Direct Steroidal Regulation and Inhibitory Mode of Action of Gonadotropin-Inhibitory Hormone (GnIH or RFRP-3) in Immortalized Hypothalamic Cell Models

by

Nicole Gojska

A thesis submitted in conformity with the requirements for the degree of Master of Science

Department of Physiology
University of Toronto

© Copyright by Nicole Gojska 2013
Direct Steroidal Regulation and Inhibitory Mode of Action of Gonadotropin-Inhibitory Hormone (GnIH or RFRP-3) in Immortalized Hypothalamic Cell Models

Nicole Gojska
Master of Science
Department of Physiology
University of Toronto
2013

Abstract

Fertility is dependent on the precisely orchestrated communication of an array of effectors within the reproductive axis, all of which impinge on gonadotropin-releasing hormone (GnRH) neurons. A novel reproductive inhibitor was identified in avian species and growing evidence suggests that the functional mammalian homologue, RFamide-related peptide-3 (RFRP-3 or GnIH) can inhibit GnRH neuronal activity and gonadotropin release. To date, the regulation and effects of RFRP-3 at the hypothalamic level are poorly understood. We established an Rfrp-expressing neuronal cell model to investigate the mechanisms of transcriptional regulation of the genes for RFRP and the RFRP receptor, GPR147 by dexamethasone and estradiol. We show that the RFRP system is a direct target for stress-associated transcriptional regulation. Further, employing a novel GnRH-secreting cell line, we report that GnRH neurons express Gpr147 and RFRP-3 represses the transcription of GnRH. These data further our understanding of the level and regulatory effects at which RFRP-3 modulates reproduction.
Acknowledgments

First and foremost I would like to thank my supervisor, Dr. Denise Belsham. The opportunity to work under your guidance and support was truly a privilege and has taught me so much about myself, both as a researcher and person. Prior to commencing my graduate studies I was asked about the fundamental qualities that a researcher must posses to be successful and after two years of being apart of your lab I feel that you have instilled such qualities in me, along with an ignited passion for scientific exploration. Dr. Belsham you are a role model for all aspiring researchers in the way you dedicate yourself to your students. Thank you for creating such a wonderful, yet challenging and stimulating research environment for my fellow lab mates and I to carry out our studies. Despite all the hard work, it has been a very enjoyable and rewarding process, so thank you again for all your support and commitment.

I would like to also thank my Supervisory committee members, Dr. Theodore Brown and Dr. David Lovejoy. Your insight, guidance, and dedication throughout my research project have been instrumental.

To all the members, past and present of the Belsham lab, you are all incredible!!! I never expected to meet so many amazing friends while completing my graduate studies. Thank you all for your technical advice and support, as well as making the completion of my Masters such a memorable time in my life.

Last, but certainly not least I would like to thank my family and boyfriend, Michael. You are my rock and without your incredible support, understanding, and love none of this would be possible. Words cannot express how grateful I am to have you all in my life!
Table of Contents

List of Tables and Figures..................................................................................................................vii

List of Abbreviations ..........................................................................................................................vii

Chapter 1: General Introduction ........................................................................................................1

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

1.2 The Hypothalamic-Pituitary-Gonadal Axis ................................................................................3

  1.2.1 GnRH: Hypophysiotropic Regulator of the HPG axis ..........................................................4

1.3 Regulators of GnRH synthesis and release .................................................................................7

  1.3.1 Gonadal Steroids .......................................................................................................................7

    1.3.1.1 Estrogens ...............................................................................................................................7

    1.3.1.2 Androgens .............................................................................................................................9

    1.3.1.3 Progesterone .........................................................................................................................9

  1.3.2 Innervating Neuronal Systems ...............................................................................................10

    1.3.2.1 γ-aminobutyric acid ............................................................................................................10

    1.3.2.2 Glutamate ............................................................................................................................11

    1.3.2.3 Kisspeptin ............................................................................................................................12

1.4 Gonadotropin-Inhibitory Hormone: A negative regulator of avian reproduction ....................13

  1.4.1 Discovery and Initial Characterization .....................................................................................13

  1.4.2 Identification of the GnIH-R ....................................................................................................14

  1.4.3 GnIH action in the pituitary .....................................................................................................14

  1.4.4 GnIH action in the brain ..........................................................................................................15

  1.4.5 GnIH action in the gonads .......................................................................................................15

1.5 RFRP-3: The mammalian homologue of avian GnIH ..............................................................16

  1.5.1 Initial Characterization of RFRP-3 in the mammalian HPG axis ...........................................16

  1.5.2 Role of RFRP-3 in the pituitary ..............................................................................................17

  1.5.3 Role of RFRP-3 in the brain .....................................................................................................18

  1.5.4 Role of RFRP-3 in the gonads ..................................................................................................18

1.6 Regulation of GnIH/RFRP-3 in vertebrates ............................................................................19

  1.6.1 Circadian and Seasonal Changes ............................................................................................19

  1.6.2 Steroidal Regulation ...............................................................................................................20
1.6.3 The Stress-Response ................................................................. 21

1.7 Hypothalamic cell models in the study of neuroendocrine function .................. 22
  1.7.1 Immortalized rat embryonic hypothalamic cell lines (rHypoE-xx) ............ 23
  1.7.2 Immortalized adult, non-clonal GnRH cell model (mHypoA-GnRH/GFP) .... 23

1.8 Study Hypotheses, and Aims .......................................................... 24

Chapter 2: Materials and Methods .................................................................. 27

2.1 Cell Culture and reagents .................................................................... 28
2.2 Semi-quantitative reverse transcriptase-polymerase chain reaction (RT-PCR) ... 28
2.3 Quantitative real time RT-PCR ......................................................... 29
2.4 Enzyme Immunoassay (EIA) ............................................................ 31
2.5 In silico analysis ............................................................................... 31
2.6 Statistical analysis ........................................................................... 31

Chapter 3: Glucocorticoid receptor-mediated regulation of Rfrp and Gpr147 gene expression in an immortalized hypothalamic cell line ..................................................... 32

3.1 Abstract .......................................................................................... 33
3.2 Introduction ..................................................................................... 33
3.3 Results ........................................................................................... 35
  3.3.1 rHypoE-23 neurons express Rfrp and Gpr147 mRNA, in addition to ERs, GR, and other key reproductive receptors ......................................................... 35
  3.3.2 Dexamethasone, but not E\textsubscript{2} has stimulatory effects on Rfrp and Gpr147 mRNA expression in rHypoE-23 neurons ........................................... 37
  3.3.3 Potential autocrine regulation of Rfrp and Gpr147 mRNA expression .......... 37
  3.3.4 GR antagonist, RU-486 abolishes the dexamethasone-mediated mRNA expression changes in rHypoE-23 neurons ......................................................... 40
  3.3.5 Dexamethasone-mediated induction of Rfrp and Gpr147 genes is independent of de novo protein synthesis in hypothalamic neurons .................................. 43
3.4 Discussion ....................................................................................... 43

Chapter 4: RFRP-3 mediated transcriptional suppression of GnRH mRNA levels in a novel GnRH-secreting cell mode ................................................................. 50

4.1 Abstract .......................................................................................... 51
4.2 Introduction ..................................................................................... 51
4.3 Results ........................................................................................... 54
4.3.1 Characterization of Gpr147 mRNA expression in mHypoA-GnRH/GFP neurons .........................................................................................................................54
4.3.2 RFRP-3 induces neuronal activation in mHypoA-GnRH/GFP neurons .................................................................................................................................54
4.3.3 RFRP-3 mediates the direct and transient suppression of GnRH mRNA levels .........................................................................................................................57
4.3.4 Transcriptional blockade attenuates the RFRP-3 mediated suppression of the GnRH gene .........................................................................................................................57
4.3.5 RFRP-3 is unable to attenuate SNP-induced GnRH secretion in mHypoA-GnRH/GFP neurons .........................................................................................................................60
4.3.6 In silico analysis of putative miRNA binding sites in the mouse GnRH mRNA sequence .........................................................................................................................63

4.4 Discussion .............................................................................................................................63

Chapter 5: General Discussion ........................................................................................................69

5.1 Overall conclusions .................................................................................................................70
5.2 Study limitations .......................................................................................................................75
5.3 Future directions .......................................................................................................................78
5.4 Concluding Remarks .................................................................................................................80

References .........................................................................................................................................81
List of Tables and Figures

Table 2.1 List of primers used for cell screening and real time RT-PCR..........................30

Figure 1.1 A schematic of the mammalian hypothalamic-pituitary-gonadal axis...................5

Figure 1.2 Schematic summarizing the current mechanism through which RFRP-3 may participate in the central regulation of the HPG axis.................................................................25

Figure 3.1 Characterization of the gene expression profile of Rfrp-expressing, rHypoE-23 neurons.........................................................................................................................36

Figure 3.2 Dexamethasone increases Rfrp and Gpr147 transcript levels in rHypoE-23 neurons.........................................................................................................................38

Figure 3.3 Effect of RFRP-3 treatment on Rfrp and Gpr147 transcript levels in rHypoE-23 neurons.........................................................................................................................39

Figure 3.4 Dexamethasone upregulation of Rfrp and Gpr147 mRNA expression in rHypoE-23 neurons is blocked by GR antagonist, RU-486.................................................................41

Figure 3.5 Effects of protein synthesis blockade on dexamethasone-mediated mRNA expression changes in rHypoE-23 neurons..................................................................................42

Figure 3.6 Schematic of the rat Rfrp and Gpr147 promoter-luciferase constructs..................48

Figure 4.1 Gene expression profile of a adult-derived, non-clonal GnRH-secreting cell line, mHypoA-GnRH/GFP...........................................................................................................55

Figure 4.2 RFRP-3 induces cFos mRNA expression in the mHypoA-GnRH/GFP neurons.......56

Figure 4.3 RFRP-3-mediated regulation of GnRH mRNA expression in mHypoA-GnRH/GFP neuronal cells................................................................................................................58

Figure 4.4 Effects of transcription blockade on RFRP-3-mediated suppression of GnRH mRNA expression in mHypoA-GnRH-GFP neurons.................................................................59

Figure 4.5 Effects of RFRP-3 on GnRH secretion in the presence of SNP in mHypoA-GnRH/GFP neurons..........................................................................................................................61

Figure 4.6 In silico analysis of mouse GnRH mRNA sequence for potential miRNA binding sites .................................................................................................................................62

Figure 5.1 Representative model summarizing the proposed mechanism by which dexamethasone directly regulates RFRP and GPR147 mRNA levels in rHypoE-23 neurons......72

Figure 5.2 Representative model summarizing the proposed mechanism involved in the regulation of GnRH neuron activity by RFRP-3 in mHypoA-GnRH/GFP neurons ..............76
# List of Abbreviations

<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>AC</td>
<td>adenyl cyclase</td>
</tr>
<tr>
<td>Act D</td>
<td>actinomycin D</td>
</tr>
<tr>
<td>ACTH</td>
<td>adrenocorticotropic hormone</td>
</tr>
<tr>
<td>ANOVA</td>
<td>analysis of variance</td>
</tr>
<tr>
<td>AR</td>
<td>androgen receptor</td>
</tr>
<tr>
<td>ARC</td>
<td>arcuate nucleus</td>
</tr>
<tr>
<td>AVPV</td>
<td>anteroventral periventricular</td>
</tr>
<tr>
<td>bp</td>
<td>base pair</td>
</tr>
<tr>
<td>cAMP</td>
<td>cyclic adenosine monophosphate</td>
</tr>
<tr>
<td>CHO</td>
<td>Chinese hamster ovary</td>
</tr>
<tr>
<td>CHX</td>
<td>cycloheximide</td>
</tr>
<tr>
<td>C-LHIH</td>
<td>C-luteinizing hormone inhibitory hormone</td>
</tr>
<tr>
<td>CNS</td>
<td>central nervous system</td>
</tr>
<tr>
<td>CRE</td>
<td>cAMP response element</td>
</tr>
<tr>
<td>CRH</td>
<td>corticotropin-releasing hormone</td>
</tr>
<tr>
<td>DHT</td>
<td>dihydrotestosterone</td>
</tr>
<tr>
<td>DMH</td>
<td>dorsomedial hypothalamus</td>
</tr>
<tr>
<td>DNA</td>
<td>deoxyribonucleic acid</td>
</tr>
<tr>
<td>dNTP</td>
<td>deoxyribonucleotide triphosphate</td>
</tr>
<tr>
<td>DRB</td>
<td>5,6-Dichlorobenzimidazole riboside</td>
</tr>
<tr>
<td>$E_2$</td>
<td>17β-estradiol</td>
</tr>
<tr>
<td>Acronym</td>
<td>Full Term</td>
</tr>
<tr>
<td>---------</td>
<td>-----------</td>
</tr>
<tr>
<td>EDTA</td>
<td>ethylenediaminetetraacetic acid</td>
</tr>
<tr>
<td>EIA</td>
<td>enzyme immunoassay</td>
</tr>
<tr>
<td>ERα</td>
<td>estrogen receptor alpha</td>
</tr>
<tr>
<td>ERβ</td>
<td>estrogen receptor beta</td>
</tr>
<tr>
<td>ERK</td>
<td>extracellular signal-regulated kinase</td>
</tr>
<tr>
<td>FBS</td>
<td>fetal bovine serum</td>
</tr>
<tr>
<td>FSH</td>
<td>follicle-stimulating hormone</td>
</tr>
<tr>
<td>GABA</td>
<td>γ-aminobutyric acid</td>
</tr>
<tr>
<td>GCs</td>
<td>glucocorticoids</td>
</tr>
<tr>
<td>GnIH</td>
<td>gonadotropin-inhibitory hormone</td>
</tr>
<tr>
<td>GnRH</td>
<td>gonadotropin-releasing hormone</td>
</tr>
<tr>
<td>GnRH-GFP</td>
<td>GnRH-green fluorescence protein</td>
</tr>
<tr>
<td>GPCR</td>
<td>G-protein coupled receptor</td>
</tr>
<tr>
<td>GR</td>
<td>glucocorticoid receptor</td>
</tr>
<tr>
<td>GT1</td>
<td>GnRH T-antigen</td>
</tr>
<tr>
<td>HH</td>
<td>hypogonadotropic hypogonadism</td>
</tr>
<tr>
<td>HPA</td>
<td>hypothalamic-pituitary-adrenal</td>
</tr>
<tr>
<td>HPG</td>
<td>hypothalamic-pituitary-gonadal</td>
</tr>
<tr>
<td>ICV</td>
<td>intracerebroventricular</td>
</tr>
<tr>
<td>IV</td>
<td>intravenous</td>
</tr>
<tr>
<td>kDa</td>
<td>kilodaltons</td>
</tr>
<tr>
<td>LH</td>
<td>luteinizing hormone</td>
</tr>
<tr>
<td>LHRIF</td>
<td>luteinizing hormone release inhibitory factor</td>
</tr>
<tr>
<td>MAPK</td>
<td>mitogen-activated protein kinase</td>
</tr>
<tr>
<td>Acronym</td>
<td>Full Form</td>
</tr>
<tr>
<td>---------</td>
<td>-----------</td>
</tr>
<tr>
<td>ME</td>
<td>median eminence</td>
</tr>
<tr>
<td>miRNA</td>
<td>microRNA</td>
</tr>
<tr>
<td>mRNA</td>
<td>messenger RNA</td>
</tr>
<tr>
<td>mPOA</td>
<td>medial preoptic area</td>
</tr>
<tr>
<td>MS</td>
<td>medial septum</td>
</tr>
<tr>
<td>MSDS</td>
<td>medial septum/diagonal band of Broca</td>
</tr>
<tr>
<td>NPVF</td>
<td>neuropeptide VF</td>
</tr>
<tr>
<td>NTC</td>
<td>non-template control</td>
</tr>
<tr>
<td>OVLT</td>
<td>organum vasculosum of lamina terminalis</td>
</tr>
<tr>
<td>OVX</td>
<td>ovarioectomized</td>
</tr>
<tr>
<td>P</td>
<td>progesterone</td>
</tr>
<tr>
<td>PR</td>
<td>progesterone receptor</td>
</tr>
<tr>
<td>PBS</td>
<td>phosphate buffer solution</td>
</tr>
<tr>
<td>PCR</td>
<td>polymerase chain reaction</td>
</tr>
<tr>
<td>PKC</td>
<td>protein kinase C</td>
</tr>
<tr>
<td>PLC</td>
<td>phospholipase C</td>
</tr>
<tr>
<td>PVN</td>
<td>paraventricular nucleus</td>
</tr>
<tr>
<td>RFRP</td>
<td>Arginine (R)-phenylalanine (F) amide-related peptide</td>
</tr>
<tr>
<td>RNA</td>
<td>ribonucleic acid</td>
</tr>
<tr>
<td>RT-PCR</td>
<td>real-time polymerase chain reaction</td>
</tr>
<tr>
<td>SCN</td>
<td>suprachiasmatic nucleus</td>
</tr>
<tr>
<td>SV40</td>
<td>simian virus 40</td>
</tr>
<tr>
<td>T-Ag</td>
<td>T Antigen</td>
</tr>
<tr>
<td>TF</td>
<td>transcription factor</td>
</tr>
</tbody>
</table>
Chapter 1
Introduction
1.1 General Introduction

Reproduction is necessary for species survival and is therefore under the control of a myriad of neuronal, endocrine, and peripheral modulators to ensure reproductive success. The maintenance of reproductive function involves the orchestration of this complex array of neuromodulators and peripheral signals that operate at both the central and peripheral levels to communicate the physiological status of an organism. This intricate network of neuroendocrine pathways is what defines the hypothalamic-pituitary-gonadal (HPG) axis. Due to interconnectivity and crosstalk of many physiological circuits, including the HPG axis, severe challenges to the dynamic internal homeostatic mechanisms that govern one system are reflected at another level of physiologic control. Due to the large amount of energy required for reproductive development and maintenance, perturbations in metabolism or stress-related disorders result in reproductive compromise, defined by temporary or permanent infertility (1-7). In turn, reproductive pathophysiologies do not solely manifest within the HPG axis; rather, reproductive diseases are implicated in altering psychological, behavioural and metabolic status (8). The identification of the central mechanisms, whether neuronal, hormonal, or environmental that are implicated in communicating the physiological status of an organism is indispensible for advancing our knowledge of normal reproductive biology, as well as pathophysiological conditions.

The search for inhibitory reproductive effectors at the level of the central nervous system (CNS), particularly the endocrine hypothalamus have been sought out for decades. However, it was not until 2000, when Tsutsui et al. identified a novel avian neuropeptide termed gonadotropin-inhibitory hormone (GnIH) that such a molecule was successfully isolated (9). GnIH inhibits gonadotropin release from the pituitary gland in all studied avian species and a functional homologue, RFamide-Related Peptide-3 (RFRP-3) has been identified and characterized in mammals, including humans (10-12). RFRP-3 has pronounced inhibitory effects at all levels of the reproductive axis in many mammalian species and represents a novel neuropeptidergic system that integrates an array of environmental or steroid-induced signals to modulate reproductive status (9, 11, 13-17). To date, in vivo studies have been limited in the ability to dissect the direct effects of RFRP-3, mainly due to the complexity and heterogeneity of the hypothalamus. To address this, the central aim of this thesis is to delineate the molecular mechanisms involved in the direct steroidal regulation of RFRP and GPR147 biosynthesis, as
well as to examine whether RFRP-3 directly modulates GnRH neurons using two distinct in vitro models. Overall, this study aims to expand on the role of RFRP neurons in integrating steroid-induced physiological changes at the hypothalamic level, particularly the GnRH neuronal network.

1.2 The Hypothalamic-Pituitary-Gonadal Axis

The HPG axis is based on the paradigm of negative and positive feedback mechanisms, as well as the interconnectivity of multiple organs, which is a unifying principle for all endocrine systems. In 1932, Moore and Price proposed a reciprocal interplay between the testes and pituitary gland (18, 19). They described the importance of steroid feedback on the pituitary, as well as the necessity of the gonadotropin hormones, luteinizing hormone (LH) and follicle-stimulating hormone (FSH) for steroidogenesis, gonadal development, and function. Walter Hohlweg and Karl Junkmann expanded on these initial studies, including the CNS as an integral modulator of reproductive mechanisms (18). It became apparent that gonadal steroids feed onto neuronal pathways, which in turn act on the anterior pituitary to regulate the release of the gonadotropin hormones. The gonadotropins further act to control gonadal functions such as, follicular development, ovulation, spermatogenesis, and steroidogenesis. G.W. Harris later deemed the hypothalamus to be the control site and essential component of the reproductive axis, integrating a multitude of stimuli (20). Indeed, electrode stimulation of either the hypothalamus or the anterior pituitary induces successful ovulation in rabbits (20). Intriguingly, it was further suggested that the hypothalamus was not only necessary for the control of reproduction, but the existence of hypothalamic factors that regulate the pituitary were indispensable in reproductive function (18, 19, 21). In fact, the hypothalamus is composed of a multitude of unique neuronal populations, each expressing an array of neuropeptides and neurotransmitters necessary for incorporating central and peripheral modulatory signals. The hypothalamus is subdivided into specific nuclei that are interconnected to effectively regulate centrally-mediated physiological processes and overall homeostasis (22). Of interest, the discrete population of cell bodies that express and secrete GnRH, the master endocrine molecule that controls the release of the gonadotropic hormones (23, 24). GnRH neurons are localized mainly to the preoptic area (POA) in rodents and the mature peptide is secreted as pulses from the nerve terminals into the hypophyseal portal system, acting as a neuroendocrine factor to stimulate the release of LH and
FSH via its G-protein-coupled receptor (GPCR), GnRH-R (25-28). In females, the basal levels of gonadotropins regulate folliculogenesis, corpus luteum formation, and steroidogenesis, whereby higher levels of LH induce ovulation (29, 30). In males, LH is responsible for Leydig cell differentiation and the production of testosterone, while FSH is the main modulator of spermatogenesis. (Figure 1.1) (30, 31).

