct from other hypothalamic NPY neurons. This finding suggests NPY neuronal heterogeneity. Together, studies in vivo strongly suggest a heterogeneity amongst hypothalamic NPY cell populations, which aligns with our observations in vitro that NPY-expressing cell lines respond uniquely to hormonal treatments, and together these
results begin to describe the cellular basis for the multifaceted control of NPY in energy homeostasis and reproduction (see Figure 6.1).
Figure 6.1. Summary of findings.

(1) Chapter 3 describes the anorexigenic action of estrogen in NPY neuronal cell lines, mHypoE-42 and mHypoA-2/12. Here, estrogen decreases NPY secretion through membrane-bound ER-α and through the PI3K and AMPK signaling pathways. (2) In Chapter 4, leptin differentially regulates NPY neuronal cell lines. Leptin decreases NPY secretion in the NPY-GFP and mHypoA-59 cell lines, which was also mediated through the PI3K and AMPK pathways. Prolonged leptin exposure prevents the decrease in NPY secretion and phospho-AMPK, indicating NPY neurons are susceptible to leptin-induced leptin resistance. Conversely, leptin increases NPY secretion in the mHypoE-38 cell line, occurring through the MAPK and PI3K pathways. (3) In Chapter 5, KCl-stimulated mHypoE-38 media directly placed onto GT1-7 cells increases GnRH mRNA levels through the Y1 receptor subtype. These studies provide additional evidence that NPY neurons play a role in both reproductive function and appetite regulation, which may be mediated by spatially segregated circuitries projecting to medial preoptic GnRH neurons or feeding related nuclei including the PVN, LH and VMH.
6.2 Limitations

The strength of using immortalized hypothalamic clonal cell lines is their capacity to demonstrate the cause-and-effect relationships of specific neuromodulators. However, when taking this reductionist approach, scientists must avoid overstating their conclusions since immortalized cell lines lack the heterogeneous and complex neuronal architecture observed in vivo. The cells used in this study were isolated from both embryonic and adult hypothalamic sources. The embryonic cell lines were derived from mice at days embryonic 15 (E15), E17 and E18 during significant hypothalamic neurogenesis (219). At this time of development, hypothalamic circuits are not fully formed, which could result in functionally or phenotypically underdeveloped cells compared to mature adult neurons. However, numerous studies using these embryonic neuronal cell models have found that these neurons function similar to native adult neurons (54, 216, 236-241, 339). In addition, I corroborated these studies by using a combination of clonal and mixed adult hypothalamic cell lines. Collectively, the use of multiple neuronal phenotypes from both embryonic and adult sources has allowed scientists to analyze the direct effect of neuromodulators on individual neuronal cell populations.

Although cell lines allow for the elucidation of molecular processes, a major disadvantage is that the immortalizing gene SV40 T-antigen can interfere with cellular processes. This might affect the pathways studied. Preliminary data from our laboratory using the shRNA knockdown of T-antigen demonstrates that T-antigen expression increases the basal activity of cellular signaling kinases AKT, Jak2 and STAT3 (Belsham, unpublished data). As a result, I have attempted to reduce the basal activity of
these signaling kinases using serum starvations, low glucose and/or pH modifications during experimentation. Importantly, I found that T-antigen expression did alter key genes of interest including ER-α and NPY in our hypothalamic cell lines. Additional studies completed by May et al. demonstrate that T-antigen expression in murine embryonic fibroblasts have only 379 genes altered (out of 22,600 probes in total) altered compared to nontransformed cells, with the majority of these genes involved with proliferation and nucleotide synthesis (340). Together, these studies show that immortalized cell lines comprise a useful tool for unraveling complex signaling pathways and for studying the direct action of neuromodulators on specific neuronal phenotypes. Of course, researchers should design studies carefully in order to optimize experimental conditions and to avoid overstating conclusions.

In Chapter 3, the membrane impermeable conjugate, E₂-BSA, was used to analyze the role of cell membrane-bound ERs in the regulation of NPY secretion. However, recent studies have demonstrated that E₂-BSA data should be interpreted cautiously. This study analyzed the forms of commercially available E₂-BSA conjugates and found that 3-5 % of E₂-BSA is available in the free unconjugated E₂ form (341). Taguchi et al. have also provided evidence that E₂-BSA can induce ERE-driven luciferase gene activity in neuroblastoma cells (341). This finding suggests the involvement of nuclear ER. However, other studies have demonstrated that E₂-BSA does not increase the transcription of ERE-based reporter genes in human neuroblastoma cells (342). These studies suggest that E₂-BSA does not enter the cell and is unable to activate nuclear ER. For this reason, it is advisable to filter E₂-BSA to remove free E₂ or to use the newly generated and more stable E₂ conjugate, E₂ dendrimers (EDCs), produced by the Katzenellenbogen lab (343).
Pharmacological inhibitors have been widely used in the study of signal transduction and have provided invaluable insight into the function of signaling kinases in various cell types. However, a substantial body of evidence indicates that inhibitors can have non target effects, which are independent of inhibiting intended proteins. Although these actions are varied, the inhibitors used in our studies have been demonstrated to act on several unintended proteins. For example, LY294002 can potentially inhibit glycogen synthase kinase and casein kinase 2; H89 can inhibit non-PKA kinases including MAPK and calcium signaling pathways; U0126 can also inhibit calcium signaling and calmodulin-dependent kinase (344). As a result, although pharmacological inhibitors are valuable tools scientists use to dissect intracellular signaling mechanisms, the potential of the inhibitors to act on a variety of other cellular processes limits the conclusions that can be drawn. Additional studies utilizing RNA interference or the introduction of dominant negative versions of protein kinases could be used to study the role of specific signaling pathways. Although these methods can be accompanied by transfection difficulties and inadequate construct expression, the results of these techniques have an extremely high specificity compared to the results of techniques using pharmacological agents. This high specificity allows for much more decisive conclusions.