1.2.1 GnRH: Hypophysiotropic Regulator of the HPG axis

The decapeptide, GnRH or luteinizing-hormone-releasing hormone (LHRH) was originally isolated in mammals by the independent laboratories of Andrew Schally and Roger Guillemin (23, 24). To ensure proper function and connectivity, GnRH neurons undergo a unique and precisely orchestrated developmental pattern (32). Originating in the olfactory placode, GnRH neurons migrate along vomeronasal nerves, crossing the cribiform plate into the medial forebrain and than caudally into the hypothalamus (33, 34). The small heterogeneous population of 800-1000 GnRH neurons takes residence mainly in the medial preoptic area (mPOA) of the mammalian hypothalamus (35-38). Retrograde tracing and immunochemical staining studies have shown that up to 70% of GnRH neurons extend long processes towards the external neurosecretory zone of the median eminence (ME). The ME, located at the base of the hypothalamus represents an important interface between neuronal pathways and endocrine function (39). A small population of GnRH neuronal terminals is also found near the organum vasculosum of the lamina terminalis (OVLT), a region outside the blood-brain-barrier (40-42); thus, suggesting GnRH neurons play a multifaceted role in integrating a diverse array of physiological signals. Failure of GnRH neuronal migration and the development of the intricate neuronal network, results in hypogonadotropic hypogonadism (HH). HH is mainly characterized by low gonadotropin and sex steroid levels, as well as delayed or complete absence of sexual maturity, caused by the underlying inability to activate the pulsatile secretion of GnRH at the time of puberty (43). The underlying mechanisms of HH mostly involve genetic mutations in genes involved in GnRH neuron migration, secretion, and action at the pituitary. Interestingly, approximately 60% of HH causes are associated with the well-documented genetic disorder Kallman syndrome, where genes encoding proteins involved in GnRH neuronal migration are mutated (44, 45).
In brief, neuropeptidergic, hormonal, metabolic, and environmental changes are reflected in alterations in GnRH neuronal activity. GnRH neurons represent the final modulatory pathway governing the HPG axis. GnRH is secreted in a pulsatile manner into the hypophyseal system of the ME, which vascularizes the anterior pituitary. There, GnRH acts by interacting with its GPCR, GnRH-R found on the gonadotropes to stimulate the release of the gonadotropic hormones, LH and FSH. LH and FSH are released into general circulation where they further act on the gonads to mediate gonadal development and maintenance, gametogenesis, as well as steroidogenesis. The gonadal steroids participate in a feedback mechanism at all levels of the HPG axis, regulating hormonal levels.
With such a firmly established role in fertility and reproductive success it is imperative to understand the nature and regulation controlling the GnRH gene and peptide. GnRH is one of the oldest and most phylogenetically conserved peptides, found in species ranging from *Saccharomyces cerevisiae* (yeast) to humans (46). In mammals, two forms of the GnRH gene exist, GnRH-I and GnRH-II. Both genes are produced as a prohormone and share a common structure encompassing 4 exons that encode a signal peptide, the mature GnRH peptide, and a GnRH-associated protein (GAP) (47-49). The coding sequence for mouse GnRH-I maps to chromosome 14, while a homologous region is located on chromosome 8 in humans (50).

Mammalian GnRH also referred to as GnRH-I, is the gene encoding the decapeptide that is recognized as the fundamental endocrine molecule regulating reproductive activity. GnRH is required for normal reproductive function, evident in the hypogonadal mouse model (32, 51, 52). Of interest, these animals display a recessive mutation in the GnRH-I gene, which results in an underdevelopment of the reproductive tract in both sexes, sterility, and remarkably reduced plasma levels of the gonadotropic hormones, all of which is consistent with the HH phenotype. Interestingly, GnRH-II is the more conserved and evolved form of GnRH, originally isolated in chickens (53). In humans, GnRH-II is expressed in all structures of the brain stem and hypothalamus, as well as in the periphery (54). Due to its disperse localization patterns, it has been suggested that GnRH-II is most likely involved in neuronal networks integrating energy homeostasis and reproductive behaviour (55, 56). Recent studies also suggest GnRH-II can play a complementary role to GnRH by stimulating LH and FSH *in vivo*, even though GnRH-II receptors are silenced in humans (57). Therefore, more studies focusing on the functional role of GnRH-II are necessary in vertebrates.

As mentioned previously, neuroendocrine control of reproduction requires the pulsatile secretion of GnRH, a phenomenon that is initiated at the time of puberty and results in episodic gonadotrophic release from the anterior pituitary (58-60). Wildt *et al.* initially demonstrated that pulsatile perfusions of GnRH can initiate puberty in female rhesus monkeys (61). Moreover, stimulation of a normal menstrual cycle and the recovery from abnormal ovarian anatomy in a patient with Kallman syndrome can be achieved solely with the administration of GnRH every 2 hours (62). The pulsatile nature of GnRH, ranging from every 30 minutes (min) to 2 hours (h) in different species, has been extensively studied. Of interest, the episodic mode of the GnRH neuronal network is an intrinsic feature of the GnRH neuron, independent of glial or neuronal
innervation (63, 64). Remarkably, in vitro studies utilizing the clonal, immortalized GnRH T-antigen (GT1) cell lines reported that individual GnRH neurons rhythmically release GnRH and the synchronization of this secretion is mediated by cell-to-cell contact mechanisms (65). The central importance of GnRH and the finely orchestrated phasic pattern required for reproductive function is undisputed; hence, the study of factors modulating GnRH synthesis and secretion is of great relevance (66, 67).

1.3 Regulators of GnRH synthesis and release

1.3.1 Gonadal Steroids

The gonadal steroids represent one of the most essential regulatory pathways in the HPG axis, particularly at the level of the CNS. The three main sex steroid classes, including estrogens, androgens, and progestogens initiate both stimulatory and/or inhibitory mechanisms in the hypothalamus, particularly directly or indirectly at the level of the GnRH neuron. All three classes of sex steroids are synthesized mainly in the gonads; though, extraglandular biosynthesis is found in the adrenal glands, placenta, and regions of the brain (68).

1.3.1.1 Estrogens

Estrogen plays a quintessential role at all levels of the reproductive axis, displaying multifaceted effects required to maintain and achieve reproductive and developmental competence (46). Estrogen is primarily synthesized in the ovaries, though extragonadal sites exist (68). In females, de novo synthesis of the precursor hormone, androstenedione from cholesterol occurs in the thecal cells of the ovaries (69). Due to its weak steroidogenic activity, androstenedione crosses the membrane into neighboring granulosa cells where it is readily converted by P450 aromatases and 17β-hydroxysteroid dehydrogenases into estrone and testosterone, respectively. Estrone is further converted into 17β-estradiol (E2) by 17β-hydroxysteroid dehydrogenase. Conversely, P450 aromatases catalyze the formation of E2 from testosterone. E2 is the most physiologically active estrogen and exerts its effects through binding and activating the nuclear estrogen receptors (ERs) (70-74) or a GPCR, GPR30 (75, 76). The ERs, ERα and ERβ are members of the superfamily of nuclear receptors, which act as transcription factors (TF) to regulate genes containing an estrogen response element (ERE) (70-73). ERα knockout (ERαKO) mouse models
display significant gonadal dysfunction, as well as impaired steroidal feedback. Homozygous deletions result in infertility and display high circulating levels of gonadotropin and steroid hormones, implicating ERα signaling as the main driver of negative feedback (77-80). On the contrary, disruption of ERβ-mediated signaling, as observed in the ERβKO mouse model results in subfertility, though steroid and gonadotropin hormone levels are comparable to controls (81-83). Although ERβ may be involved in other processes, its essential role in estrogen feedback is disputable; however, it is plausible that redundancy exists in ER signaling and ERβ may influence other E2-mediated effects at the central level.

It is well established that E2 has a bimodal effect on the hypothalamus, exerting positive and negative feedback mechanisms on both the GnRH neuronal network directly and via synaptic cell bodies (84). For the majority of the female cycle, both in vivo (85-87) and in vitro (88-90) studies have shown that basal E2 levels inhibit GnRH synthesis and secretion. Conversely, during the late follicular phase, E2 levels rise and initiate a stimulatory feedback system leading to an increase in GnRH pulse frequency and release, known as the GnRH surge (91-93). Despite the necessity of E2 signaling in the regulation of GnRH, the mechanisms by which this occurs are controversial. Although initial studies in the guinea pig reported the existence of E2-responsive GnRH neurons (94), subsequent reports in vivo have established that many mammalian species show little to no co-expression of GnRH and ERs in native GnRH neurons (95-97). These studies put forth the hypothesis that E2 regulation, both inhibitory and stimulatory occurs strictly through innervating afferent circuits. However, immunocytochemical studies have shown that GnRH neurons in vivo express ERβ (98, 99). Similarly, the GT1-7 cell model expresses both ERα and ERβ (89, 100). Herbison et al. also reported ERα and ERβ mRNA expression in both prepubertal and adult GnRH neurons using single cell RT-PCR (101). Further, 10-20% of GnRH neurons contain nuclear ERβ immunoreactivity in humans and the human GnRH gene contains a functional ERE, suggesting a potential direct ER-mediated mechanism (102, 103). In line with a direct regulatory role, in vitro studies indicate that E2 directly suppresses GnRH mRNA levels at low concentrations through an ERα-mediated signaling cascade (89, 104). Together, these studies provide credence for direct estrogen-mediated regulation of GnRH neurons. Though, the significance in vivo and precise cellular signaling cascades remain to be determined.
1.3.1.2 Androgens

Testosterone is an androgen produced mainly in the Leydig cells of the testis in males; though, small amounts are produced in the ovaries (105). Testosterone is converted intracellularly into dihydrotestosterone (DHT) in most androgen-sensitive cells containing the $5\alpha$-reductase type 2 enzyme (106). Androgens, particularly DHT, operate both at the level of the pituitary to limit the release of the gonadotrophic hormones (107) and the hypothalamus to restrict GnRH neurosecretion (108, 109). Despite the importance of androgens, the presence of the androgen receptor (AR) directly on GnRH neurons is controversial and in vivo studies have been unsuccessful in detecting co-expression of GnRH and AR (110). Intriguingly, in vitro models have demonstrated the expression of functional ARs, as well as the $5\alpha$-reductase type 2 enzyme (106, 111). Similarly, DHT treatment induces GnRH repression through an indirect tethering interaction with Oct-1 at the level of the GnRH 5’ regulatory region (111, 112). Thus, within the hypothalamus it is apparent that androgens are integral regulators of GnRH synthesis and peptide availability. Though, like estrogens our knowledge regarding the precise mechanisms in vivo are incomplete.

1.3.1.3 Progesterone

Progesterone (P) is the predominant gonadal steroid present during the luteal phase of the menstrual cycle and is secreted by the corpus luteum; however, de novo synthesis of progesterone exists in the central nervous system (113-115). Elevated circulating levels of P exhibit a potent and rapid inhibition of the GnRH and LH surge (116). The rapid suppression by P is dependent on prior estrogen exposure, leading to an increase in the progesterone receptor (PR) (116). Interestingly, the E$_2$-stimulated increase in PR expression is necessary for stimulatory estrogen feedback, driving the LH surge (117, 118). Further, E$_2$ induces the hypothalamic synthesis of progesterone, referred to as the “pre-ovulatory progesterone” surge that has been implicated in the activation of hypothalamic neuropeptidergic circuits mediating GnRH neuronal activity (117, 119). P has also been implicated in the maintenance of low GnRH levels during the wake period of early puberty (120); however, the mechanisms by which P exerts its effects are still controversial, similar to that of other gonadal steroids. Native GnRH neurons have not been found to possess the nuclear PR in ewes or rodents (121), though a small population of 20% of GnRH neurons expresses immunoreactive PR in the guinea pig (122). In


Vivo studies have demonstrated that the PR antagonist, RU-486 partially (123) or completely (116) blocks P-mediated inhibition of GnRH secretion. Further, P-induced suppression of GnRH neurosecretion persists in the presence of GABA and glutamate AMPA/NMDA receptor antagonists, bicuculline and CNQX/AP5, respectively (116, 123). These findings demonstrate that P-induced regulation is independent of other neuropeptidergic or neurotransmitter inputs. Intriguingly, a recent study using the transgenic GnRH-GFP mouse model has shown that GnRH neurons express a membrane bound progesterone receptor, progesterone receptor membrane component 1 (PgRMC1) (123). The PgRMC1 receptor is implicated in the rapid actions of P, mediating the reduction in calcium influx and inhibition of neuronal firing in GnRH neurons (123, 124). These studies highlight the importance of P signaling at the central hypothalamic level and in maintaining overall normal reproductive states; however, the receptor-specific mechanisms remain under investigation.

1.3.2 Innervating Neuronal Systems

The primary mediators of GnRH biosynthesis and neurosecretion, apart from direct steroidal feedback are innervating hypothalamic circuits (125). It has been well documented that steroid-responsive neuronal populations, expressing ERα, ERβ, AR, and PR receive hormonal input from the gonads and make synaptic contacts with subpopulations of GnRH neurons in the POA to drive normal reproduction (126-128). The most commonly studied neuronal circuits, include γ-aminobutyric acid, glutamate, kisspeptin, and more recently GnIH/RFRP-3. It is important to mention other neuropeptides that are involved in the direct regulation of GnRH and conveying steroidal feedback such as, neuropeptide Y (129), noradrenaline (130), and neurotensin (95, 131).

1.3.2.1 γ-aminobutyric acid

γ-aminobutyric acid (GABA) is the predominant inhibitory neurotransmitter in the CNS of all vertebrates (132). Early studies by Flugge et al. demonstrated that a population of E2-responsive neurons is present in the mPOA, identifying GABA as a primary candidate in relaying E2 feedback directly to GnRH neurons (133-136). Native GnRH neurons express functional GABA_A receptors and are responsive to treatments with GABA or bicuculline, a GABA_A receptor antagonist (137, 138). E2 stimulates a 50% increase in GABA release in the mPOA,
which coincides with a decrease in LH secretion and pulse frequency (135, 136). Intriguingly, studies in female rhesus monkeys and mice have shown that GABA directly suppresses GnRH release only following the onset of puberty (139, 140). Exposure to 30 – 100 µM of GABA depolarizes GnRH neurons in immature female mice, yet induces hyperpolarization in the adult, as recorded by perforated-patch cell recordings (141). Further, it has been reported that blockade of endogenous GABA<sub>A</sub> receptor signaling by 20 µM bicuculline increases neuronal firing in 80% of GnRH neurons in both sexes (137). Collectively, these studies demonstrate the prominent role of GABA as an E<sub>2</sub>-responsive neuronal population and critical reproductive neuronal factor (142).

1.3.2.2 Glutamate

Glutamate, like GABA is an amino acid (aa) neurotransmitter and the principal excitatory signal in the neuroendocrine hypothalamus (143). Stimulation of GnRH neurons with pulsatile NMDA, a glutamate agonist for 16-30 weeks results in the onset of precocious puberty and functional activation of the HPG axis in female rats (144, 145). Glutamatergic neurons make synaptic contacts within hypothalamic nuclei controlling neuroendocrine functions including, the arcuate nucleus (ARC), paraventricular nucleus (PVN), and the mPOA (143). In situ hybridization studies show that 80% of GnRH neurons co-express NMDA-R mRNA, a specific subtype of glutamate receptor (146). Glutamate treatment results in a rapid NMDA-mediated induction of GnRH mRNA levels and LH release (146-148). Further, NMDA-R antagonist, MK801 causes a delay in puberty, inhibits the LH surge, and decreases cytoplasmic GnRH mRNA levels in rats and primates (148, 149). In contrast, in vitro studies employing the GT1-7 cell line demonstrated that NMDA treatment results in the transient cyclic guanosine monophosphate/nitric oxide (NO)-mediated repression of GnRH gene expression (150). Of interest, it has also been suggested that glutamatergic neurons are E<sub>2</sub>-responsive and may provide stimulatory feedback onto GnRH neurons prior to the surge (151); however, E<sub>2</sub> treatment in the presence of MK801 fails to block E<sub>2</sub>-mediated changes in GnRH activity (146). Overall, it has been established that glutamate neurons play a larger role in mediating the basal stability of GnRH mRNA levels, rather than participating in E<sub>2</sub>-mediated stimulatory feedback (125, 146, 151).
1.3.2.3 Kisspeptin

Kisspeptin (Kiss) is the product of the KISS-1 gene and was originally identified as a tumor metastasis suppressor (152). Kiss belongs to the RFamide group of peptides, like GnIH and RFRP-3 (discussed in sections 1.4 and 1.5) and is the natural ligand for the GPCR, GPR54 (153, 154). The Kiss/GPR54 system has an established role in the neuroendocrine regulation of GnRH secretion, whereby mutations in the GPR54 gene result in low levels of plasma gonadotropic and gonadal hormones, delayed puberty, and defective sexual organ development in both humans and mice (153, 154). KISS-1 mRNA is expressed throughout the CNS, particularly the ARC and anteroventral periventricular (AVPV) hypothalamic nuclei, as well as the placenta (155, 156). Accordingly, GPR54 expression is highest in the hypothalamus and hippocampus (157). Using in situ hybridization and a transgenic GPR54 LacZ knock-in mouse model, many groups have identified that 60 - 90% of GnRH neurons express GPR54, implicating Kiss neurons as upstream mediators of GnRH (157). Intriguingly, intraperitoneal and ICV injection of kisspeptin-10 (Kiss-10) or kisspeptin-54 (Kiss-54), variants of the cleaved endogenous Kiss gene product, increases both LH and FSH levels, as well as induces neuronal activation and secretion of GnRH in rodent models (158-161). Furthermore, infusion of an antimetastin antibody into the POA of ovariectomized (OVX) rats suppresses the LH surge and leads to abnormal estrous cyclicity (162). In vitro studies utilizing the GT1-7 and GN11 cell models corroborate in vivo data by demonstrating the direct stimulatory effect of Kiss-10 on GnRH mRNA synthesis and secretion (163). Interestingly, the central actions of Kiss are blocked by the administration of acyline, a GnRH-R antagonist, verifying its role as an essential upstream afferent mediator of GnRH neurons (158).

Due to the anatomical distribution of Kiss in the hypothalamus, its role in steroidal feedback has been extensively studied. Smith et al. identified that 99.8 % and 98.7 % of Kiss neurons in the ARC and AVPV, respectively, express ERα; however, only 25% of ARC and 30% AVPV Kiss neurons co-express ERβ (127). E2 demonstrates anatomically specific effects on Kiss; attenuating both Kiss biosynthesis and cell number in the ARC, while simultaneously inducing gene expression and cell number in the AVPV. Intriguingly, knockout models bearing deletions in ERα and ERβ have revealed that the duality of E2 regulation on Kiss neurons is mediated by ERα (127). Of interest, both the stimulatory and inhibitory effects of E2 on Kiss are absent in the
ERαKO, but not the ERβKO mouse. Due to the importance of Kiss populations in steroidal feedback, the involvement of the Kiss/GPR54 signaling cascade in pubertal onset is well documented (160, 164-166). However, a recent study by Mayer and Boehm has called into question the necessity of Kiss signaling in pubertal onset. Mice with ablated Kiss neurons, KissIC/R26-DTA demonstrate a reduced ovarian mass, but otherwise normal fertility and litter size; suggesting that normal reproductive development can occur in the absence of Kiss (167). Conversely, acute ablation of Kiss neurons in adult mice causes infertility, verifying the essential role of Kiss neuronal signaling in the integration of physiological cues to GnRH neurons in the adult. Overall, these studies corroborate the indispensible role of Kiss and GPR54 signaling in the neuroendocrine regulation of mammalian reproduction.

1.4 Gonadotropin-Inhibitory Hormone: A Negative Regulator of Avian Reproduction

Until recently, GnRH was believed to be the sole hypophysiotropic factor regulating gonadotropin synthesis and release (23, 24). Early attempts isolating an inhibitory hypothalamic factor that attenuates LH release had been made, though none were successful. In 1975, Johansson et al. isolated a new entity, C-luteinizing hormone inhibitory hormone (C-LHIH) from porcine hypothalamic fragments, which could inhibit LH release at concentrations of 100-500 nanograms (168). However, the precise function of this peptide was undetermined. Similarly, Hwan and Freeman isolated a 12 kilodaltons (kDa) peptide from rat hypothalamus termed, LH release-inhibiting factor (LHRIF), which inhibited the actions of GnRH both in cultured anterior pituitaries and in vivo (169). Though, after these studies the search for such an inhibitory hypothalamic peptide was abandoned for decades.

1.4.1 Discovery and Initial Characterization

In 1977, Price and Greenberg identified the first cardioexcitatory peptide containing the sequence, Arg-Phe-NH₂ (RFamide) at the C-terminal in the ganaglia of Macrocallista nimbosa (170). The first vertebrate RFamide peptide was identified in chickens, playing a critical role in attenuating blood pressure and altering neuronal firing activity (170, 171). These findings were among the first to provide credence for the potential role of RFamide peptides in mammalian neuroendocrine function at the level of the CNS and periphery (172, 173).
In 2000, a previously unknown RFamide neuropeptide was identified and isolated from the Japanese quail (*Coturnix japonica*) (9). The dodecapeptide (SIKPSAYLPLRFamide) was localized to the diencephalon, particularly the hypothalamic PVN of the quail (9, 174) and other avian species (175, 176). Additionally, some immunoreactive fibers were present in the POA and ME. Tsutsui *et al.* further demonstrated that this novel peptide significantly suppresses the release of LH in a rapid and dose-dependent manner in cultured pituitaries (9). Due to this unique inhibitory role within the avian hypothalamo-hypophysial system, the neuropeptide was named GnIH (176, 177).

### 1.4.2 Identification of the GnIH-R

The receptor for GnIH, GPR147 (OT7T022 or NPFFR1) belongs to the family of GPCRs displaying a characteristic seven transmembrane domain structure (10). GPR147 exhibits a vast expression pattern in the CNS, the anterior pituitary gland, and the reproductive organs (176, 178, 179). GPCR signaling mechanisms are well characterized and couple with three main subunits, $G_{\alpha q}$, $G_{\alpha s}$, and $G_{\alpha i}$, each exhibiting a unique signaling cascade. Initial studies in mammals reported on the ability of GnIH to interact with previously characterized neuropeptide FF receptors, NPFFR1 (GPR147) and NPFFR2 (180); however, GnIH displays the highest affinity and signal potency with GPR147, even compared to neuropeptide FF (10, 181). COS-7 cells transfected with GPR147 confirmed the receptor ligand specificity to C-terminal amidated (LPXRF-amide) GnIH products in quail (182). Upon activation, GPR147 triggers the inhibition of cyclic adenosine monophosphate (cAMP) production by coupling with the $G_{\alpha i}$ subunit, which inactivates adenylyl cyclase (AC) (10, 181). These seminal findings substantiate the idea that GnIH exerts an overall inhibitory role in the reproductive axis; however, the precise molecular mechanisms that link this inhibitory signaling cascade to the overall inhibition of the HPG axis are not well established at the hypothalamic level.

### 1.4.3 GnIH Actions in the Pituitary

The inhibitory role of GnIH at the level of the gonadotropes has been well documented. In the pituitary, GPR147 immunoreactive cells co-localize with both LHβ and FSHβ mRNA in quail and chickens (179). Further, the favourable neuroanatomical positioning of GnIH terminals at the ME permits the suppression of gonadotropin synthesis and release, as well as alters the
sensitivity of the pituitary to GnRH stimulation. GH3 cells co-transfected with the predominant GnRH-R in chicken pituitaries, cGnRH-III-R and GPR147 demonstrated a dose-dependent reduction in GnRH-induced cAMP response element (CRE) luciferase activity, when simultaneously stimulated with GnIH and GnRH. Indeed, GnIH is implicated in reducing cAMP production and cytoplasmic calcium mobilization initiated by the ligand-activated GnRH-R, Ga_s and Ga_q pathways (183, 184). Furthermore, the ratio of cGnRH-III-R to GPR147 strongly dictates the inhibitory ability and sensitivity of GnIH in vitro; hence, as the ratio of GnIH-R/cGnRH-III-R increases the CRE-luciferase activity decreases. In accordance with in vitro studies, intravenous (IV) injection of quail GnIH results in a rapid reduction of plasma LH concentrations in sparrows (175). Thus substantial evidence exists indicating that GnIH signals through a classical inhibitory GPCR pathway, exerting its anti-gonadotropic effects by antagonizing the GnRH signaling cascade at the pituitary (183, 184).

1.4.4 GnIH Actions in the Brain

The highest expression of GPR147 and immunoreactive GnIH cell bodies is found in the hypothalamus, a feature conserved across all avian species (176, 177, 185, 186). GnIH axon terminals are found in close proximity to GnRH-I and GnRH-II neurons in the European Starling brain, suggesting a conserved central mechanism through which GnIH controls gonadotropin release and reproductive behaviours (176, 177). In situ hybridization studies further reveal that approximately 80.5% and 89.4% of GnRH-I and GnRH-II neurons respectively, co-express GPR147 mRNA (176). ICV injection of GnIH rapidly reduces LH plasma levels, as well as significantly reduces copulation solicitations in female white crowned sparrows (178). Though these studies are in accordance with a hypophysiotropic effect of GnIH, the direct GnIH-mediated inhibition of GnRH activity at the level of the GnRH neuron, remains poorly understood; however, it is evident that the inhibitory role of GnIH is partly mediated at the hypothalamic level.

1.4.5 GnIH Actions on the Gonads

Many neuropeptides involved in the HPG axis function directly at the level of the gonads (187). Bentley et al. identified both GnIH and Gpr147 mRNA in the ovary, testes, epididymis, and vas deferens of many avian species (187, 188). Peripheral GnIH administration significantly reduces...
LH and FSH mRNA levels, as well as induces testicular apoptosis, particularly in germ cells and Sertoli cells in males (189). In addition, GnIH suppresses spermatogenic activity, which also leads to a decrease in the overall diameter of the seminiferous tubules and a failure to develop adult plumage, a sign of immaturity in avian species (189). GnIH further inhibits LH-induced testosterone secretion both *in vivo* and *in vitro* (188). These studies indicate that along with the indirect role of GnIH on steroidogenesis through central mechanisms, GnIH may evoke direct inhibition in the gonads (187-189). Taken together, these studies reveal a potential paracrine and/or autocrine role for GnIH at the gonadal level, regulating the reproductive organs during periods of quiescence or poor physiological status. The precise functional significance and molecular mechanism of GnIH at the level of peripheral organs in avian species is yet to be confirmed.

### 1.5 RFRP-3: The mammalian homologue of avian GnIH

#### 1.5.1 Initial Characterization of RFRP-3 in the mammalian HPG axis

Due to the importance of central mechanisms in regulating the reproductive axis, further confirming the role of GnIH in mammals was necessary. Hinuma *et al.* utilized the gene database, GenBank/EMBL to classify and isolate human, bovine, and rodent complementary DNA encoding the novel RFamide peptide in mammalian species (10). The mammalian *Rfrp* gene (also known as *Npvf*) encodes a prohormone that generates two physiologically active RFRPs, RFRP-1 and -3. The N-terminal portions of GnIH and RFRP peptides show diversity in aa sequence and length among species; however, the minimum sequence, PNLPQRFamide is conserved and has the same biological activity as the full-length peptide (181). Studies in Chinese hamster ovary (CHO) cell lines expressing various orphan GPCRs, reported on the ability of both RFRP-1 and RFRP-3 to bind to GPR147 (10, 181). Indeed, GPR147 was found to bind exclusively to RFRP-1 and RFRP-3 ligands in rat, bovine, and human species inhibiting cAMP production (10). Expression of GPR147 is localized to the hypothalamus, pituitary, and gonads in mammals (11, 15, 16). Interestingly, following these initial reports, studies regarding this novel neuropeptidergic system focus on RFRP-3 as the functional homologue of GnIH, whereby the physiological role of RFRP-1 remains largely unexplored.
To determine whether RFRP-3 represents the functional mammalian homologue of avian GnIH, Kriegsfeld et al. characterized the distribution of RFRP-3 cell bodies and fibers in rodents (16). Immunohistochemical studies revealed that RFRP-3 immunoreactive fibers and cell bodies are concentrated in the dorsomedial nucleus of the hypothalamus (DMH) with terminals extending to the ME and medial brain regions such as, the diagonal band of Broca, POA, and anterior hypothalamus (16). RFRP-3-terminals have been visualized in the neurosecretory zone of the ME in hamsters, sheep, and primates, though this feature is disputed in rats (190). Nevertheless, both IV and ICV injection of RFRP-3 results in a rapid and potent repression of LH release. Centrally, a large portion of RFRP-3 axonal terminals project onto GnRH cells, suggesting a direct inhibitory role in the hypothalamus (16). Therefore, as seen in avian studies, RFRP-3 displays a similar neuroanatomical distribution in the CNS and potentially functions as a hypophysiotropic neuroendocrine molecule to modulate the mammalian reproductive axis.

1.5.2 Role of RFRP-3 in the Pituitary

Due to the similarities in the neuroanatomical distribution of RFRP-3 across all studied species, many functional studies have investigated the target tissues and direct effects of RFRP-3. Peripheral injections of both GnIH and RFRP-3 suppress LH release (12, 14, 191). Moreover, RFRP-3 effectively suppresses GnRH-stimulated LHβ and FSHβ gene transcription, inhibits LH release, as well as attenuates GnRH-stimulated ERK phosphorylation (12, 16, 192-194). Smith et al. recently set precedence for the role of RFRP-3 as a neurohormone, demonstrating its presence exclusively in the hypophyseal portal circulation of ewes (16). Further, it was reported that RFRP-3 has a short peptide half-life (6 min) and is secreted as pulses, suggesting that RFRP-3 directly opposes the actions of GnRH at the pituitary and is indisputably a neuroendocrine modulator of reproduction in this species (16). Whether this is a conserved feature in all mammals requires further investigation. Impairment of RFRP-3 signaling upon the central administration of a novel GPR147 receptor antagonist, RF9 (10^{-6} M), results in the stimulation of LH release in vivo (14). Conversely, recent studies in male Syrian hamsters reported a dose-dependent increase in both LH and testosterone release following ICV injection of RFRP-3; therefore, the role of RFRP-3 as a mediator of pituitary function is likely species and sex-specific, implicating the necessity of other peripheral hormones in its actions (195). Overall, the precise mechanisms involved in the role RFRP-3 at the level of the gonadotropes remain to be
investigated; however, it is likely that the central neuroendocrine effect of RFRP-3 is a more conserved feature of this neuropeptidergic system.

1.5.3 Role of RFRP-3 in the Brain

The ability of RFRP-3 to influence GnRH neurons is now a well-accepted feature of this neuropeptide circuit. RFRP-3 neurons directly appose GnRH-expressing cell bodies in the POA (126, 176, 196). RFRP-3-immunoreactive fibers extend to a large percentage of GnRH neurons, approximately 40% in Syrian hamsters (12). The same relationship is observed within the ovine and murine hypothalamus, whereby 63-75% of Rfrp-expressing neurons are in direct apposition to GnRH cell bodies (196). In accordance with avian studies (176), Rizwan et al. demonstrated that subpopulations of GnRH and AVPV Kiss neurons express Gpr147 (197). Moreover, RFRP-3 (1 µM) evokes a direct and rapid suppression of neuronal firing rate in 41% of GnRH neurons in mice, whereby the addition of GABA or glutamate receptor antagonists has no effect; thus, suggesting a direct RFRP-3-mediated mechanism that is independent of upstream afferent effectors (198). Unexpectedly, RFRP-3 simultaneously causes no change or an increase in neuronal activity in 47% and 12% of GnRH neurons, respectively. Such heterogeneity in GnRH neurons has previously been reported in the GnRH-secreting, GT1-7 model (199). Further, RFRP-3 causes hyperpolarization of vesicular glutamate receptor transporter 2 (vGluT2)-expressing GnRH neurons via postsynaptic mechanisms, involving the potassium channel, where it also attenuates Kiss-mediated activation of GnRH neurons in the medial septum/diagonal band of Broca (MSDS) (200). Central RFRP-3 infusion (25ng/h) results in a dramatic suppression of c-Fos expression, a physiological marker for neuronal activation in GnRH and AVPV neurons during an E2-induced surge in rats (192). Collectively, these results indicate that the central effects of RFRP-3 significantly contribute to the inhibitory mode of action of this neuropeptide system and are likely mediated through the suppression of GnRH neural activity. Currently, there is a lack of studies reporting on the cellular mechanisms involved in the RFRP-3-mediated hypothalamic regulation of GnRH.

1.5.4 Role of RFRP-3 in the Gonads

A potential role of GnIH in the reproductive organs was established in avian species. In the mammalian testis, Rfpr and Gpr147 gene expression is found exclusively in the seminiferous
tubules, an area involved in the formation of the gametes (201). Immunohistochemical studies described the presence of RFRP-3 in the ovaries of mice, concentrated mainly in the granulosa and thecal cells of large antral follicles (202). Interestingly, GnIH treatments (1 – 100 ng) cause a suppression of GnRH-R expression in ovaries in vitro. Further, treatment of primary human granulosa-lutein cells with RFRP-3 (10 – 100 nM) inhibits gonadotropin-induced progesterone production, as well as attenuates the expression of StAR, the enzyme responsible for the rate-limiting step in steroidogenesis (203). Overall, these studies suggest that RFRP-3 may participate in autocrine or paracrine mechanisms in the reproductive organs; however further studies are required to determine the functional significance and implications of RFRP gonadal expression.

1.6 Regulation of RFRP-3/GnIH in vertebrates

Reproductive success is reflected in an organism’s ability to maintain homeostasis during fluctuating and severe environmental or physiological challenges (204). The GnIH/RFRP-3 neuropeptidergic circuit has emerged as a modulator of reproductive function. Further, there is mounting evidence supporting the influential role of Rfrp neurons in steroid and environmental-induced reproductive effects (13, 126, 205-208).

1.6.1 Circadian and Seasonal Changes

Reproduction, like other physiological processes depends on the precisely timed coordination of a myriad of effectors. Photoperiodic, or day length changes are one of the most critical factors required for reproductive adaptations in many species (204). Such adaptations allow animals to remain sexually quiescent during the winter or short day (SD) lengths, whereby long day (LD) lengths stimulate reproductive activity and behaviour (209-211). Melatonin, a hormone produced by the pineal gland in response to the length of night provides the entrainment of environmental cues and endogenous circadian rhythms (212). Ubuka et al. demonstrated that the absence of endogenous melatonin, achieved by pinealectomy (removal of the pineal gland) and orbital enucleation (removal of the eyes), results in a significant decrease in GnIH mRNA in the PVN of avian species (213, 214). Upon exogenous administration of melatonin to animals that underwent both a pinealectomy and orbital enucleation, GnIH levels are significantly recovered (214). Furthermore, GnIH neurons express melatonin receptor 1C (Mel 1C) and ICV administration of melatonin stimulates hypothalamic GnIH mRNA levels in vivo (177, 214). Interestingly,
endogenous gonadal expression of GnIH and GPR147 are also dependent on seasonal changes, whereby GnIH reduces LH and FSH-stimulated testosterone secretion in male European starlings only during the non-breeding season (215). Similarly, in photoperiodic mammals, such as Syrian hamsters SD length results in reproductive regression. Contrary to avian studies, sexually quiescent hamsters exposed to consistent SD lengths demonstrate testicular regression and low expression levels of GPR147, as well as melatonin-independent RFRP-3 release (204, 216). In contrast, sheep are classified as SD breeders and demonstrate low GnIH expression throughout the SD breeding season, accompanied by a significant increase in GnIH expression during LD periods; suggesting maximal activity of GnIH during the sexually quiescent period in ewes (217). Therefore, contention exists whether GnIH operates in photoperiodic vertebrates mainly due to the species-specific effects that exist. However, it is plausible that GnIH/RFRP-3 acts only in the initial suppression of reproductive function in some mammals, while also contributing to the maintenance of a reproductive quiescent state in others (204).

In addition to photoperiod, the circadian clock is an essential component of reproductive survival in both photoperiodic and non-photoperiodic mammals. The circadian clock is responsible for daily rhythmic changes and the ovulatory cycle in females. The master regulator governing the daily biological changes is found within the suprachiasmatic nucleus (SCN) of the hypothalamus (218, 219). Though the precise molecular mechanisms linking the GnRH system, E$_2$ feedback, and the SCN are poorly understood, the inhibitory nature of the RFRP system makes it a potential hypothalamic system involved in the precisely-timed LH surge. Immunoreactive fibers from the SCN extend to the DMH, where the mammalian Rfrp neurons reside. Of interest, RFRP cell number and activity decrease at the time of the LH surge (220). Overall, these studies suggest a possible role for Rfrp neurons to receive SCN inputs and further communicate alterations in hormonal levels to GnRH neurons by inhibition or disinhibition of the RFRP-3 circuit.

### 1.6.2 Steroidal Regulation

The gonadal steroids play a critical role in regulating numerous neuropeptidergic and neurotransmitters systems, providing the fine-tuning and feedback necessary to maintain normal reproductive function. To date, studies investigating the role of gonadal steroids on the RFRP system have yielded inconsistent results. Initial studies reported that 40% of RFRP-3-
immunoreactive neurons in female Syrian hamsters co-express ERα, while Rfrp neurons in males co-stained for the AR. Further, E₂ treatment stimulates the expression of c-Fos in Rfrp-expressing neurons, suggesting a potential role in E₂-mediated feedback (185). Subsequent studies in mice reported that E₂ facilitates the moderate repression of Rfrp mRNA levels in the DMH in both sexes through ERα (126, 221). Similar studies in ewes observed no effect (222), while studies in prepubertal rats reported an increase in Rfrp mRNA levels upon E₂ treatments (223). Furthermore, the initial co-expression of AR with Rfrp-expressing neurons, as reported in Syrian hamsters (12) was not detected in the mouse hypothalamus (126). Overall, the mechanisms governing steroid regulation of RFRP-3 neurons remain unclear; however, it is likely that the discrepancies reported in vivo are in part due to species and sex-specific differences, as well as heterogeneity within the Rfrp neuronal population (126).

1.6.3 The Stress-Response

Stress can be defined as any stimuli that “challenge homeostasis” within an organism (224). Exposure to a myriad of stressors, whether acute or chronic have been causally linked to the suppression of the HPG axis, eventuating in reproductive compromise (2, 225, 226). Stress activates the hypothalamic-pituitary-adrenal (HPA) axis, resulting in the release of corticotropin-releasing hormone (CRH) and adrenocorticotropic hormone (ACTH) from the hypothalamus and pituitary, respectively. ACTH further mediates the release of glucocorticoids (GCs) from the adrenal gland. The stress response, particularly GCs act centrally to inhibit GnRH biosynthesis and release (199, 227-230) and at the pituitary to suppress gonadotropic hormone synthesis and secretion (3, 231). More recently, acute and chronic stressors have been implicated in mediating reproductive dysfunction through the RFRP pathway in mammals. Rfrp neurons in the rat hypothalamus co-express the CRH receptor 1(CRH-R1) and the GC receptor (GR) (13). Acute or chronic immobilization in male rats results in approximately a 2-fold increase in Rfrp mRNA levels and cell number in the DMH. Further, adrenalectomy abrogates the stress-induced increase in Rfrp expression, suggesting a prominent role for the ligand-bound GR in an Rfrp-mediated stress response. Studies in vitro, using hypothalamic N39 cells also demonstrated that 100 nM CRH treatments increase Gpr147 mRNA (232). Overall, the present data suggest that Rfrp is under the immediate control of stress hormones, thereby representing one of several pathways facilitating the stress-induced suppression of the reproductive axis.
1.7 Hypothalamic Cell Models in the Study of Neuroendocrine Function

The maintenance of internal homeostasis plays a fundamental role in many biological processes, including reproduction. The neuroendocrine hypothalamus represents the integrative core of the CNS, made up of a highly specialized array of unique neuropeptidergic and neurotransmitter systems that process peripheral signals to control a variety of coordinated physiological events (233). Over the past two decades, neuroendocrinologists have focused on dissecting the neuronal circuits mediating reproductive biology and much progress has been made regarding the neuromodulators involved; nonetheless, due to the technical limitations of in vivo and electrophysiological approaches, dissecting direct effects is challenging and confounded by the heterogeneous nature of the hypothalamus. In vivo approaches are indispensable in studying how a myriad of stimuli, such as hormones and peptides affect the overall physiological state or particular tissue of an organism. However, in vivo models limit our ability to dissect the direct regulatory mechanisms involved in hormonal or neuropeptidergic effects at the cellular and molecular level, due to the presence of many afferent and glial connections. Therefore, the development of a number of novel hypothalamic cell models has bridged observations seen in vivo and further defined the specific molecular mechanisms governing homeostasis.

Historically, scientists have tried to generate immortalized cell models to overcome the limitations seen with in vivo studies, as well as the difficulty associated with primary cell culture (234). In 1990, Mellon et al. successfully generated clonal, mature, neurosecretory GnRH cell lines, known as the GT1 (235). The GT1 cell model was generated by targeted tumorigenesis, whereby the oncogene, simian virus 40 (SV40) T antigen (T Ag) was directed by the 5’ regulatory region of the GnRH gene, resulting in exclusive expression in GnRH neurons. The tumor, containing GnRH neurons and associated glial cells was excised and cells were further dispersed, cultured, and passaged over a 6 month period, resulting in three distinct homogeneous subclones: GT1-1, GT1-3, and GT1-7. These neuronal cells display neuron-specific markers, secrete GnRH in a pulsatile manner, and respond to a complement of hormones and neuromodulators; thereby, serving as an indispensable in vitro model of GnRH neurons. This novel technique has led to the generation of a multitude of models that allow the study of the direct effects of an array of stimuli on the CNS (233, 236-238).
1.7.1 Immortalized rat embryonic hypothalamic cell lines (rHypoE-xx)

Although GT1 cells contribute immensely to reproductive cell biology, there remained a limited availability of other neuroendocrine cell models, which also contribute to the unique and heterogeneous hypothalamus (239). To address this, Belsham et al. generated and characterized 38 immortalized, embryonic, hypothalamic cell lines of mouse origin (233). In 2009, 33 rat, clonal, hypothalamic neuronal cell models were subsequently developed (240). Briefly, murine cell models were generated through the retroviral transfer of SV40 T-Ag into primary hypothalamic cultures that were isolated on E15, E17, and E18. These heterogeneous neuronal cultures were serially diluted until clonal cell populations were established. The cell lines are designated as mHypoE-xx (mouse hypothalamic embryonic – clone number) or rHypoE-xx (rat hypothalamic embryonic- clone number). Both murine models have been extensively characterized for the presence of neuron-specific markers, as well as the endogenous expression of a multitude of neuropeptides, receptors, and processing enzymes. Each cell line displays a unique expression profile and neuronal morphology. To date, the murine cell lines have been used to dissect the precise signaling cascades initiated by hormonal and peptide stimulation, which are involved in the overall maintenance or perturbation of energy homeostasis, as well as reproductive function (89, 150, 240-262). Overall, these cell models are an indispensable tool to further our knowledge regarding the complex molecular mechanisms governing gene regulation, neurosecretory responses, and neuronal activity at the hypothalamic level.