6.3 Future directions of study

This thesis describes the cellular mechanisms that leptin, E₂ and NPY act through to regulate neuropeptide mRNA and secretion levels from hypothalamic neuronal cell lines. Although these studies identify important relationships between signal kinase activity and neuronal function, several new questions arose during the completion of these studies that warrant further investigation.
Studies have demonstrated that E\(_2\) deficient mice have decreased leptin responsiveness, suggesting an interplay between estrogen and leptin signaling, although how this occurs is unclear (345). Our group and others have found co-expression of ER (both ER-\(\alpha\) and ER-\(\beta\)) and Ob-R in NPY hypothalamic cell lines and our group is currently in the process of confirming this finding *in vivo* (346). Additionally, Thorn *et al.* has demonstrated that E\(_2\) can modulate Ob-R mRNA in specific E\(_2\)-responsive tissue (347). Most recently, preliminary studies from our laboratory indicate that the biphasic regulation of NPY mRNA by E\(_2\) in the mHypoE-38 cell line can be attenuated with leptin co-treatment (Belsham, unpublished data). Utilizing the NPY-expressing cell lines that are responsive to hormonal treatments identified in this thesis, I could determine whether or not E\(_2\) and leptin co-treatments can modify the NPY secretory responses to the individual hormonal treatments. These experiments will allow us to determine whether leptin or E\(_2\) pre/co-treatments can enhance the negative or positive effect on NPY release.

In order to identify signaling pathways involved in potential E\(_2\) and leptin cross-talk, assays which contain high throughout signaling kinase analysis of protein phosphorylation such as the R&D Systems Proteome Array profiler could be utilized. These studies are of immense interest given the identification of the identical signaling mechanisms involved in the leptin and E\(_2\) regulation of NPY secretion in hypothalamic cell lines and given the paucity of information known about the interaction of these hormones in the hypothalamus.

Although leptin is linked to the neuroendocrine regulation of the reproductive axis, the direct regulation of the GnRH neuron by leptin has not been described. To date, leptin receptors have not conclusively been found on GnRH neurons *in vivo* (264). This may be due to technological limitations and inadequate staining sensitivity. Using RT-PCR, I
have found Ob-R mRNA in the newly generated GnRH-GFP immortalized cell lines and our group is currently in the process of confirming these findings in vivo using a newly available Ob-R antibody and GnRH-GFP hypothalamic slices (Belsham lab, unpublished data). Additionally, I have preliminary data that demonstrates that leptin treatments can activate the newly generated GnRH-GFP cell model by measuring cFos mRNA levels in response to leptin (Belsham, unpublished data). Future studies could further analyze the involvement of leptin action on the GnRH-expressing cell lines in these newly generated GnRH-GFP cell populations by examining the GnRH secretory responses, transcriptional changes and signaling mechanisms involved. In addition, E<sub>2</sub> has been demonstrated to enhance the GnRH neuronal responses to NPY (20). Further experimentation could determine whether leptin requires the steroid hormone, E<sub>2</sub>, to exert this stimulatory effect on GnRH neuronal cell lines. These studies would indicate that like E<sub>2</sub>, leptin might modulate the reproductive axis through multiple mechanisms.

I found that prolonged treatment with leptin prevented the leptin-mediated regulation of the AMPK phosphorylation and NPY secretion. To fully understand the cellular mechanisms of leptin resistance and obesity, additional mechanistic studies could be completed to decipher how hyperleptinemia results in desensitization in NPY neuronal cell lines. Initially, experiments could determine the minimum concentration that is required to induce leptin resistance in NPY neuronal cell lines. Dose response curves using 0.1 pM to 100 nM leptin treatments could be used over a 4, 8 and 24 h time course. Identifying the minimum length of exposure and concentration of leptin that is required to induce leptin resistance would be valuable information for future experimental paradigms. Leptin signaling is under the negative feedback control of SOCS3 and PTP1B (335, 336). Thus, in order to begin to understand the potential role of these negative
regulators in hyperleptinemic conditions, studies could examine the effect of the overexpression of SOCS3 and PTP1B and its effect on the signaling kinases, AMPK, in NPY-neuronal hypothalamic cell lines. The commencement of leptin resistance with SOCS3 and PTP1B overexpression would suggest such a mechanism. Through Western blot analysis, studies could also examine whether or not prolonged leptin exposure increases Ob-R degradation (285). Recent studies demonstrate that Ob-R can be degraded via an ubiquitin-dependent endocytosis mechanism (183, 285). Studying leptin resistance in NPY neuronal cell populations would therefore allow for a detailed description of neuronal resistance in hyperleptinemic cases.

The differential regulation of NPY secretory responses from the immortalized NPY-synthesizing cell lines should be confirmed in vivo. Initial studies should attempt to identify bona fide gene markers specific to each NPY subpopulation. The identification of cellular markers specific to each NPY subpopulation could be completed using microarray analysis or RT-PCR. These newly identified cellular markers would allow for the confirmation of these NPY subpopulations in vivo using IHC double-labeling techniques. Ideally, NPY subpopulations from the ARC that project to GnRH neurons in the anterior hypothalamus would display mHypoE-38 specific markers, and NPY subpopulations that project to feeding related nuclei including the PVN and LH from the ARC would selectively express markers identified in the NPY-GFP and mHypoA-59 cell lines. The completion of these studies in vivo would substantially strengthen our hypothesis of NPY neurons acting as the integration centre for peripheral hormones to regulate food intake and reproduction.
Chapter 7 – References
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