1.7.2 Immortalized adult-derived, non-clonal GnRH cell model (mHypoA-GnRH/GFP)

Due to the rare and disperse nature of GnRH neurons in the intact hypothalamus, studying the direct effects of modulators is confounded. Although clonal, GnRH-secreting and murine embryonic cell models are available, it is imperative to understand how neurons of embryonic or tumoral origin defer from the mature, adult heterogeneous population of GnRH neurons. Belsham et al. observed the ability of ciliary neurotrophic factor (CNTF) to induce neurogenesis, thereby enabling the generation of an array of adult-derived, immortalized cell lines, which was previously difficult due to the lack of neurogenesis in mature neurons (241). In brief, hypothalami from 2 month old transgenic GnRH-GFP mice [generated by Dr. Suzanne Moenter (University of Michigan, Ann Arbor, MI), and available through The Jackson Laboratory, Bar
Harbor, ME] were isolated and primary hypothalamic culture was treated with 10 mg/mL recombinant rat CNTF. Neurons were incubated with virus-containing medium comprising of the SV40 T-Ag and a neomycin resistance gene. Cells were sorted by fluorescence activated cell sorting (FACS) using a BD FACS Aria cell sorter, protocol previously described (263). This cell model is labeled as mHypoA-GnRH/GFP (mouse hypothalamic adult- GnRH-GFP) and has been characterized for neuron specific markers, as well as the endogenous expression of relevant reproductive and metabolic receptors and enzymes (McFadden et al., manuscript submitted). Furthermore, neurons display neurosecretory properties and respond to a plethora of stimuli. Overall, the immortalization protocol described above likely generates a non-clonal, adult-derived GnRH cell line representative of the whole heterogeneous population of native GnRH neurons.

1.8 Study Hypotheses and Aims

To date, GnIH has an established role in mediating avian reproduction and behaviour; however, the molecular mechanisms and signaling that govern this novel neuropeptidergic system in mammals remains unclear. It has been established by both in vivo and in vitro studies that the RFRP system regulates LH synthesis and release at both the level of the hypothalamus and pituitary (6-12); yet, the direct regulation of RFRP and GPR147 biosynthesis is not well defined. There is mounting evidence supporting the influential role of RFRP in steroid-induced reproductive effects (13, 126, 205-208); corroborated by the endogenous expression of GR, CRH-R1, ERs and AR in Rfrp-expressing cells in vivo (Figure 1.2). However, due to a lack of appropriate models dissecting direct effects on the RFRP system are challenging. Therefore, Chapter 3 of the present thesis utilized a rat, embryonic hypothalamic cell line, rHypoE-23 that demonstrates strong endogenous expression of Rfrp, as well as other relevant reproductive receptors with the aim of testing the general hypothesis that Rfrp-expressing neurons are direct targets of steroidal regulation, particularly E2 and GC stimulation.

To test this hypothesis the study will be subdivided into 3 main aims. **Aim 1** will focus on characterizing an Rfrp-expressing cell line. In **Aim 2** it will be established whether synthetic GC, dexamethasone and/or E2 directly regulate Rfrp or Gpr147 gene expression. Lastly, **Aim 3** will
Figure 1.2 Schematic summarizing the current mechanism through which RFRP-3 may participate in the central regulation of the HPG axis. This working model suggests that estradiol (E2) and glucocorticoids (GCs) are involved in the direct regulation of Rfrp- and Gpr147-expressing neurons in the DMH. Furthermore, a large subpopulation of Rfrp neurons projects to the preoptic area (POA), where they directly appose GnRH neurons, potentially mediating the synthesis and release of GnRH. Some Rfrp-expressing neurons also project to the AVPV, where they may innervate kisspeptin (Kiss) neurons, which have been found to express the RFRP receptor, GPR147.
delineate the specific receptor-mediated transcriptional mechanisms involved in the observed GC-induced changes in gene expression profiles in the \textit{Rfrp}-expressing neuronal cell line.

It is well accepted that RFRP-3 has central effects targeting GnRH neurons, whereby central administration of RFRP-3 decreases LH release (12, 193) and results in the hyperpolarization of a subset of GnRH neurons (198). However, the mechanisms through which this neuropeptide directly alters GnRH biosynthesis and secretion remain unclear. \textbf{Chapter 4} of this thesis will test the overall hypothesis that RFRP-3 suppresses both GnRH biosynthesis and secretion in the novel, adult-derived, GnRH-secreting cell line, mHypoA-GnRH/GFP.

The second hypothesis will be tested with three aims: \textbf{Aim 1} will establish the presence of GPR147 expression in GnRH neurons \textit{in vitro}. In \textbf{Aim 2} we will elucidate the transcriptional mechanisms involved in the direct regulation of GnRH gene expression by RFRP-3. \textbf{Aim 3} will further establish the potential involvement of RFRP-3 in modulating GnRH neurosecretory potential.
Chapter 2
Materials and Methods
2.1 Cell Culture and Reagents

Immortalized hypothalamic neuronal cells were cultured in monolayer in Dulbecco’s Modified Eagles Medium (DMEM) supplemented with 5% fetal bovine serum (FBS) (Sigma-Aldrich, Oakville, Canada) and 1% penicillin/streptomycin (Gibco, Burlington, Canada). Furthermore, both rHypoE-23 and mHypoA-GnRH/GFP neurons were maintained at 37°C with 5% CO2, a methodology previously described (260, 264). Dexamethasone and E₂ (Sigma-Aldrich, Oakville, Canada) were dissolved in absolute ethanol to a stock concentration and stored at -20°C prior to mRNA studies. RU-486, a GR antagonist and cycloheximide (CHX), a protein synthesis inhibitor were purchased from Sigma-Aldrich and reconstituted to a stock concentration in absolute ethanol and water, respectively. By using water in subsequent dilutions, the final ethanol concentration in vehicle controls and treated neurons was minimized to nanomolar concentrations. RFRP-3 (Sigma-Aldrich, Oakville, Canada) was dissolved in water to a stock concentration and stored at -80°C prior to mRNA studies. For c-Fos mRNA studies, cell culture medium was replaced with serum-free DMEM containing only 1% penicillin/streptomycin for a minimum of 4 hours prior to treatments. Transcription inhibitors, actinomycin D (Act D) and 5,6-Dichlorobenzimidazole riboside (DRB) were purchased from Sigma-Aldrich (Oakville, Canada) and Tocris Bioscience (Ellisville, MO), respectively and reconstituted in dimethyl sulfoxide (DMSO; Sigma-Aldrich) to a stock concentration.

2.2 Reverse Transcriptase-Polymerase Chain Reaction (RT-PCR)

Cells were grown to approximately 85-90% cell confluence in 100 mm culture plates. Total RNA was isolated by the guanidium isothiocyanate phenol chloroform extraction method. Subsequent to isolation, RNA concentration and purity ratios were measured using a NanoDrop 2000c spectrophotometer, followed by treatment with Turbo DNase (Ambion) prior to amplification. RT-PCR was performed using the QIAGEN One-Step RT-PCR kit, according to manufacturers protocol. Briefly, 200 ng of template DNase treated RNA was combined with 1 × one step RT-PCR buffer, 0.4 mM deoxynucleotide triphosphates (dNTPs), one-step enzyme mix and 0.6 mM of both the reverse and forward gene-specific primers. PCR products were separated and visualized on a 2% agarose gel containing 0.5ug/mL ethidium bromide and run alongside a 100bp ladder (Fermentas, Burlington, Canada). The primers, designed using Integrated DNA Technologies PrimerQuest and NCBI Primer-Blast, are listed in Table 2.1.
2.3 Quantitative real time RT-PCR

Hypothalamic neuronal cell lines were grown in 60 mm culture plates to 80-85% confluence. Prior to steroidal treatments in rHypoE-23 neurons, cell culture medium was changed to phenol red-free DMEM (HyClone), supplemented with 5% charcoal-stripped FBS, protocol previously described (89, 260, 261, 265), and 1% penicillin/streptomycin (Gibco, Burlington, Canada) for a minimum of 2 h. rHypoE-23 cells were treated with vehicle, dexamethasone (20 nM), and/or E₂ (10 nM). Total RNA was harvested at the indicated time points. For antagonist and inhibitor studies, rHypoE-23 neurons were pretreated with 50 nM RU-486, 1ug/mL CHX, or vehicle for one hour prior to dexamethasone (20 nM) treatment and RNA was isolated at 8 and 24 h time points. mHypoA-GnRH/GFP neurons were treated with vehicle, 10 nM RFRP-3, or 100 nM RFRP-3 and RNA was harvested over a 24 h time course at indicated time points. For subsequent inhibitor studies, mHypoA-GnRH/GFP neurons were pre-incubated with either DMSO, 10 ug/mL Act D, or 60 µM DRB for 1 h prior to RFRP-3 (100 nM) treatments and total RNA was isolated at 1, 2, and 4 h. For both cell models, total RNA from treated and control cell culture plates was isolated using the guanidium isothiocyanate phenol chloroform extraction method. Subsequently, RNA concentration and purity were measured with the NanoDrop 2000c spectrophotometer. Reverse transcription was performed with 2 ug of total RNA that was treated with Turbo DNase (Ambion) prior to use of the High Capacity cDNA Reverse Transcription kit, according to manufacturer’s protocol (Applied Biosystems). Further, 50 - 100 ng of cDNA template was amplified using SYBR green PCR master mix for real-time RT-PCR containing, 0.3 × SYBR green dye, 1 × PCR buffer, 3 mM MgCl₂, 2 mM dNTPs, 1 × ROX reference dye, 0.3 µM gene-specific primers, and 0.2 U of Platinum Taq DNA polymerase (Invitrogen, Burlington, Canada). The primers used are listed in Table 2.1. All primer products were verified with sequencing. Samples were run in triplicate on the Applied Biosystems Prism 7000 real-time PCR machine. In brief, all genes were run on the real-time PCR machine according to the following protocol conditions: 50 C for 2 min, 95 C for 10 min; 40 cycles for 15 sec at 95 C, 60 C for 1 min. Analysis of qRT-PCR data was performed using the standard curve method and normalized to 18S ribosomal RNA (18S) or histone 3a.
### Table 2.1 List of primers used for cell screening and real time RT-PCR

<table>
<thead>
<tr>
<th>Gene Name</th>
<th>Primer Sequence</th>
<th>Amplicon Size (bp)</th>
<th>Annealing Temp (°C)</th>
</tr>
</thead>
<tbody>
<tr>
<td>RFamide-related peptide (RFRP)</td>
<td>F: TTA TTG ACT TTA GCA ACT TCA AGC</td>
<td>150</td>
<td>60</td>
</tr>
<tr>
<td></td>
<td>R: AGT GAC ACT TCT TTC CTT TAC CCC</td>
<td></td>
<td></td>
</tr>
<tr>
<td>GPR147</td>
<td>F: AAC ACC CTG GTC TGC TTC ATT GTG</td>
<td>82</td>
<td>60</td>
</tr>
<tr>
<td></td>
<td>R: TGA CGG CCA GGT TGA GGA TAA ACA</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Estrogen receptor α (ERα)</td>
<td>F: AAT TCT GAC AAT CGA CGC CAG</td>
<td>345</td>
<td>60</td>
</tr>
<tr>
<td></td>
<td>R: GTG TT CAA CAT TCT CCC TCC</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Estrogen receptor β (ERβ)</td>
<td>F: ATC TGT CCA GCC ACG AAT CAG TGT</td>
<td>114</td>
<td>60</td>
</tr>
<tr>
<td></td>
<td>R: TCT CCT GGA TCC ACA CTT GAC CAT</td>
<td></td>
<td></td>
</tr>
<tr>
<td>G-protein coupled estrogen receptor (GPR30)</td>
<td>F: TCA GCA GTA CGT GAT TGC CCT CTT</td>
<td>103</td>
<td>60</td>
</tr>
<tr>
<td></td>
<td>R: AGC TGA TGT TCA CCA CCA GGA TGA</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Glucocorticoid receptor (GR)</td>
<td>F: AAC CTC AAT AGG TCG ACC AGC GTT</td>
<td>305</td>
<td>60</td>
</tr>
<tr>
<td></td>
<td>R: CCC GCC AAA GGA GAAAGC AAG TTT</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Androgen receptor (AR)</td>
<td>F: CTT GGT GAG CTG GTA GAA GCG C</td>
<td>560</td>
<td>60</td>
</tr>
<tr>
<td></td>
<td>R: ACG TCC TGG AAG CCA TTG AGC C</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Galanin</td>
<td>F: TCG GGA TGC CAA CAA AGG AGA AGA</td>
<td>112</td>
<td>60</td>
</tr>
<tr>
<td></td>
<td>R: GTG AGG CCA TGC TGG TCG CTA AAT</td>
<td></td>
<td></td>
</tr>
<tr>
<td>c-Fos</td>
<td>F: CAA CGA GCC CTC CTC CGA CT</td>
<td>68</td>
<td>60</td>
</tr>
<tr>
<td></td>
<td>R: TGC CTT CTC TGA CTG CTC ACA</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Gonadotropin-releasing hormone (GnRH)</td>
<td>F: CGT TCA CCC CTC AGG GAT CT</td>
<td>51</td>
<td>60</td>
</tr>
<tr>
<td></td>
<td>R: CTC TTC AAT CAG ACT TTC CAG AGC T</td>
<td></td>
<td></td>
</tr>
<tr>
<td>18S</td>
<td>F: GTA ACC CGT TGA ACC CCA TT</td>
<td>151</td>
<td>60</td>
</tr>
<tr>
<td></td>
<td>R: CCA TCC AAT CGG TAG TAG CG</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Histone 3a</td>
<td>F: CGC TTC CAG AGT GCA GCT ATT</td>
<td>72</td>
<td>60</td>
</tr>
<tr>
<td></td>
<td>R: ATC TTC AAA AAG GCC AAC CAG AT</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>
2.4 Enzyme Immunoassay (EIA)

mHypoA-GnRH/GFP neurons were grown in 24-well culture plates to 85-90% cell confluence. Cell culture medium was changed to serum-free, low glucose DMEM, supplemented with 1% penicillin/streptomycin 4 h prior to treatments. mHypoA-GnRH/GFP neurons were treated with water vehicle, 100 μM sodium nitroprusside (SNP), or SNP in the presence of 100 nM RFRP-3 for 1 h. Media and total protein were collected 1 h following treatments and samples were immediately dried, while protein was centrifuged for 10 min at 14,000 rpm and 4°C. Supernatant from cell lysates was then stored at –80°C. To quantify changes in GnRH secretion, 100 μl of media sample was rehydrated and a LH-RH EIA kit (Phoenix Pharmaceuticals) was followed according to manufacturers protocols. The LH-RH EIA kit shows 100% cross-reactivity with human, rodent, and porcine GnRH, as well as has a detectable range of 0-25 ng/ml.

2.5 In silico analysis

The web tool, Microinspector was used to determine potential microRNA (miRNA) binding sites on the mouse GnRH mRNA template (http://bioinfo.uniplovdiv.bg/microinspector/+).

2.6 Statistical Analysis

Data are presented as the mean ± SEM for the number of independent experiments indicated. Data analyses were performed using GraphPad Prism or SigmaStat (Systat Software, Inc.,Chicago, IL). Statistical significance was established by one- or two-way ANOVA, as indicated, followed by Bonferroni’s post hoc test.
Chapter 3

Glucocorticoid Receptor-mediated Regulation of RFRP and GPR147 Gene Expression in an Immortalized Hypothalamic Cell Line
3.1 Abstract

The maintenance of internal homeostatic mechanisms governing reproduction requires the precisely coordinated integration of an array of neurochemical pathways. Despite the importance of reproductive success, the central effectors that communicate environmental and steroid-induced alterations remain incomplete. A novel RFamide peptide, termed GnIH has emerged as a key modulator of avian reproduction. However, the functional role of the mammalian homologue, RFRP-3 remains poorly understood. There is growing evidence that the RFRP neuronal circuit is influenced by perturbations to physiological homeostasis, particularly the activation of the HPA axis. To date, it has not been reported that the Rfrp gene is under direct transcriptional regulation by GCs, in the presence or absence of the gonadal steroid, estradiol. Here, we investigated the regulation of transcription of Rfrp and Gpr147 genes by steroids in a hypothalamic RFRP-expressing cell model. Real time RT-PCR studies show that the synthetic GC, dexamethasone increases both Rfrp and Gpr147. We show that dexamethasone acts directly on the nuclear GR to mediate GC-dependent transcriptional changes. Further, GC-mediated changes in gene expression profiles are independent of de novo protein synthesis, whereby incubation of neurons with a protein synthesis inhibitor, CHX fails to attenuate dexamethasone-induced changes in Rfrp and Gpr147 mRNA levels. Here, we report that estradiol has no significant effect on Rfrp or Gpr147 expression over a 24 h time course. Overall, our results suggest that Rfrp-expressing neurons serve as potential upstream mediators of stress hormone-associated effects, whereby GR-dependent mechanisms are involved in the direct transcriptional increase of Rfrp and Gpr147 genes.

3.2 Introduction

Stress, whether acute or chronic, challenges the internal physiological homeostasis of an organism. The physiology of stress has been causally linked to the suppression of the HPG axis, resulting in reproductive compromise (2, 225, 226). Numerous studies in vertebrates, including humans have demonstrated that the activation of the HPA axis is dependent on the rapid release of neuropeptides, particularly CRH from the hypothalamus and ACTH from the pituitary. The activation of the HPA axis results in elevated levels of GCs from the adrenal cortex, leading to a global suppression of LH release (1, 266). Chronic administration of GCs, cortisol or
coritosterone, in humans and rodents, respectively, results in the inhibition of cyclicity and ovulatory activity in female rats (226, 267), as well as causes an inhibition of overall sexual maturation (2, 267). The stress response, particularly the actions of CRH and GCs, act both directly at the hypothalamic level to inhibit GnRH biosynthesis and secretion (199, 227-230) and at the pituitary to suppress gonadotrophic hormone synthesis and release into circulation (3, 231).

In 2000, a previously unknown RFamide neuropeptide, GnIH was characterized and isolated from the Japanese quail (Coturnix japonica), demonstrating a unique ability to suppress LH release (9). In avian species, GnIH has a confirmed inhibitory role within the HPG axis in vivo (178, 179, 185, 206, 268), though its role in mammals is still under investigation. The mammalian homologue to avian GnIH, RFRP-3 demonstrates potent and sustained inhibitory actions on gonadotropic hormone secretion (12, 17, 191, 192, 220, 269, 270), as well as GnRH neuronal activation and signaling (194, 198, 200). Mammalian RFRP-3 is encoded by the Rfrp (also referred to as Npyf) gene and generates a preprohormone, which contains 2 physiologically active RFamide-related peptides, RFRP-1 and RFRP-3 (10). The receptor for GnIH/RFRP-3, GPR147, is a GPCR that is highly expressed in regions of the hypothalamus, pituitary and gonads (11, 12, 17). Immunohistochemical studies have further revealed that RFRP-3 cell bodies are concentrated in the DMH with terminals extending to the ME and medial regions, including the diagonal band of Broca, POA, and anterior hypothalamus (12, 271). Of interest, Rfrp neurons have been found to coexpress ERα, GR, and CRH-R1 (12, 13, 126, 221). Therefore, as this body of evidence suggests RFRP-3 and GPR147 pose as a potential modulatory neuronal system operating at multiple levels of the HPG axis. To date, the regulation of Rfrp and Gpr147 synthesis by GCs, in the presence or absence of gonadal steroids has not been directly assessed at the cellular level.

The central hypothalamic mechanisms involved in the stress-mediated suppression of GnRH release, whether direct or through innervating neuronal systems remains poorly understood. Due to the inhibitory nature of RFRP-3 and the expression of Gpr147 on GnRH neurons (197), this neuronal network poses as a potential mediator of stress-induced mechanisms directly influencing GnRH synthesis and release at the level of the GnRH neuron. Of interest, Kirby et al. demonstrated that 53% of Rfrp-positive neurons co-express GR and both acute and chronic stress cause a drastic increase in Rfrp mRNA expression and perotein levels (13). Similarly, GnIH
immunoreactive cell bodies are greatly induced by capture-handling stress in house sparrows (*Passer domesticus*) in a season-dependent manner over the avian reproductive cycle (186).

In the present study, we sought to investigate whether GCs alone or in the presence of the gonadal steroid, E₂ can directly act on Rfrp neurons to regulate transcriptional changes and to further delineate the receptor-mediated mechanisms involved. Due to heterogeneous nature and complex architecture of the *in vivo* hypothalamus, dissecting the direct effects of GCs on particular neuronal systems is confounded and hence, very challenging. Therefore, to ascertain GC-mediated effects on Rfrp and Gpr147 gene expression, we employed a rat embryonic-derived immortalized hypothalamic cell line, embryonic rat hypothalamic-23 (rHypoE-23). This cell line has been previously characterized for the expression of neuron-specific markers (233, 240), as well as demonstrates strong endogenous expression of Rfrp and other key reproductive receptors. We establish that the GC receptor agonist, dexamethasone has direct effects on Rfrp and Gpr147 mRNA levels in rHypoE-23 neurons. Furthermore, we elucidate the transcriptional mechanisms and GR-mediated changes in the RFRP/GPR147 system *in vitro*. Taken together, these findings provide the first line of evidence that dexamethasone directly augments GR-mediated transcriptional changes of Rfrp and Gpr147 genes in an Rfrp-expressing hypothalamic cell line.

### 3.3 Results

#### 3.3.1 rHypoE-23 neurons express Rfrp and Gpr147 mRNA, in addition to ERs, GR, and other key reproductive receptors

We have previously reported the generation of an array of clonal hypothalamic neuronal cell lines that demonstrate unique expression profiles of neuropeptides and relevant receptors (233, 240). With the aim of characterizing a representative Rfrp-expressing cell line, we conducted RT-PCR to further determine the gene expression profile of the rHypoE-23 neurons. In accordance with immunohistochemical studies, we demonstrate that rHypoE-23 neurons show high expression of Rfrp mRNA (*Figure 3.1*), as well as synthesize the GR, both nuclear ERs
Figure 3.1. Characterization of the gene expression profile of Rfrp-expressing, rHypoE-23 neurons. (A) RT-PCR results of relevant reproductive neuropeptides and receptors in the whole hypothalamus (positive control) and indicated hypothalamic cell line. (+) indicates the presence of a gene, and (-) indicates the absence or weak expression of a gene. (B) Representative RT-PCR screening of Rfrp, amplicon size is 150 bp. Total RNA was isolated and used in the One-Step RT-PCR Qiagen kit with gene-specific primers. Products were visualized on a 2% agarose gel with whole hypothalamus (positive control) and a non-template control (NTC; negative control).
and the GPCR, GPR30. We also report the presence of the AR mRNA in rHypoE-23 neurons. The expression of \textit{Rfrp}, as well as the presence of steroid receptors, suggests that this cell line is suitable for the study of direct hormonal regulatory mechanisms involved in RFRP biosynthesis at the cellular level. Moreover, coexpression of \textit{Rfrp} and \textit{Gpr147} in rHypoE-23 neurons indicates the potential for RFRP-3 autoregulatory mechanisms, a regulatory pathway that has not yet been explored.

### 3.3.2 Dexamethasone, but not E\textsubscript{2} has stimulatory effects on Rfrp and Gpr147 mRNA expression in rHypoE-23 neurons

The presence of GR, as well as both nuclear ERs prompted investigation into the potential direct regulation of \textit{Rfrp} and \textit{Gpr147} by the synthetic GR, dexamethasone and/or the main gonadal steroid, E\textsubscript{2} in this neuronal model. Neurons were treated with vehicle, 20 nM dexamethasone, 10 nM E\textsubscript{2}, or co-treatment of dexamethasone with E\textsubscript{2} over a 24 h time course. We found that dexamethasone alone or in the presence of E\textsubscript{2} induces a 2.98 and 2.47 fold change in \textit{Rfrp} mRNA levels after 24 hours of treatment: vehicle (0.49 ± 0.05) vs. dexamethasone (1.48 ± 0.22), P<0.05; vehicle (0.49 ± 0.05) vs. dexamethasone + estradiol (1.23 ± 0.37), P<0.05 (Figure 3.2A). Similarly, \textit{Gpr147} levels are significantly augmented after 8 and 24 hours following 20 nM dexamethasone treatment [8 h, vehicle (0.78 ± 0.06) vs. dexamethasone (1.44 ± 0.20), P<0.01; 24 h, vehicle (0.80 ± 0.14) vs. dexamethasone (1.47 ± 0.05), P<0.01] (Fig. 3.2B). E\textsubscript{2} alone has no significant effect on \textit{Rfrp} or \textit{Gpr147} mRNA levels (Figure 3.2A-B). These results clearly demonstrate that both the \textit{Rfrp} and \textit{Gpr147} genes are regulated directly by GCs and not by the gonadal steroid, E\textsubscript{2} in the hypothalamic rHypoE-23 neuronal cell line.

### 3.3.3 Potential autocrine regulation of Rfrp and Gpr147 mRNA expression by RFRP-3

The co-expression of \textit{Rfrp} and \textit{Gpr147} in rHypoE-23 neurons prompted the investigation into potential autoregulatory mechanisms involved in the fine-tuning of the RFRP/GPR147 system. Neurons were treated with vehicle, 10 nM RFRP-3, or 100 nM RFRP-3 over a 24 h time course and RNA was isolated at indicated time points. We found that RFRP-3 did not exert any changes on \textit{Rfrp} gene expression; however, a moderate dose-dependent increase was observed at 1 h
Figure 3.2. Dexamethasone increases Rfrp and Gpr147 transcript levels in rHypoE-23 neurons. Cells were treated with 10nM Estradiol, 20 nM Dexamethasone, co-treatment of estradiol (10 nM)/dexamethasone (20nM), or vehicle. RNA was collected at specified time points and changes in Rfrp (A) or Gpr147 (B) mRNA levels were quantified using qRT-PCR. mRNA levels were normalized to 18S. Data are expressed as mean ±SEM (n=5-6 independent experiments). * P<0.05, **P<0.01 vs. vehicle control. Statistical significance was determined by two-way ANOVA with Bonferroni’s post hoc test.
Figure 3.3. Effect of RFRP-3 treatment on Rfrp and Gpr147 transcript levels in the rHypoE-23 neurons. Cells were treated with vehicle, 10nM RFRP-3, or 100 nM RFRP-3. Total RNA was collected over a 24 h time course at specified time points and changes in Rfrp (A) or Gpr147 (B) mRNA levels were quantified using qRT-PCR. mRNA levels were normalized to the internal control, 18S. Data are expressed as mean ±SEM (n=4-5 independent experiments). * P<0.05 vs. time-matched vehicle control. Statistical significance was determined by two-way ANOVA with Bonferroni’s post hoc test.
following 100 nM RFRP-3 treatment: vehicle (0.70 ± 0.14) vs. 10 nM RFRP-3 (1.14 ± 0.11) vs. 100 nM RFRP-3 (1.21 ± 0.10), P>0.05 (Figure 3.3A). Gpr147 mRNA levels were not significantly changed upon 10-100 nM RFRP-3 treatment over a 24 h time course (Figure 3.3B). These results demonstrate that RFRP-3 does not independently participate in any autoregulatory mechanisms within 24 h in this Rfrp-expressing neuronal cell model.

3.3.4 GR antagonist, RU-486 abolishes the dexamethasone-mediated mRNA expression changes in rHypoE-23 neurons

We further investigated whether the induction of Rfrp and Gpr147 mRNA levels by dexamethasone in rHypoE-23 neurons was a GR-mediated mechanism. RU-486, a GR antagonist was used to block the activity of the GR. rHypoE-23 neurons were pre-incubated with 50 nM RU-486 or water vehicle for 1 hour prior to dexamethasone treatment. Neurons pre-treated with RU-486 failed to demonstrate modified levels of Rfrp mRNA upon dexamethasone treatment after 24 hours: 50 nM RU-486 (0.585 ± 0.14) vs. RU-486 + dexamethasone (0.72 ± 0.16), P>0.05 (Figure 3.4A). In contrast, control neurons exhibit a 2.75-fold increase of Rfrp mRNA levels in response to dexamethasone treatment, as compared to controls (water, 0.63 ± 0.09 vs. dexamethasone, 1.75 ± 0.36; P<0.01). Therefore, pretreatment with RU-486 significantly blocks the dexamethasone-mediated increases of Rfrp mRNA: dexamethasone: (1.75 ± 0.36) vs. RU-486 + dexamethasone (0.72 ± 0.16), P<0.05. RU-486 exerted similar effects on dexamethasone-mediated upregulation of Gpr147 mRNA levels, whereby total RNA was isolated at 8 and 24 hours following dexamethasone treatment. qRT-PCR results demonstrate that pre-incubation of neuronal cells with RU-486 blocks the augmentation of Gpr147 mRNA levels initially seen with dexamethasone treatment at 8 hours: vehicle (0.77 ± 0.07) vs. dexamethasone (1.43 ± 0.14), P<0.01; RU-486 (0.93 ± 0.14) vs. RU-486 + dexamethasone (0.92 ± 0.15), P>0.05 (Figure 3.4B), as well as 24 hours: vehicle (0.88 ± 0.05) vs. dexamethasone (1.66 ± 0.27) P<0.01; RU-486 (0.78 ± 0.15) vs. RU-486 + dexamethasone (0.94 ± 0.05), P>0.05 (Figure 3.4C). RU-486 treatment alone has no effect on basal mRNA levels of Rfrp or Gpr147. These findings indicate that dexamethasone increases both Rfrp and Gpr147 through a GR-dependent mechanism, and further demonstrate that the rHypoE-23 neuronal cell model is directly responsive to GCs.
Figure 3.4. Dexamethasone upregulation of Rfrp and Gpr147 mRNA expression in rHypoE-23 neurons is blocked by GR antagonist, RU-486. Relative mRNA levels for Rfrp (A) and Gpr147 (B and C) for 8 and 24 h with dexamethasone (20 nM) in the presence or absence of RU-486 (50 nM) or RU-486 treatment alone. mRNA levels were normalized to the internal control, 18S. Results shown are expressed as mean ±SEM (n=4-5 independent experiments). **, P<0.01 vs. control or dexamethasone. Statistical significance was determined by one-way ANOVA with Bonferroni’s post hoc test.
Figure 3.5. Effects of protein synthesis blockade on dexamethasone-mediated mRNA expression changes in rHypoE-23 neurons. Cells were pre-treated with CHX (1μg/mL) for 1 hour and followed by dexamethasone or vehicle treatment for 24 h. Rfrp (A) and Gpr147 (B) mRNA expression was quantified using real-time RT-PCR and levels were normalized to the internal control, 18S. Results are expressed as mean ±SEM (n=5-6 independent experiments). *, P<0.05, **, P<0.01, ***, P<0.001 vs. vehicle or CHX control. Statistical significance was determined by one-way ANOVA with Bonferroni’s post hoc test.
3.3.5 Dexamethasone-mediated induction of Rfrp and Gpr147 genes is independent of de novo protein synthesis in hypothalamic neurons

To determine whether the upregulation of Rfrp and Gpr147 mRNA is dependent on de novo protein synthesis, rHypoE-23 neurons were pre-exposed to CHX (1 μg/mL) for 1 hour, followed by 20 nM dexamethasone treatments. Total RNA was isolated at indicated time points and analyzed using real time RT-PCR. Despite the presence of CHX, dexamethasone significantly induces Rfrp mRNA after 24 hours: CHX (0.34 ± 0.03) vs. CHX + dexamethasone (1.08 ± 0.12), P<0.05 (Figure 3.5A). Though, neurons treated with dexamethasone alone demonstrate significantly higher levels of Rfrp mRNA than those incubated with both CHX and dexamethasone: dexamethasone (2.47 ± 0.28) vs. CHX + dexamethasone (1.08 ± 0.12), P<0.01. Similarly, CHX had no effect on the dexamethasone-mediated upregulation of Gpr147 mRNA after 24 hours in rHypoE-23 neurons (Figure 3.5B): vehicle (0.65 ± 0.09) vs. dexamethasone (1.37 ± 0.23), P<0.01; CHX (0.72 ± 0.07) vs. CHX + dexamethasone (1.27 ± 0.12), P<0.05. Our results indicate that CHX decreases the basal levels of Rfrp mRNA, suggesting that CHX may be inhibiting the synthesis of proteins, such as gene enhancers responsible for the stability and basal regulation of Rfrp mRNA levels. Furthermore, these findings demonstrate that de novo protein synthesis is not required for the effects of dexamethasone on Rfrp and Gpr147 biosynthesis, supporting a direct transcriptional mechanism.

3.4. Discussion

The RFRP neuronal circuit has emerged as an important mediator of reproductive function within the mammalian HPG axis, yet the direct regulation of Rfrp and Gpr147 gene biosynthesis is not well defined. There is mounting evidence supporting the influential role of Rfrp in steroidal and environmental-induced reproductive effects (13, 126, 205-208); however, due to heterogeneity of the hypothalamus and numerous innervating neuronal networks, dissecting direct and indirect effects on the RFRP system in vivo, are challenging and confounded. In the present study, we used an embryonic hypothalamic cell line that demonstrates strong endogenous expression of Rfrp mRNA, as well as relevant reproductive receptors, with the aim of delineating GC-mediated effects on the RFRP system, in the presence or absence of E2. The expression profile of rHypoE-23 neurons presently described is corroborated by in vivo studies
demonstrating the co-expression of \( Rfrp \) with the GR, ERs and AR (12, 126, 221, 222); though, the latter has only been identified in male Syrian hamsters (12) and at very low (3%) levels in male and female mice with no effect on RFRP expression (126). Furthermore, we describe for the first time that \( Rfrp \)-expressing neurons also synthesize \( Gpr147 \) mRNA, indicative of a potential autoregulatory mechanism controlling RFRP synthesis or secretion. This mechanism is substantiated by the presence of autocrine mechanisms in other neuropeptidergic systems, such as GnRH. GnRH receptor (GnRH-R) has been found to be expressed on GnRH neurons \textit{in vivo} and in GnRH-secreting cell models, allowing for a concentration and time-dependent ultra short feedback loop to mediate both the neuronal activity and peptide secretion in a subpopulation of neurons (272, 273). The presence of \( Gpr147 \) in \( Rfrp \)-expressing neurons warrants further investigation into potential autocrine feedback mechanisms responsible for the fine-tuning of \( Rfrp \) mRNA synthesis and peptide availability. Preliminary data suggests that RFRP-3 treatment only moderately increases \( Rfrp \) gene expression following 1 h of treatment; however no significant changes were reported within 24 h of treatment (Figure 3.3). These results suggest that RFRP-3 may require other effectors to participate in autocrine mechanisms, a longer incubation period, or may not directly regulate the RFRP system in rHypoE-23 neurons. Further studies are required to investigate the involvement of RFRP-3 in any potential autoregulatory mechanisms in this hypothalamic cell model. Overall, given the co-expression of \( Rfrp \) with sex steroid and glucocorticoid receptors, the rHypoE-23 cell model is strongly representative of \( Rfrp \)-expressing neurons localized to the DMH.

\( E_2 \) is the most potent estrogen and its main role is to regulate reproductive function at the level of the hypothalamus and pituitary (91). Previous studies investigating the role of estradiol in the regulation of RFRP-3 have generally yielded inconsistent results. It is noteworthy, that \( E_2 \) treatment alone did not exert any changes in \( Rfrp \) or \( Gpr147 \) gene expression, nor did it demonstrate additive or antagonizing effects in the presence of dexamethasone in rHypoE-23 neurons in the present study. Initial studies in ovariectomized Syrian hamsters found that subcutaneous \( E_2 \) injections induce FOS activation in \( Rfrp \) neurons after 3 and 6 h, suggesting a role of \( Rfrp \) gene expression and peptide levels in estrogen feedback (12). Subsequent studies report that \( E_2 \) facilitates the suppression of \( Rfrp \) mRNA levels in mice (126, 221), yet similar studies in ewes found no response (222) and investigations in prepubertal rats reported a stimulation of \( Rfrp \) mRNA (223). There are several possible explanations for these observed
differences. E$_2$-mediated effects can be highly sensitive to dose and developmental stage, whereby as reported by Iwasa et al. lower E$_2$ concentrations during the prepubertal stage can cause a stimulatory effect, while higher concentrations during adulthood, a time also marked by decreases in Rfrp cell number, result in the suppression of Rfrp mRNA (126, 223). Another factor to consider is the existence of heterogeneous subpopulations within Rfrp neurons, a common feature in neuropeptidergic systems both in vivo and in vitro (199, 274-276). In vitro, this notion is supported by observations that GT1-7 cells are comprised of two morphologically distinct subpopulations of GnRH neurons, type I and II (199). Moreover, the two populations of neurons display differential sensitivity and regulation of both GnRH mRNA and secretion upon GC exposure. Heterogeneity within the Rfrp neurons is consistent with recent studies by Poling et al. showing the presence of two distinct high and low-expressing Rfrp neuronal populations in the DMH, each revealing unique developmental and steroidal induction patterns (126).

Interestingly, other studies have also shown that subpopulations of cells can vary in the ratios of ERs present in response to specific treatments, which has been associated with alterations in both ER-responsiveness and signaling in a cell-specific manner (260, 277). It is therefore conceivable that functional heterogeneity exists among the Rfrp neuronal population, particularly in regards to the sensitivity of cells to steroidal regulation. Moreover, this may also underlie the differences in ER expression profiles, reported by others and in the present study. Thus, rHypoE-23 neurons are likely representative of an Rfrp-expressing subpopulation indirectly mediated by E$_2$, thereby supporting our findings that demonstrate no direct estradiol-mediated effect on gene expression profiles. Because we did not examine the effect of E$_2$ on protein synthesis, we cannot rule out the possibility that E$_2$ is regulating the peptide availability of RFRP-3 or GPR147 through translational or post-translational mechanisms in this hypothalamic cell model. Overall, it remains unclear whether the RFRP system serves as a direct target for the integration of sex steroid-dependent feedback.

In contrast to E$_2$, previous reports examining the effects of stress on the RFRP/GPR147 neuronal system are convincing and indicate that Rfrp neurons are both responsive and direct targets of GCs (13, 186). Similar to these findings, we report that dexamethasone evoked a significant increase in both Rfrp and Gpr147 mRNA expression at 8 and 24 h following treatment in this hypothalamic cell model. It is well documented in literature that the physiological consequences of stress on the HPG axis, in humans and nonprimate species are regulated mainly by circulating
levels of GCs. Administration of GCs decreases LH release in rats and humans (1, 2, 266), as well as directly represses GnRH gene expression by acting at the level of the proximal promoter (227, 229). We report that the GR is required for the GC-induced transcriptional regulation of Rfrp and Gpr147 genes, whereby a potent GR antagonist, RU-486 abolishes the initial effects observed upon dexamethasone treatment. The involvement of GCs in an RFRP-mediated stress response aligns with evidence that adrenalectomized rats fail to demonstrate changes in Rfrp mRNA expression in response to stress, as compared to sham controls (13). Given the parallel roles of RFRP-3 and stress in reducing responsiveness of the pituitary to GnRH (194), leading to reductions in LH release (16, 193, 269), as well as acting at the level of the GnRH neuron to decrease neuronal activity (198), it is plausible that this neuronal network serves to mediate GC-dependent suppression of the HPG axis. Interestingly, ICV injection of RFRP-3 was found to stimulate the release of ACTH and oxytocin, as well as induced anxiety-related behaviours during an open-field test in rats (207). Consistent with the RFRP-mediated stress-related behaviours, Rfrp neurons have been shown to be in close apposition to CRH and oxytocin neurons (196), both of which are responsible for the maintenance of homeostatic control throughout the stress response (230, 278). Therefore, Rfrp neurons may act directly on GnRH neurons or indirectly through other stress-related neuropeptidergic systems, like CRH. Presently, we cannot rule out the involvement of CRH in the direct regulation of Rfrp and Gpr147 during the stress response, as we did not examine these effects; however, Kirby et al. found that 13% of Rfrp neurons express the CRH-R1 and studies in hypothalamic N39 cells have shown that CRH increases Gpr147 mRNA through the CRH-R1 (13, 232). Nevertheless, our results are consistent with a direct role for GCs in the regulation of Rfrp and Gpr147 gene expression through a GR-dependent signaling mechanism in hypothalamic Rfrp-expressing neurons.

Gene regulation can occur at different levels, most commonly through transcriptional activation of the 5’ regulatory region, modulation of mRNA stability, and the rate of translation. To address which regulatory pathway predominates in the regulation of gene expression in rHypoE-23 neurons upon GC exposure, we further investigated the necessity of new protein synthesis on GC-mediated changes in Rfrp and Gpr147 mRNA levels. Using an inhibitor of protein synthesis, CHX, we are the first to report that the stimulation of gene expression of both Rfrp and Gpr147 by GCs is independent of de novo protein synthesis and potentially due to transcriptional activation of specific promoter elements. This is consistent with results from other studies.
showing GR-mediated mechanisms in the hypothalamus. Delayed GC actions are generally mediated by the activation of the classical steroid hormone response involving the GR, a member of the nuclear receptor superfamily (279). Ligand-bound GRs translocate into the nucleus and initiate an intracellular cascade leading to the activation or suppression of target genes. Commonly, target genes contain a glucocorticoid response element (GRE) or other DNA-binding TF elements, allowing for direct DNA binding or tethering mechanisms through associated TFs (12, 280, 281). GnRH-secreting, GT1-7 neurons show direct repression of GnRH following GC treatment by virtue of a GR-containing multiprotein complex at a distal and proximal negative GRE (229, 280). Similarly, studies in LβT2 gonadotrope cells found that dexamethasone upregulates mouse GnRH-R mRNA through the recruitment of GR to an AP-1 site, simultaneously bound with TFs, c-Jun and c-Fos (282). Of interest, a search of the rat Rfrp promoter indicates the presence of a potential GRE, as well as other commonly associated TF binding elements within the first 1500 base pairs (bp) of the 5’ flanking region (Figure 3.6A). This observation is supported by similar findings by Kirby et al. in the rat Rfrp promoter (13). Similarly, the rat Gpr147 promoter reveals a classic GRE sequence within 1500 bp of the transcriptional start site (Figure 3.6B), supporting the direct GR-mediated transcriptional changes reported for both Rfrp and Gpr147 genes in the present study. Future studies will map regions within the 5’ regulatory regions of Rfrp and Gpr147 to localize sequences that are required and sufficient for the GR-mediated transcriptional changes in gene expression observed in rHypoE-23 neurons.

Taken together with previous findings, the present study suggests that rHypoE-23 neurons are representative of functional Rfrp-expressing hypothalamic neurons originating from the DMH, which are directly regulated by GCs. Here we demonstrate for the first time that the synthetic GC, dexamethasone stimulates the transcription of Rfrp and Gpr147 through a GR-dependent mechanism, as a result of transcriptional activation, rather than de novo protein synthesis in rHypoE-23 neurons. Because RFRP-3 has been found to regulate both neuroendocrine and behavioural mechanisms (16, 178, 191, 198, 206, 270, 283), the stress-mediated transcriptional regulation of both Rfrp and Gpr147 may further modulate or cross talk with innervating neural pathways to exert effects within the HPG axis (13, 282). Therefore, in line with the inhibitory role of RFRP-3 at the hypothalamic level (197, 198), we suggest that the RFRP/GPR147 system...
Figure 3.6. Schematic of the rat RFRP and GPR147 promoter-luciferase construct (pGL2). Rat RFRP (A) and GPR147 (B) contain transcription factor binding sites at indicated positions relative to the transcription start site.
adds a new level of complexity to the fine-tuning of the mammalian HPG axis and is a direct target for stress-mediated transcriptional changes at the hypothalamic level, potentially altering reproductive function and status in response to a myriad of stressors.
Chapter 4

RFRP-3-mediated Transcriptional Suppression of GnRH mRNA levels in a Novel GnRH-secreting Cell Model
4.1 Abstract

Reproduction is coordinated by the actions of various neuropeptides and peripheral hormones, all of which converge on GnRH neurons, residing at the pinnacle of the HPG axis. Recently, a novel hypothalamic neuropeptide, GnIH has emerged as a potent inhibitory modulator of neuroendocrine function in avian species. In mammals, RFRP-3 displays similar inhibitory functions to GnIH, though its distinct role in the HPG axis is not well defined. To date, there is a paucity of studies focusing on the direct regulatory effects of RFRP-3 on GnRH neurons. To address this, we analyzed the dose-dependent and direct effect of RFRP-3 on GnRH biosynthesis and secretion in a newly established cell model of GnRH neurons, mHypoA-GnRH/GFP that were found to express the RFRP-3 receptor, GPR147. Incubation of mHypoA-GnRH/GFP neurons with 100 nM RFRP-3 results in c-Fos activation and a transient attenuation of GnRH mRNA expression by approximately 60% for up to 4 h. To evaluate whether RFRP-3-induced suppression of GnRH mRNA levels was owing to mRNA stabilization or transcriptional regulation, we evaluated the actions of transcription inhibitors, actinomycin D and 5,6-Dichlorobenzimidazole riboside (DRB). Consistent with a transcriptional mechanism, both actinomycin D and DRB abolished the repression of the GnRH gene by RFRP-3. No significant changes in GnRH secretion were observed upon co-incubation of RFRP-3 with a NO donor, sodium nitroprusside. Our studies support a novel central mechanism for RFRP-3-mediated regulation at the level of the GnRH neuron via RFRP-3-induced suppression of GnRH synthesis, resulting in the limited availability of the GnRH transcript in mHypoA-GnRH/GFP neurons.

4.2 Introduction

Reproduction is controlled by the HPG axis, consisting of a complex array of neurochemical inputs that communicate information regarding the physiological status of an organism. The discrete population of GnRH neurons represents the final modulatory pathway governing the release of the gonadotropic hormones, LH and FSH from the anterior pituitary gland (23, 24). The amount of GnRH that is synthesized and further released into the hypophyseal portal circulation is dictated by a multiplicity of physiological regulators (46, 125, 284). GnRH gene expression and secretion are mediated by direct feedback from gonadal steroids (89, 91, 101, 103, 284, 285), as well as innervating hypothalamic neuronal systems (137, 151, 161-163, 286).
Gaining an in-depth understanding of how neuropeptidergic circuits are regulated and in turn mediate the HPG axis is critical in both normal and pathophysiological reproductive states.

Until recently, the decapeptide GnRH, originally isolated from mammals, was believed to be the sole hypophysiotropic mediator of gonadotropin synthesis and release (23, 24). Early attempts isolating an inhibitory hypothalamic factor with the ability to directly inhibit LH release had been made, though none were successful (168, 169). In 2000, Tsutsui et al. identified a novel avian neuropeptide, termed GnIH for its unique ability to rapidly and dose-dependently inhibit gonadotropic hormone release in cultured pituitaries (9). In avian species, GnIH neurons originate from the PVN and project to several brain regions, including the POA, the medial septum/diagonal band of Broca (MSDS), and the external layer of the ME. The favorable neuroanatomical positioning allows GnIH to regulate gonadotropin synthesis and release, as well as the sensitivity of the pituitary to GnRH stimulation (9, 183, 184). Furthermore, GnIH functions centrally to mediate the activity of both GnRH-I and-II neurons, thereby altering GnRH release and reproductive behaviours (178, 268, 287). Thus, GnIH plays a critical role in mediating avian reproduction and behaviour, however its actions in the mammalian HPG axis are still under investigation.

In mammals, GnIH homologues are encoded by the Rfrp gene, which generates two physiologically active RF-amide related peptides, RFRP-1 and RFRP-3(10). Rfrp-expressing neurons are found within regions of the DMH and PVN in rodents, ewes, hamsters, and humans (11, 12, 17, 196). RFRP-3 acts mainly through the GPR, GPR147 (OT7T022) that is expressed in regions of the hypothalamus, pituitary, and gonads (11, 15, 16). The conserved neuroanatomical distribution of RFRP-3 and GPR147 across all studied species, suggests that this novel neuropeptide acts both at the level of the hypothalamus and pituitary. Regarding the latter, peripheral injections of both quail GnIH and RFRP-3 were found to suppress LH release (14, 191). Moreover, RFRP-3 effectively suppresses GnRH-stimulated LHβ and FSHβ gene transcription, inhibits LH release, as well as further attenuates GnRH-stimulated ERK phosphorylation (194, 288). RFRP-3 can also be measured in the ovine hypophyseal portal circulation, exhibiting pulsatility and a short peptide half-life (6 min) (16). Contrary to these findings, no RFRP-terminals are found within the secretory zone of the median eminence in rats (190). Although species-specific differences exist regarding the hypophysiotropic actions of
RFRP-3, it seems likely that this novel neuropeptide operates both at the level of the gonadotrope and hypothalamus to mediate mammalian reproduction. Interestingly, unlike the pituitary effects, the central actions of RFRP-3 are the most conserved and consistent feature of this neuropeptide circuit. As in avian species, immunocytochemical studies have observed that RFRP-immunoreactive fibers are in direct apposition to GnRH neurons in rodents, ewes, and humans (11, 12, 17, 222). ICV injection of RFRP-3 decreases LH release \textit{in vivo} (12, 193), as well as results in the hyperpolarization and inhibition of neuronal firing in subpopulations of GnRH neurons (198). Recent studies in mice have also demonstrated that 33% of GnRH neurons \textit{in vivo} express GPR147 (197). Therefore, it is well accepted that RFRP-3 has central effects targeting GnRH neurons; however, the mechanisms through which this neuropeptide directly alters GnRH biosynthesis and secretion remain unclear.

To date, most mammalian studies have focused on the ability of GnIH/RFRP-3 to act as a hypophysiotropic factor inhibiting the actions of GnRH at the level of the pituitary, though the molecular mechanisms that govern RFRP-3-mediated changes in the synthesis and secretion of GnRH at the level of the GnRH neuron remain to be investigated. In the present study, our aim was to determine whether RFRP-3 treatment could directly alter hypothalamic GnRH biosynthesis or release and to further delineate the cellular mechanisms involved in this regulation. Due to the complex architecture and heterogeneous nature of the hypothalamus, as well as the lack of adult, non-clonal GnRH-secreting cell lines, we proceeded to use a newly established and characterized GnRH neuronal model, the mHypoA-GnRH/GFP, previously described (McFadden et al., manuscript submitted). We establish that mHypoA-GnRH/GFP neurons express \textit{Gpr147} and demonstrate c-Fos activation upon RFRP-3 treatment. Furthermore, we provide the first line of evidence to suggest that RFRP-3 can act directly on GnRH neurons \textit{in vitro} to mediate GnRH biosynthesis, occurring through transcriptional activation rather than disruption of mRNA stability.
4.3 Results

4.3.1 Characterization of Gpr147 mRNA expression in mHypoA-GnRH/GFP neurons

We have previously reported the generation and characterization of a non-clonal, adult-derived GnRH-synthesizing hypothalamic cell line (McFadden et al., manuscript submitted). Briefly, this cell line was immortalized through retroviral transfection methods utilizing the simian virus 40 T antigen on primary hypothalamic culture isolated from a 2 month old transgenic GnRH-GFP mouse, followed by fluorescence activated cell sorting (FACS) to generate a non-clonal GnRH cell model, using a protocol previously established (251). mHypoA-GnRH/GFP neurons express a multitude of key reproductive receptors and neuron specific markers (Figure 4.1A). The regulation of GnRH by RFRP-3 is mediated through the actions of its receptor, GPR147. With the aim of further characterizing the mHypoA-GnRH/GFP model, we conducted RT-PCR to determine whether the receptor for RFRP-3 is present on GnRH neurons in vitro. Here we report that the novel GnRH-secreting cell model, mHypoA-GnRH/GFP expresses Gpr147 mRNA (Figure 4.1B), whereas Rfrp mRNA was not expressed (Figure 3.1B). Importantly, the expression of Gpr147 in mHypoA-GnRH/GFP neurons provides evidence for the role of RFRP-3 in the direct regulation of GnRH synthesis and secretion in vitro.

4.3.2 RFRP-3 induces neuronal activation in mHypoA-GnRH/GFP neurons

c-Fos and its protein equivalent have become the most widely used marker for the anatomical identification of neuronal activation (289). In response to a myriad of stimuli, c-Fos mRNA changes have been reported to peak at 30 and 60 min after acute exposure. mHypoA-GnRH/GFP neurons were treated with vehicle or RFRP-3 (100 nM) and total RNA was collected at 15, 30, and 60 min following treatments. A sample was collected at 0 min, serving as a negative control to report basal c-Fos levels in the hypothalamic neuronal cell line. mHypoA-GnRH/GFP neurons displayed a robust increase in c-Fos mRNA levels at 30 and 60 min following RFRP-3 treatment (Figure 4.2), [30 min, vehicle (0.95 ± 0.08) vs. RFRP-3 (1.55 ± 0.09), P<0.001; 60 min, vehicle (0.69 ± 0.06) vs. RFRP-3 (1.20 ± 0.16), P<0.001]. These observations show that mHypoA-
Figure 4.1. Gene expression profile of an adult-derived, non-clonal GnRH-secreting cell line, mHypoA-GnRH/GFP (A) RT-PCR results of relevant reproductive neuropeptides and receptors in the whole hypothalamus (positive control) and indicated hypothalamic cell line. (+) indicates the presence of a gene, and (-) indicates the absence or weak expression of a gene. (B) Representative RT-PCR screening of Gpr147. In brief, total RNA was isolated and amplified using the One-Step RT-PCR Qiagen kit with gene-specific primers. Products were visualized on a 2% agarose gel with whole hypothalamus (positive control) and a non-template control (NTC; negative control).
**Figure 4.2. RFRP-3 induces c-Fos mRNA expression in the mHypoA-GnRH/GFP neurons.**

Cell culture media was changed to serum free, low glucose media for 4 h prior to treatment with vehicle (white bars) or 100 nM RFRP-3 (black bars). RNA was harvested at 0, 15, 30, and 60 min following treatment and changes in c-Fos were quantified using real-time RT-PCR and mRNA levels were normalized to the internal control, histone 3a. The *dotted line* represents basal c-Fos mRNA levels at 0 min (negative control). Results are expressed as mean ±SEM (n=5-6 independent experiments). ***, P<0.001 vs. time-matched vehicle control. Statistical significance was determined by two-way ANOVA with Bonferroni’s post hoc test.
GnRH/GFP neurons are activated in response to RFRP-3 treatment, confirming that these cells are representative of RFRP-3-responsive GnRH neurons.

4.3.3 RFRP-3 mediates the direct and transient suppression of GnRH mRNA levels in mHypoA-GnRH/GFP neurons

There is compelling evidence that GnRH neurons are directly innervated by RFRP-3-immunoreactive fibers, eventuating in a suppression of GnRH neuronal activity (17, 197, 198, 200). Indeed, the presence of GPR147 in mHypoA-GnRH/GFP neurons and neuronal activation upon RFRP-3 prompted investigation into RFRP-3-mediated changes in GnRH gene expression. To assess the role of RFRP-3 on the regulation of GnRH biosynthesis, we explored the dose-dependent effects of RFRP-3 treatments on mHypoA-GnRH/GFP neurons. Using real time RT-PCR, we measured changes in GnRH mRNA for 24 h following vehicle, 10 nM, or 100 nM RFRP-3 treatment. RNA was isolated at indicated time points. 100 nM RFRP-3 was found to suppresses GnRH mRNA expression by 59%, 59%, and 55% at 1, 2, and 4h, respectively following treatment (Figure 4.3) [1 h, vehicle (1.27 ± 0.12) vs. 100 nM RFRP-3 (0.76 ± 0.15), P<0.05; 2 h, vehicle (1.44 ± 0.08) vs. 100 nM RFRP-3 (0.86 ± 0.18), P<0.05; 4 h, vehicle (1.38 ± 0.09) vs. 100 nM RFRP-3 (0.76 ± 0.06), P<0.05]. No significant changes in GnRH mRNA levels were observed with 10 nM RFRP-3; although, a trend suggesting modest transcriptional suppression of the GnRH gene was evident up to 4 h. Indeed, these results clearly demonstrate that GnRH neurons are not only responsive to RFRP-3, but show a repression of GnRH biosynthesis in a rapid and transient manner in mHypoA-GnRH/GFP neurons.

4.3.4 Transcription blockade attenuates the RFRP-3-induced suppression of GnRH gene expression

To determine whether RFRP-3-mediated transcriptional changes are a result of mRNA stability mechanisms or require new RNA synthesis, mHypoA-GnRH/GFP neurons were exposed to RNA polymerase II gene transcription inhibitors, Act D (10 µg/mL) and DRB (60 uM). Given the significant changes in gene expression observed with 100 nM RFRP-3, this dose was chosen for subsequent analysis regarding the mechanisms involved in the regulation of GnRH gene expression and release. Neurons were treated with Act D, DRB, or DMSO vehicle for 1 h prior
Figure 4.3. RFRP-3-mediated regulation of GnRH mRNA expression in mHypoA-GnRH/GFP neuronal cells. Cells were treated with vehicle (white bars), 10 nM RFRP-3 (gray bars), and 100 nM RFRP-3 (black bars) over a 24 h time course. Total RNA was collected at indicated time points and changes in GnRH mRNA levels were assessed using real-time RT-PCR. mRNA expression levels were normalized to the internal control, histone 3a. Data are expressed as mean ± SEM (n=4-6 independent experiments). *, P<0.05 vs. time-matched vehicle control. Statistical significance was determined by two-way ANOVA with Bonferroni’s post hoc test.
Figure 4.4. Effects of transcription blockade on RFRP-3-mediated suppression of GnRH mRNA expression in mHypoA-GnRH-GFP neurons. Cells were pre-treated for 1 h in the presence or absence of Act D (10 ug/mL) or DRB (60 uM), followed by treatment with 100 nM RFRP-3, or vehicle over a 4 hr time course. GnRH mRNA expression was determined using real-time RT-PCR and levels were normalized to the internal control, histone 3a. Results shown are expressed as mean ± SEM (n=5-8 independent experiments). *, P<0.05 vs. vehicle control. Statistical analysis was calculated by two-way ANOVA. White bars, control vehicle (H20, DMSO in the presence or absence of transcription inhibitors, Act D or DRB); black bars, treatment (RFRP-3 in the presence or absence of Act D or DRB).
to RFRP-3 treatments. RNA was collected at 1, 2, and 4 h subsequent to RFRP-3 treatment. As compared with DMSO vehicle, RFRP-3 significantly represses GnRH mRNA levels at 1 h; however, pretreatment with transcription inhibitors attenuated RFRP-3 mediated changes in gene expression (Figure 4.4A) [1h, DMSO: (0.81 ± 0.04) vs. DMSO + RFRP-3 (0.32 ± 0.05) P<0.05; Act D (1.47 ± 0.18) vs. Act D + RFRP-3 (1.48 ± 0.18) P>0.05; DRB (1.31 ± 0.28) vs. DRB + RFRP-3 (1.28 ± 0.16) P>0.05]. Similar effects were observed at 2 h after treatment, [2h, DMSO: (1.14 ± 0.16) vs. DMSO + RFRP-3 (0.55 ± 0.09) P<0.05; Act D (1.45 ± 0.24) vs. Act D + RFRP-3 (1.55 ± 0.18) P>0.05; DRB (0.88 ± 0.09) vs. DRB + RFRP-3 (0.98 ± 0.19) P>0.05], as well as 4 h following RFRP-3 treatment [4 h, DMSO: (0.89 ± 0.08) vs. DMSO + RFRP-3 (0.32 ± 0.07) P<0.05; Act D (1.32 ± 0.25) vs. Act D + RFRP-3 (1.43 ± 0.12) P>0.05; DRB (1.56 ± 0.17) vs. DRB + RFRP-3 (1.54 ± 0.29) P>0.05], whereby transcription inhibitors attenuated the suppressive effect of RFRP-3 on the GnRH gene (Figure 4.4B-C). Act D and DRB alone were shown to increase basal GnRH mRNA levels at 1 and 4 h, respectively in this neuronal cell line, suggesting that the transcription inhibitors may be attenuating the synthesis of a suppressor or labile mRNA destabilizer responsible for the basal regulation of the GnRH transcript (246, 290). Overall, these findings suggest that RFRP-3-induced repression of GnRH mRNA levels is mediated at the level of transcription rather than mRNA stabilization in mHypoA-GnRH/GFP neurons.

4.3.5 RFRP-3 is unable to attenuate SNP-induced GnRH secretion in mHypoA-GnRH/GFP neurons.

Finally, we examined whether RFRP-3 can suppress GnRH-induced secretion in this novel cell model. In studies analyzing the signaling cascade of RFRP-3, the inhibitory effect of RFRP-3 was mediated by the coupling of GPR147 with G\textsubscript{ai}, activating an inhibitory AC/cAMP/PKA-dependent pathway, as well as hindering an intracellular calcium influx (191, 194). A preliminary analysis suggested that RFRP-3 could not reduce basal GnRH secretion alone (unpublished data), a common feature of RFRP-3-mediated mechanisms (194, 197, 291). Subsequent studies used a nitric oxide donor, sodium nitroprusside (SNP) previously shown to induce basal GnRH secretion \textit{in vitro} (292, 293). Therefore, by stimulating GnRH secretion we could highlight the potential inhibitory effect of RFRP-3. Neurons were serum starved for 4 h prior to treatment with water vehicle, 100µM SNP, or co-treatment of SNP and 100 nM RFRP-3.
Figure 4.5. Effects of RFRP-3 on GnRH secretion in the presence of SNP in mHypoA-GnRH/GFP neurons. Cell culture media was changed to serum free, low glucose media for 4 h prior to treatment with water vehicle, SNP (100 µM), or SNP (100 µM) + RFRP-3 (100 nM). Media and total protein was collected after 1 hour, and GnRH levels were measured by a GnRH-specific EIA and further normalized to total protein levels. Results are expressed as mean ±SEM (n=3 independent experiments). *, P<0.05 vs. vehicle control. Statistical significance was determined by one-way ANOVA with Bonferroni’s post hoc test.
Figure 4.6. *In silico* analysis of mouse GnRH mRNA sequence for potential miRNA binding sites. Nucleotide sequence and accession number were obtained from GenBank and were subsequently analyzed by MicroInspector web tool for the presence of known mouse-specific (mmu) miRNAs. (A) Mouse GnRH nucleotide sequence with the predicted location of miRNA binding sites. (B) List of all the miRNAs predicted to bind to the mouse GnRH sequence.
Media and total protein were isolated 1 h following incubation and changes in GnRH secretion were measured with a GnRH-specific EIA. SNP significantly upregulates GnRH release in mHypoA-GnRH/GFP neurons; though, there is no significant inhibition observed upon the addition of RFRP-3 (100 nM) to cultured cells (Figure 4.5) [vehicle (1.00 ± 0.08) vs. SNP (1.57 ± 0.09) P<0.05; vehicle (1.00 ± 0.08) vs. SNP + RFRP-3 (1.48 ± 0.11) P<0.05; SNP (1.57 ± 0.09) vs. SNP + RFRP-3 (1.48 ± 0.11) P>0.05. These results suggest that upon co-treatment with SNP, a potent NO donor RFRP-3 only slightly suppresses GnRH-induced secretion, resulting in no significant changes in the overall amount of GnRH peptide secretion in this hypothalamic cell line.

4.3.6 In silico analysis of putative miRNA binding sites in the mouse GnRH mRNA sequence

Although RFRP-3 mediates the transcriptional suppression of GnRH gene expression, the rapidity of the observed response can potentially be due to the RFRP-3-mediated synthesis of miRNAs that are responsible for the turnover of the GnRH transcript. We employed the web tool, MicroInspector to scan the mouse GnRH mRNA sequence for putative miRNA binding sites (294). Analysis of the predicted miRNA binding sites suggests RFRP-3 can mediate the availability of GnRH mRNA through the activation and transcription of specific miRNAs targeting the GnRH gene (Figure 4.6).

4.3 Discussion

Numerous innervating neuropeptidergic and neurotransmitter circuits mediate the synthesis and release of GnRH in response to gonadal steroids, metabolic status, and stress. The RFamide peptides, particularly kisspeptin and GnIH/RFRP-3 have emerged as important regulators of mammalian reproduction (12, 126, 222). Although the role of RFRP-3 at the level of the pituitary is disputed in some species, the ability of RFRP-3 to influence GnRH neurons is a well-accepted feature of this neuropeptidergic system. The actions of RFRP-3 are mainly mediated by GPR147, though a second receptor, GPR74 has been characterized; however, RFRP-3 binds principally with GPR147 and shows a lower affinity for GPR74 (10, 181). The aims of the present study were to delineate the mechanisms involved in the potential direct regulation of GnRH mRNA
and secretion by RFRP-3 in a novel adult, non-clonal GnRH-secreting cell line, mHypoA-GnRH/GFP. We are the first to report that RFRP-3 is involved in the rapid and direct transcriptional repression of the GnRH gene. Our data also reveal that RFRP-3 does not induce changes in GnRH release upon co-incubation with a potent NO donor, SNP, previously reported to augment the induction of GnRH release in vitro (292, 293). Therefore, the present study supports the central inhibitory role of RFRP-3 within the HPG axis, observed mainly through the suppression of GnRH gene expression at the level of the GnRH neuron.

RFRP-3 neurons directly appose GnRH-expressing neurons in the POA in vivo (126, 176, 196). Initial characterization of RFRP-3 in mammals found that RFRP-3-immunoreactive fibers extend to a large percentage of GnRH neurons, approximately 40% in Syrian hamsters (12). The same relationship was observed within the ovine hypothalamus, whereby 63% of RFRP-expressing neurons are in direct apposition to GnRH cell bodies within the POA (196). In accordance with avian studies (176), Rizwan et al. demonstrated that subpopulations of GnRH neurons express Gpr147, whereas Gpr74 mRNA was not found to colocalize with GnRH in mice (197).

Interestingly, ICV injection of a potent GPR147 antagonist, RF9 causes an induction of LH release, supporting a central mechanism for RFRP-3; however, the same study failed to report modifications in LH plasma levels upon ICV injection of RFRP-3 (291). Similarly, a recent study reported a stimulatory effect of RFRP-3 on LH release following central administration in male Syrian hamsters. These data indicate that the inhibitory actions of RFRP-3 within the in vivo hypothalamus are not only species specific but also strongly dependent on the steroidal and seasonal status of an animal (16). Further investigations into such mechanisms are required.

Overall, the expression of Gpr147 in mHypoA-GnRH/GFP neurons corroborates in vivo observations that subpopulations of GnRH neurons co-express Gpr147 and that RFRP-3 regulates GnRH activity directly at the GnRH neuron. The mHypoA-GnRH/GFP cell model is representative of the heterogeneous population of GnRH neurons in vivo and is a suitable model to investigate the regulatory role of RFRP-3 on GnRH synthesis and secretion.

A number of studies have reported the electrophysiological effect of RFRP-3 on central neurons, particularly GnRH. Previous work confirmed that RFRP-3 (1 µM) evokes a direct and rapid suppression of neuronal firing rate in 41% of GnRH neurons in the mouse, whereby the addition of GABA or glutamate receptor antagonists has no effect, suggesting a direct mechanism (198). The same study also reported that 47% and 12% of GnRH neurons demonstrate no change or an
increase in neuronal activity upon RFRP-3 treatment, respectively. Such heterogeneity in GnRH neurons has previously been reported in the GnRH-secreting, GT1-7 model (199). Similarly, RFRP-3 causes hyperpolarization of vesicular glutamate receptor transporter 2 (vGluT2)-expressing GnRH neurons via postsynaptic mechanisms, involving the potassium channel, where it also attenuates kisspeptin-mediated activation of GnRH neurons in the MSDS (200). Central RFRP-3 infusion (25ng/h) results in a dramatic suppression of c-Fos expression in GnRH and AVPV neurons during an estradiol-induced surge in rats (192). Collectively, these results indicate that the central effects of RFRP-3 are mediated through the suppression of GnRH neural activity. Therefore, it is plausible that RFRP-3 participates in the coordinated inhibition of a subpopulation of GnRH neurons or attenuates stimulatory signals mediating GnRH release, reducing the overall pulsatile activity of GnRH neurons during a particular stage of development or the female reproductive cycle. Contrary to these findings, we report that RFRP-3 (100 nM) treatment induces the expression levels of immediate early gene, c-Fos in mHypoA-GnRH/GFP neurons after 30 and 60 min following exposure, suggesting that mHypoA-GnRH/GFP neurons are an RFRP-3-responsive cell model. It could be argued that RFRP-3 treatment triggers the activation of the c-Fos signaling cascade, whereby alterations to GnRH gene expression are mediated indirectly through the c-Fos/c-Jun pathway acting on the imperfect AP-1 site found in the mouse GnRH promoter (295). Consistent with these findings, the phorbol ester, TPA alters GnRH gene expression through a PKC-dependent pathway in both the GT1-1 and GT1-7 cell lines (296, 297). TPA-induced activation of PKC converges on c-Fos pathways leading to the suppression of GnRH gene expression. The potential role of c-Fos in RFRP-3-mediated signaling warrants further investigation; however, our results suggest that this hypothalamic GnRH model is activated and responsive to RFRP-3 treatment.

Several studies have documented the ability of GnIH/RFRP-3 to regulate GnRH neurons (198, 200), though due to the technical limitations of in vivo and electrophysiological approaches, dissecting direct effects is challenging. This is the first study to report that incubation of mHypoA-GnRH/GFP neurons with RFRP-3 results in the rapid and transient suppression of GnRH transcript levels. Thus, RFRP-3 mediates the reproductive axis partly by limiting the accumulation of hypothalamic GnRH mRNA, leading to a decrease in the overall amount of GnRH peptide available for secretion. This direct central role of RFRP-3 corroborates previous findings demonstrating that ICV injection of RFRP-3 results in a decrease of LH plasma levels.
Given the rapid suppression of GnRH gene expression, it seemed plausible that RFRP-3 could be affecting the stability of pre-existing GnRH mRNA. Using transcriptional inhibitors, Act D and DRB, we observed the reversal of RFRP-3-induced suppression of GnRH transcript levels, consistent with the involvement of new RNA synthesis in the regulation of the GnRH gene by RFRP-3 rather than mRNA stability. In accordance with these studies, the rapid upregulation (within 6 h) of GnRH receptor mRNA at the level of the pituitary is in part due to transcriptional activation rather than mRNA stabilization (298). Similarly, Act D attenuates dexamethasone-mediated upregulation of the ob gene, whereby insulin-stimulated leptin secretion remains unaltered upon transcription blockade, suggesting that both transcriptional activation and mRNA stabilization mechanisms play a critical role in gene regulation (290). Interestingly, Act D and DRB resulted in the basal upregulation of GnRH mRNA levels in vehicle controls, as well as RFRP-3 treated neurons. Although this was an unexpected effect of these transcription blockers, Act D and DRB have both been found to induce the basal expression of the proglucagon (246), ob (290), and c-myc (299) genes. Therefore, the observed effect was not cell specific and is most likely a result of the suppression of labile mRNA destabilizers or other degradative enzymes regulating basal GnRH mRNA levels (246, 290, 299). Collectively, these studies indicate that though the stabilization of pre-existing mRNA levels plays a critical role in hormonal and neuropeptidergic gene regulation (246, 300), our results are consistent with an RFRP-3-induced transcriptional mechanism. Further investigation is required to determine whether RFRP-3 suppression of the GnRH transcript is through direct transcriptional regulation of the GnRH promoter or indirectly through the activation of mRNA destabilizers or miRNAs. Of interest, a search for putative miRNA binding sites within the GnRH mouse promoter (Figure 4.6) revealed binding sites for several miRNAs. miRNAs play an important role in protein and gene regulation within the HPG axis, for instance miR-132 and miR-212 have been implicated in regulating GnRH-stimulated gene regulation of the gonadotropic hormones in LβT2 cells (300). Future studies will investigate the regulatory role of RFRP-3 on the miRNA expression profile in mHypoA-GnRH/GFP neurons.

Although the hypophysiotropic role of RFRP-3 is unclear in mammals, studies have shown that RFRP-3 can inhibit gonadotropin release in vivo and GnRH-stimulated signaling in cultured pituitaries and LβT2 cells (12, 16, 192-194). Interestingly, all studies have reported the inability of GnlH/RFRP-3 to suppress gonadotropin release and gene expression alone, requiring either
forskolin or GnRH stimulation to evoke an inhibitory effect. Despite the central importance of RFRP-3 at the hypothalamic level, to date, little is known regarding the effects of RFRP-3 on GnRH release. We report that RFRP-3 is unable to inhibit GnRH secretion in mHypoA-GnRH/GFP neurons alone (data not shown) or in the presence of a nitric oxide donor, SNP (positive control). SNP acts on NO-responsive GnRH neurons to activate guanylyl cyclase, thereby converting GTP into cGMP (301). Rises in cGMP lead to elevations in intracellular calcium, which acts to stimulate downstream signaling cascades that eventuate in the conversion of arachidonic acid to PGE2, further activating the cAMP/protein kinase A-dependent release of GnRH into the median eminence. RFRP-3 signals mainly through GPR147 that has been found to couple with G\textsubscript{\alpha}i to suppress cAMP production, inhibit intracellular calcium immobilization, and attenuate the phosphorylation of ERK (10, 181, 183, 194). Therefore, it is plausible that the RFRP-3/GPR147 complex triggers a G\textsubscript{\alpha}i signaling cascade, inhibiting the SNP-induced calcium influx or inhibiting AC/cAMP activation, which we would predict given the inhibitory role of RFRP-3; however, such inhibition was not observed in the present study. It could be argued that the failure of RFRP-3 to attenuate SNP-induced GnRH secretion in this neuronal cell model was due to the potency of SNP as a stimulant of GnRH secretion, masking the inhibitory potential of RFRP-3 upon co-incubation. Future studies will explore different experimental conditions including other secretory stimulants at varying concentrations, to rule out SNP as a confounding factor in the ability of RFRP-3 to attenuate GnRH peptide secretion. A further consideration is that RFRP-3 functions predominantly at the transcriptional level, altering the availability of GnRH mRNA rather than regulating translational or post-translational mechanisms directly, a notion that is supported by the reported direct and transient suppression of GnRH gene expression by RFRP-3 treatment.

Collectively, the present study provides the first line of evidence that RFRP-3 is involved in the direct and rapid suppression of GnRH mRNA levels in a novel GnRH-synthesizing cell line, mHypoA-GnRH/GFP. Furthermore, we report that the blockade of new RNA synthesis abolishes RFRP-3-mediated changes in GnRH mRNA levels and that RFRP-3 fails to attenuate SNP-induced GnRH release in mHypoA-GnRH/GFP neurons. The effect of RFRP-3 on GnRH neurons appears to occur through the transcriptional repression of the GnRH gene, rather than stability or translational mechanisms. In line with previous studies, these data establish that RFRP-3 exerts its inhibitory effects in the mammalian HPG axis, partly through its direct central
role in mediating the transcriptional activity of the GnRH gene at the level of the GnRH neuronal network.
Chapter 5
General Discussion
5.1 Overall Conclusions

GnRH is well established as the master endocrine molecule governing reproductive function; however, much remains unknown regarding the precise mechanisms through which this small and dispersed neuronal population integrates a multitude of environmental and peripheral stimuli. Although numerous neuropeptidergic and neurotransmitter systems impinging on the GnRH neuronal network have been characterized, until recently there was a missing link regarding the communication of information such as photoperiod, stress, and gonadal steroids (302). The RFamide peptides, particularly Kiss and GnIH/RFRP-3 which posses an Arg-Phe-NH$_2$ C-terminal motif have emerged as important regulators of vertebrate reproduction (12, 126, 222). There is a growing body of evidence supporting the influential role of Rfrp neurons in environmental-induced reproductive effects, particularly stress (13, 126, 205-208); however, the precise molecular mechanisms of this novel neuropeptide remain in their infancy, largely due to a lack of appropriate models. To date, most findings have focused on the hypophysiotropic properties of GnIH/RFRP-3 as a neurohormone, whereby only a handful of studies have reported on the central role. Although much progress has been made dissecting the precise level and functional role that RFRP-3 operates within the HPG axis, the steroidal mechanisms that mediate RFRP-3 biosynthesis, as well as the direct regulatory effect of RFRP-3 at the level of the GnRH neuron have not been investigated. In the present thesis, steroid-mediated mechanisms governing Rfrp and Gpr147 gene expression were defined. Further, the function of RFRP-3 at the level of GnRH biosynthesis and secretion in a novel adult, non-clonal GnRH-secreting cell line was delineated.

Indeed, the RFRP/GPR147 system adds a new level of complexity to the fine-tuning of the mammalian HPG axis and therefore, has been implicated in regulating the physiological parameters associated with a myriad of stressors. GCs directly mediate GnRH gene expression at the level of the proximal promoter (199, 227-229, 280); however, the favourable neuroanatomical distribution and presence of steroid receptors on Rfrp-expressing neurons in the DMH makes this novel neuropeptide a candidate for mediating stress-induced changes upstream of GnRH. Interestingly, Rfrp neurons have been shown to project to the PVN, where they provide input to CRH and oxytocin neurons, both of which are indispensable in regulating energy balance and the stress response (230, 278). Further supporting this idea, ICV injection of RFRP-
3 stimulates ACTH and oxytocin release, as well as induces anxiety-related behaviours in rats (207). Both acute and chronic stressors have also been implicated in increasing RFRP mRNA levels (13). This growing body of evidence supports the role of the Rfrp neuronal network in stress-induced effects, though due to heterogeneity and numerous innervating neuronal networks that make up the hypothalamus, dissecting such direct effects in vivo, are challenging. To address this issue, the work this thesis (Chapter 3) sought to identify the mechanisms by which GCs and the gonadal hormone, E₂ regulate Rfrp and Gpr147 biosynthesis. Employing a rat clonal, embryonic hypothalamic cell model that displays strong endogenous expression of Rfrp mRNA, we report that upon incubation of neurons with the synthetic GC, dexamethasone both Rfrp and Gpr147 mRNA levels were significantly stimulated (Figure 3.2). Furthermore, we elucidate that the GR is required for the dexamethasone-induced transcriptional regulation of Rfrp and Gpr147 genes, whereby a potent GR antagonist, RU-486 abolished the initial observed effects (Figure 3.4). This finding is corroborated by studies performed in the rat animal model, whereby an adrenalectomy prevents GC-mediated upregulation of Rfrp gene expression (13). Further analysis also revealed that the stimulatory effect of dexamethasone at the level of gene expression was independent of de novo protein synthesis (Figure 3.5). This finding suggests that pre-existing labile proteins or complement gene products are not responsible for increasing the stability of Rfrp or Gpr147 genes in the presence of dexamethasone; instead dexamethasone modulates the expression of these genes via direct transcriptional mechanisms. Collectively, these data have identified a novel mechanism for GR-mediated signaling at the hypothalamic level, whereby Rfrp and Gpr147 genes are direct targets of GCs in this hypothalamic neuronal cell model (Figure 5.1).

Because gonadal steroids are downstream targets of HPA axis activation and also feedback to mediate GC levels (3, 231, 303), considerable crosstalk exists between the HPG and HPA axes. To partly address the involvement of the gonadal steroids in the stress-response at the level of the Rfrp-expressing neuron, E₂ alone and in the presence of dexamethasone was incubated with the neurons. Interestingly, given the potency and necessity of E₂ in the HPG axis, its role in altering neuron specific gene expression is disputable in many hypothalamic populations. Indeed, contention exists regarding the effects of E₂ on Rfrp neurons. Studies in a multitude of animal models have yielded inconsistent results, whereby some studies support the involvement of
Figure 5.1 Representative model summarizing the proposed mechanism by which dexamethasone directly regulates Rfrp and Gpr147 mRNA levels in rHypoE-23 neurons. This working model suggests that glucocorticoids are involved in the direct regulation of Rfrp- and Gpr147-expressing rHypoE-23 neurons. Further, changes are GR-dependent and do not require the synthesis of new proteins. Both genes have putative GRE consensus sequences in the 5’ regulatory region, as well as other associated TF motifs. It remains to be elucidated whether GR binds directly or indirectly to the promoter region. Solid line (reported result); dotted line (to be elucidated)
RFRP-expressing neurons in negative steroidal feedback (12, 223); however, comparable experimental studies demonstrate no effect of E$_2$ (223) or contrary findings (126, 221). We report that E$_2$ treatment did not exert any changes in gene expression profiles nor was it involved in any additive or antagonizing effects in the presence of dexamethasone in rHypoE-23 neurons over the limited time course and dose curve used in this study (Figure 3.2). Several possible explanations exist for the inconsistencies observed. Circulating levels of E$_2$ change drastically throughout reproductive development, therefore the sensitivity of E$_2$-responsive neurons may be highly dependent on dose and developmental stage (223, 304). Because we are using a clonal hypothalamic model, we cannot rule out the responsiveness of the whole heterogeneous population of *Rfrp*-expressing neurons in the DMH to E$_2$. Furthermore, the current study utilizes an *in vitro* model, where innervating neuronal and glial networks are absent; thus, the necessity of other stimuli in the E$_2$-mediated response remains unknown. Lastly, Poling *et al.* has reported heterogeneity within the *Rfrp* network, demonstrating the presence of two distinct high and low-expressing *Rfrp* neuronal populations, each revealing unique developmental and steroidal induction patterns (126). Indeed, it is plausible that the rHypoE-23 neurons represent a neuron that is non-responsive to gonadal steroids. Overall, these findings indicate that estradiol does not augment or antagonize the stimulatory effects of dexamethasone on *Rfrp* and *Gpr147* biosynthesis; however, its role in mediating the *Rfrp* neuronal system is not clear *in vitro*.

RFRP-3 neurons directly appose 40-80% of GnRH-expressing neurons in the POA *in vivo* (126, 176, 196). Moreover, studies have recently identified that subpopulations of GnRH neurons express *Gpr147* in mammals. Further, the available data indicates that RFRP-3 inhibits GnRH neuronal activity and simultaneously attenuates stimulatory signals from upstream effectors, like Kiss that target GnRH neurons (198, 200). Indeed, precedence has been established regarding the conserved role of RFRP-3 at the level of the GnRH neuron; though, the precise RFRP-3-induced inhibitory mechanisms regulating GnRH gene expression and neurosecretion have not been described. Using a novel adult, non-clonal GnRH-secreting cell model, mHypoA-GnRH/GFP the work presented in this thesis (Chapter 4) elucidated a novel mechanism through which RFRP-3 directly acts on GnRH neurons. It was reported that RFRP-3 rapidly and transiently represses GnRH mRNA levels up to 4 h following treatment (Figure 4.3). In addition, we observed that this overall suppression in GnRH mRNA was owing to altered transcriptional rate rather than the stability of pre-existing mRNA (Figure 4.4). Collectively, these findings suggest that RFRP-3-
mediated inhibition of GnRH neurons occurs partly through the direct transcriptional repression of the GnRH gene; however, whether this mechanism is mediated directly by RFRP-3 or indirectly through the activation of other transcription factors, degradative enzymes or miRNAs cannot be ruled out (Refer to Future Directions). Due to the activation of c-Fos mRNA levels upon RFRP-3 treatment (Figure 4.2), it is plausible that the immediate early gene acts to initiate a signaling cascade implicated in the attenuation of GnRH gene expression, a previously described phenomenon (295-297). Expanding our knowledge of how this novel neuropeptidergic system alters GnRH biosynthesis is critical and will enable a better understanding of how RFRP-3 modulates reproductive activity and function at the hypothalamic level.

The release of GnRH in vivo and in vitro is dependent on stimulatory factors that directly activate intracellular cascades governing peptide secretion (125). RFRP-3/GnIH binds to its GPCR, GPR147 that has been found to couple with G_\text{ai} to promote an inhibitory effect on cAMP production, calcium influx, and the phosphorylation status of the mitogen-activated protein kinase (MAPK), ERK (10, 181, 191, 194). Therefore, due to the overall inhibitory effect RFRP-3 has on gonadotropin release in vivo and in vitro (12, 16, 192-194), we sought to address whether RFRP-3 can inhibit GnRH release directly at the level of the GnRH neuron. Precedence has been established in the literature that RFRP-3 cannot inhibit gonadotropin release independent of a stimulatory effector, such as GnRH or forskolin. Similarly, preliminary data (data not shown) demonstrated the inability of RFRP-3 to inhibit GnRH secretion alone in mHypoA-GnRH/GFP neurons. To circumvent this issue, we utilized a NO donor, SNP that is recognized as a potent stimulant of GnRH neurosecretion in vitro to induce GnRH peptide release and potentially highlighting the inhibitory effect of RFRP-3 (292, 293). Unexpectedly, RFRP-3 was unable to attenuate SNP-induced GnRH release; though, further assessment of different concentrations and time courses are required (Figure 4.5). These results may be attributable to the potency of SNP as an effective stimulant of GnRH secretion, whereby the inhibitory capacity of RFRP-3 was confounded. Additionally, it is plausible that RFRP-3 can inhibit stimulatory cascades only at specific stages of development or female cycle, requiring the presence of additional stimuli, such as peripheral hormones or neuropeptides that affect the sensitivity of GnRH neurons to RFRP-3. Interestingly, the highest level of LH secretion in LβT2 cells is reported after 4 days of continuous GnRH pulses, as well as E_2 and dexamethasone treatment; therefore, leading to an overall increase in responsiveness of cells to GnRH due to a simultaneous enhancement of
GnRH-R levels (305). Though future studies are required to explore this hypothesis, it is plausible that GnRH neurons require hormonal stimulation to increase the abundance of GPR147 present on the cell surface, thereby enhancing the potential inhibitory ability of RFRP-3 at the level of GnRH peptide secretion. Moreover, RFRP-3 may act independent of neurosecretory mechanisms in GnRH neurons. Thus, in line with our previous results demonstrating a rapid inhibition of GnRH mRNA levels upon RFRP-3 treatment, these data suggest RFRP-3 is involved in the transcriptional or post-transcriptional processing of the GnRH gene, rather than at the level of secretion. Several compounds and hormones operate at a particular or multiple levels of peptide biosynthesis, occasionally resulting in opposing effects at different biosynthetic levels (290, 306). For instance, ethanol treatment causes no significant effect on GnRH mRNA levels in GT1-7 cells, rather it results in a drastic increase in pro-GnRH peptide and a simultaneous decrease in bioactive GnRH peptide; thus, acting solely at the level of posttranslational processing (306). Collectively, these data support the role of RFRP-3 at the level of GnRH gene biosynthesis, likely limiting the availability of primary transcript for translation into mature peptide; however, future studies are required to further delineate this regulatory mechanism and further rule out the inhibitory potential of RFRP-3 at the level of GnRH peptide secretion in mHypoA-GnRH/GFP neurons (Figure 5.2).

5.2 Study Limitations

The release of GnRH into the hypophyseal portal circulation drives reproductive function. A myriad of neurotransmitters, neuromodulators, and peripheral effectors impinge on GnRH neurons to mediate GnRH at a multiplicity of regulatory levels. Despite the necessity and critical importance of GnRH as an endocrine regulator, the complex architecture, heterogeneity, and lack of nuclei-specific markers within the in vivo hypothalamus has made the study of this and other associated neuroendocrine circuits challenging. Cell models provide an invaluable tool to define the intricate cellular mechanisms governing specific cell types within the endocrine hypothalamus. Further, these models not only confirm in vivo observations, but they offer the possibility to identify novel pathways, as well as verify the presence of relevant cellular
Figure 5.2. Representative model summarizing the proposed mechanism involved in the regulation of GnRH neuron activity by RFRP-3 in mHypoA-GnRH/GFP neurons. *Gpr147* is expressed in this neuronal model and RFRP-3 treatment suppresses GnRH mRNA levels. We report that RFRP-3 attenuation of GnRH mRNA levels is the result of transcriptional repression rather than the stability of pre-existing mRNA; the precise mechanism of this repression remains to be delineated. It remains to be elucidated whether RFRP-3 directly mediates secretion in mHypoA-GnRH/GFP neurons. Dotted line (to be elucidated)
components involved in an array of effector pathways that are too difficult to identify \textit{in vivo} \cite{46, 233}. As with every experimental model, cell lines are limited in the conclusions that they provide. In the first part of this thesis, a single \textit{Rfrp}-expressing cell line was used to delineate the effects of GCs and gonadal steroids on \textit{Rfrp} and \textit{Gpr147} gene expression; hence, conclusions regarding the responsiveness of \textit{Rfrp}-expressing neurons to GC and E\textsubscript{2} stimuli cannot be generalized to the \textit{Rfrp} neuronal population as a whole. Moreover, the rHypoE-23 neurons were isolated at embryonic stages of development and although this cell line has been reported to function in parallel to mature neuronal models, it is plausible that differences exist between embryonic and adult cell models, as previously described \cite{246, 250}. This issue was circumvented in the second part of the thesis, by using a novel, non-clonal GnRH cell model that is representative of the heterogeneous GnRH population, previously described (unpublished data). Future studies regarding the direct regulation of \textit{Rfrp} neurons can corroborate these findings in an adult cell line to report on any functional differences.

It can always be argued that the insertion of a foreign gene can alter the endogeneous properties of a cell \cite{46}; however, the accessibility, reliability, and reproducibility that such cell models provide far outweigh such limitations. Preliminary data reported by the Belsham lab suggests that the basal expression of some neuropeptides, including AgRP and oxytocin are elevated as a result of T-Ag integration (Belsham \textit{et al.}, unpublished observation). Furthermore, T-Ag augments basal phosphorylation status of key signaling molecules, such as Akt, AMPK, and STAT3. Though, the cell models employed in this work were generated through the retroviral transfection of the SV40 T-Ag into primary hypothalamic culture, initiative was taken to minimize the effects discussed above, primarily through the use of serum-free, low glucose media for cell treatments. Future studies must address any further functional differences that are a direct result of T-Ag transfection.

Further, these neuronal models have been dissected from the numerous innervating neural and glial circuits that make up the hypothalamus and CNS. Therefore, such \textit{in vitro} models lack chemical effectors that are required \textit{in vivo} for survival, maintenance, sensitivity, and responsiveness of a neuron. Indeed, the conclusions gained from such studies highlight the importance of direct regulatory mechanisms within a specific cell type \cite{89, 239, 246, 250, 251, 256, 260, 261}; however, interpreting these data directly to an \textit{in vivo} setting warrants caution since these models are maintained in an artificial environment.
The protein synthesis inhibitory, CHX and the transcriptional inhibitors, Act D and DRB were found to alter basal levels of Rfrp and GnRH, respectively. Little is known regarding the mechanisms of degradative enzymes and mRNA stabilizers (299); however, these studies did not address the involvement of dexamethasone or RFRP-3 in mediating such proteins and therefore, we cannot rule out their involvement in the maintenance of basal Rfrp and GnRH mRNA levels.

Lastly, the work in this thesis provides insight into the direct steroidal regulation of the Rfrp and Gpr147 genes in an Rfrp-expressing cell model, as well as the inhibitory mode of action of RFRP-3 on a novel, adult-derived GnRH-secreting cell line. However, due to the current lack of an Rfrp or Gpr147 knockout animal model, the relevance of the developmental or cycle-dependent nature of RFRP-3 signaling remains unknown.

Overall, precedence in the literature exists regarding the ability of cell models to delineate necessary molecular mechanisms, corroborate in vivo results at the cellular level, a further elucidate novel and unexpected cell targets and cascades. Cell lines, in association with other novel experimental systems can contribute to dissecting the key neuronal circuits that are involved in driving reproductive development and function, as well as narrow down specific signaling cascades or cell-types as endogenous targets for potential therapeutics or pathophysiological conditions.

5.3 Future Directions

While considerable progress has been made in elucidating the role GnIH/RFRP-3 plays in reproductive neuroendocrinology, our understanding of the central mechanisms and necessity of this novel neuropeptidergic system remain largely unknown. This thesis has corroborated the role of RFRP-expressing neurons as a direct target for GC-mediated stress responses; though, the transcriptional mechanisms involved remain to be investigated. Both the Rfrp and Gpr147 gene promoters appear to have putative GRE domains within 1500 bp of the transcription start site, as well as other known TF binding motifs commonly associated with GR tethering, suggesting a mechanism for direct transcriptional activation at the 5’ regulatory regions by the ligand-bound GR. Thus, future studies are required to determine the specific region of the 5’ regulatory region that is necessary and sufficient for the GC-mediated upregulation of Rfrp and Gpr147 mRNA levels. Reporter gene plasmids based on the pGL2 vector, containing specific regions of the rat
5’ flanking sequence must be transfected into rHypoE-23 neurons, a protocol well established in the Belsham lab (schematically represented in Figure 3.6) (246, 256, 261). Upon recognizing the necessary component of the transiently transfected promoter-reporter construct, mutagenesis studies are required to examine the precise role of a particular binding motif to dexamethasone-induced expression of Rfrp and Gpr147. In complement, a chromatin immunoprecipitation (ChIP) assay needs to be performed to deduce any potential protein interactions at the endogenous promoter (282). Further, the GC-mediated effect will be examined in an adult Rfrp-expressing neuronal model, potentially demonstrating a conserved or developmental-specific effect. Overall, these studies will contribute greatly to the underlying molecular mechanisms involved in the modulation of the RFRP neuropeptidergic circuit by stress signals, such as GC levels and will further ascertain a direct transcriptional mechanism for GC regulation in Rfrp-expressing neurons in vitro.

Furthermore, this thesis described a novel mechanism through which RFRP-3 modulates GnRH neurons, whereby RFRP-3 treatment directly suppresses GnRH transcript levels in mHypoA-GnRH/GFP neurons. It was found that the effect of RFRP-3 on the regulation of GnRH gene expression was independent of direct mRNA stability mechanisms, suggesting the potential involvement of miRNAs or degradative enzymes. Employing the same experimental protocol, a miRNA array can be used to determine whether the rapid and transient response reported by RFRP-3 is a result of the active transcription or activation of a miRNA, underlying the suppressive RFRP-3-mediated effect. As discussed, numerous putative miRNA binding motifs exist on the GnRH gene, suggesting a mechanism for RFRP-3 to suppress GnRH gene expression. Interestingly, of the reported potential miRNA binding sites, some have previously been classified in post-transcriptional mechanisms within the CNS, such as miR-134, miR-802 and miR-138-2 (307-309). Of interest, miR-138-2 is highly expressed in the brain and has been associated with regulating anxiety-related pathways (309). Because of the former findings reported in this thesis, implicating the Rfrp gene as a direct target of stress-mediated responses, this miRNA may provide a bridge in an overall RFRP-mediated stress response. In addition, miR-138-2 has been shown to inhibit the size of dendritic spines in rat hippocampal cells, thereby representing a potential inhibitory effector of GnRH gene expression. If RFRP-3 is found to induce or suppress the expression of a particular miRNA, overexpression or siRNA knockout models must be established to further confirm the necessity of the specific miRNA in RFRP-3-
mediated suppression of GnRH mRNA. miRNAs are recognized as key regulators in many biological processes and have been implicated in gene expression at other levels of the HPG axis. Therefore, future studies will expand on this possible mechanism, gaining a more in depth understanding regarding the role of RFRP-3 in GnRH gene regulation.

Finally, due to the inability of RFRP-3 to attenuate SNP-stimulated GnRH secretion, additional studies are necessary to elucidate whether RFRP-3 can act to modulate GnRH neurosecretory properties. As previously mentioned, SNP is a very potent stimulator of GnRH secretion in vitro and it is plausible that pre-incubation of neurons with RFRP-3, followed by the application of SNP may result in a different neurosecretory response. Furthermore, other pharmacological agents or peptides, such as kisspeptin that activate alternate pathways to induce GnRH release in vitro will be employed to rule out the potential of RFRP-mediated inhibition of GnRH secretion. The studies described will aid in defining the level at which RFRP-3 operates within the GnRH neuron.

5.4 Concluding Remarks

Collectively, in line with previous studies regarding the inhibitory role of RFRP-3 at the hypothalamic level (197, 198), the evidence from this thesis supports the idea that the RFRP/GPR147 system operates at the hypothalamic level as a potential neuromodulator within the mammalian HPG axis. Importantly, in line with our hypothesis, this current work suggests that Rfrp-expressing neurons are a direct target for stress-mediated transcriptional changes, potentially altering reproductive function and status in response to a myriad of stressors. Further, these data provide a novel mechanism through which RFRP-3 mediates neuroendocrine function at the level of the GnRH neuron using a novel, adult-derived GnRH-secreting cell model. Overall, these studies contribute to our understanding of Rfrp gene biosynthesis regulation, as well as highlight the involvement of RFRP-3 as a neuromodulator at the level of the GnRH neuron.
References


7. **True C, Grove KL, Smith MS** 2011 Beyond Leptin: Emerging Candidates for the Integration of Metabolic and Reproductive Function during Negative Energy Balance. Front Endocrinol (Lausanne) 2:53


16. Smith JT, Young IR, Veldhuis JD, Clarke IJ 2012 Gonadotropin-inhibitory hormone (GnIH) secretion into the ovine hypophyseal portal system. Endocrinology 153:3368-3375


26. **Green JD, Harris GW** 1947 The neurovascular link between the neurohypophysis and adenohypophysis. J Endocrinol 5:136-146

27. **Green JD, Harris GW** 1949 Observation of the hypophysio-portal vessels of the living rat. J Physiol 108:359-361

28. **Harris GW** 1948 Regeneration of the hypophysial portal vessels. Nature 162:70

29. **Channing CP, Tsafirri A** 1977 Mechanism of action of luteinizing hormone and follicle-stimulating hormone on the ovary in vitro. Metabolism 26:413-468

30. **Harris GW** 1964 Sex Hormones, Brain Development and Brain Function. Endocrinology 75:627-648


34. **Wray S, Grant P, Gainer H** 1989 Evidence that cells expressing luteinizing hormone-releasing hormone mRNA in the mouse are derived from progenitor cells in the olfactory placode. Proc Natl Acad Sci U S A 86:8132-8136


38. **Silverman AJ, Jhamandas J, Renaud LP** 1987 Localization of luteinizing hormone-releasing hormone (LHRH) neurons that project to the median eminence. J Neurosci 7:2312-2319


42. **Goldsmith PC, Ganong WF** 1975 Ultrastructural localization of luteinizing hormone-releasing hormone in the median eminence of the rat. Brain Res 97:181-193

43. **Bianco SD, Kaiser UB** 2009 The genetic and molecular basis of idiopathic hypogonadotrophic hypogonadism. Nat Rev Endocrinol 5:569-576

44. **Schwanzel-Fukuda M, Bick D, Pfaff DW** 1989 Luteinizing hormone-releasing hormone (LHRH)-expressing cells do not migrate normally in an inherited hypogonadal (Kallmann) syndrome. Brain Res Mol Brain Res 6:311-326

45. **Soules MR, Hammond CB** 1980 Female Kallmann's syndrome: evidence for a hypothalamic luteinizing hormone-releasing hormone deficiency. Fertil Steril 33:82-85


59. Clarke IJ, Cummins JT 1982 The temporal relationship between gonadotropin releasing hormone (GnRH) and luteinizing hormone (LH) secretion in ovariectomized ewes. Endocrinology 111:1737-1739


64. Levine JE, Ramirez VD 1982 Luteinizing hormone-releasing hormone release during the rat estrous cycle and after ovariectomy, as estimated with push-pull cannulae. Endocrinology 111:1439-1448


70. **Couse JF, Lindzey J, Grandien K, Gustafsson JA, Korach KS** 1997 Tissue distribution and quantitative analysis of estrogen receptor-alpha (ERalpha) and estrogen receptor-beta (ERbeta) messenger ribonucleic acid in the wild-type and ERalpha-knockout mouse. Endocrinology 138:4613-4621


95. **Herbison AE, Theodosis DT** 1992 Localization of oestrogen receptors in preoptic neurons containing neurotensin but not tyrosine hydroxylase, cholecystokinin or luteinizing hormone-releasing hormone in the male and female rat. Neuroscience 50:283-298


108. **Roselli CE, Kelly MJ, Ronneklev OK** 1990 Testosterone regulates progonadotropin-releasing hormone levels in the preoptic area and basal hypothalamus of the male rat. Endocrinology 126:1080-1086


116. **Skinner DC, Evans NP, Delaleu B, Goodman RL, Bouchard P, Caraty A** 1998 The negative feedback actions of progesterone on gonadotropin-releasing hormone secretion are transduced by the classical progesterone receptor. Proc Natl Acad Sci U S A 95:10978-10983


121. **Skinner DC, Caraty A, Allingham R** 2001 Unmasking the progesterone receptor in the preoptic area and hypothalamus of the ewe: no colocalization with gonadotropin-releasing neurons. Endocrinology 142:573-579


123. **Bashour NM, Wray S** 2012 Progesterone directly and rapidly inhibits GnRH neuronal activity via progesterone receptor membrane component 1. Endocrinology 153:4457-4469

124. **Sun J, Moenter SM** 2010 Progesterone treatment inhibits and dihydrotestosterone (DHT) treatment potentiates voltage-gated calcium currents in gonadotropin-releasing hormone (GnRH) neurons. Endocrinology 151:5349-5358


126. **Poling MC, Kim J, Dhamija S, Kauffman AS** 2012 Development, sex steroid regulation, and phenotypic characterization of RFamide-related peptide (Rfrp) gene
expression and RFamide receptors in the mouse hypothalamus. Endocrinology 153:1827-1840


132. Dichter MA 1980 Physiological identification of GABA as the inhibitory transmitter for mammalian cortical neurons in cell culture. Brain Res 190:111-121


137. Han SK, Todman MG, Herbison AE 2004 Endogenous GABA release inhibits the firing of adult gonadotropin-releasing hormone neurons. Endocrinology 145:495-499

139. **Keen KL, Burich AJ, Mitsushima D, Kasuya E, Terasawa E** 1999 Effects of pulsatile infusion of the GABA(A) receptor blocker bicuculline on the onset of puberty in female rhesus monkeys. Endocrinology 140:5257-5266

140. **Mitsushima D, Hei DL, Terasawa E** 1994 gamma-Aminobutyric acid is an inhibitory neurotransmitter restricting the release of luteinizing hormone-releasing hormone before the onset of puberty. Proc Natl Acad Sci U S A 91:395-399

141. **Han SK, Abraham IM, Herbison AE** 2002 Effect of GABA on GnRH neurons switches from depolarization to hyperpolarization at puberty in the female mouse. Endocrinology 143:1459-1466


143. **van den Pol AN, Wuarin JP, Dudek FE** 1990 Glutamate, the dominant excitatory transmitter in neuroendocrine regulation. Science 250:1276-1278


146. **Ottem EN, Godwin JG, Petersen SL** 2002 Glutamatergic signaling through the N-methyl-D-aspartate receptor directly activates medial subpopulations of luteinizing hormone-releasing hormone (LHRH) neurons, but does not appear to mediate the effects of estradiol on LHRH gene expression. Endocrinology 143:4837-4845


150. **Belsham DD, Wetsel WC, Mellon PL** 1996 NMDA and nitric oxide act through the cGMP signal transduction pathway to repress hypothalamic gonadotropin-releasing hormone gene expression. EMBO J 15:538-547


Thompson EL, Patterson M, Murphy KG, Smith KL, Dhillon WS, Todd JF, Ghatei MA, Bloom SR 2004 Central and peripheral administration of kisspeptin-10 stimulates the hypothalamic-pituitary-gonadal axis. J Neuroendocrinol 16:850-858

the regulation of preovulatory luteinizing hormone surge and estrous cyclicity in female rats. Endocrinology 146:4431-4436


168. Johansson KN, Greibrokk T, Currie BL, Hansen J, Folkers K 1975 Factor C-LHIH which inhibits the luteinizing hormone from basal release and from synthetic LHRH and studies on purification of FSHRH. Biochem Biophys Res Commun 63:62-68


184. Shimizu M, Bedecarrats GY 2010 Activation of the chicken gonadotropin-inhibitory hormone receptor reduces gonadotropin releasing hormone receptor signaling. Gen Comp Endocrinol 167:331-337


186. **Calisi RM, Rizzo NO, Bentley GE** 2008 Seasonal differences in hypothalamic EGR-1 and GnIH expression following capture-handling stress in house sparrows (Passer domesticus). Gen Comp Endocrinol 157:283-287


190. **Rizwan MZ, Porteous R, Herbison AE, Anderson GM** 2009 Cells expressing RFamide-related peptide-1/3, the mammalian gonadotropin-inhibitory hormone orthologs, are not hypophysiotropic neuroendocrine neurons in the rat. Endocrinology 150:1413-1420


192. **Anderson GM, Relf HL, Rizwan MZ, Evans JJ** 2009 Central and peripheral effects of RFamide-related peptide-3 on luteinizing hormone and prolactin secretion in rats. Endocrinology 150:1834-1840

193. **Johnson MA, Tsutsui K, Fraley GS** 2007 Rat RFamide-related peptide-3 stimulates GH secretion, inhibits LH secretion, and has variable effects on sex behavior in the adult male rat. Horm Behav 51:171-180


208. Clarke IJ, Smith JT 2010 The role of kisspeptin and gonadotropin inhibitory hormone (GnIH) in the seasonality of reproduction in sheep. Soc Reprod Fertil Suppl 67:159-169


211. Pevet P 1988 The role of the pineal gland in the photoperiodic control of reproduction in different hamster species. Reprod Nutr Dev 28:443-458

212. Tsutsui K, Bentley GE, Bedecarrats G, Osugi T, Ubuka T, Kriegsfeld LJ 2010 Gonadotropin-inhibitory hormone (GnIH) and its control of central and peripheral reproductive function. Front Neuroendocrinol 31:284-295


220. **Gibson EM, Humber SA, Jain S, Williams WP, 3rd, Zhao S, Bentley GE, Tsutsui K, Kriegsfeld LJ** 2008 Alterations in RFamide-related peptide expression are coordinated with the preovulatory luteinizing hormone surge. Endocrinology 149:4958-4969


226. **Smith ER, Johnson J, Weick RF, Levine S, Davidson JM** 1971 Inhibition of the reproductive system in immature rats by intracerebral implantation of cortisol. Neuroendocrinology 8:94-106


239. **Belsham DD** 2007 Hormonal regulation of clonal, immortalized hypothalamic neurons expressing neuropeptides involved in reproduction and feeding. Mol Neurobiol 35:182-194


243. **Cheng H, Isoda F, Belsham DD, Mobbs CV** 2008 Inhibition of agouti-related peptide expression by glucose in a clonal hypothalamic neuronal cell line is mediated by glycolysis, not oxidative phosphorylation. Endocrinology 149:703-710

244. **Cheung S, Fick LJ, Belsham DD, Lovejoy DA, Thompson M** 2011 Interfacial behavior of immortalized hypothalamic mouse neurons detected by acoustic wave propagation. Analyst 136:4412-4421


247. **Dhillon SS, Belsham DD** 2011 Leptin differentially regulates NPY secretion in hypothalamic cell lines through distinct intracellular signal transduction pathways. Regul Pept 167:192-200


249. **Dhillon SS, Gingerich S, Belsham DD** 2009 Neuropeptide Y induces gonadotropin-releasing hormone gene expression directly and through conditioned medium from mHypoE-38 NPY neurons. Regul Pept 156:96-103


252. **Fick LJ, Cai F, Belsham DD** 2009 Hypothalamic preproghrelin gene expression is repressed by insulin via both PI3-K/Akt and ERK1/2 MAPK pathways in immortalized, hypothalamic neurons. Neuroendocrinology 89:267-275

253. **Fick LJ, Fick GH, Belsham DD** 2011 Palmitate alters the rhythmic expression of molecular clock genes and orexigenic neuropeptide Y mRNA levels within immortalized, hypothalamic neurons. Biochem Biophys Res Commun 413:414-419


259. Mayer CM, Belsham DD 2010 Central insulin signaling is attenuated by long-term insulin exposure via insulin receptor substrate-1 serine phosphorylation, proteasomal degradation, and lysosomal insulin receptor degradation. Endocrinology 151:75-84


270. Johnson MA, Fraley GS 2008 Rat RFRP-3 alters hypothalamic GHRH expression and growth hormone secretion but does not affect KiSS-1 gene expression or the onset of puberty in male rats. Neuroendocrinology 88:305-315


Kisspeptin neurons in the arcuate nucleus of the ewe express both dynorphin A and neurokinin B. Endocrinology 148:5752-5760


281. **Johansson-Haque K, Palanichamy E, Okret S** 2008 Stimulation of MAPK-phosphatase 1 gene expression by glucocorticoids occurs through a tethering mechanism involving C/EBP. J Mol Endocrinol 41:239-249


283. **Sethi S, Tsutsui K, Chaturvedi CM** 2010 Age-dependent variation in the RFRP-3 neurons is inversely correlated with gonadal activity of mice. Gen Comp Endocrinol 168:326-332


292. **Mahachoklertwattana P, Black SM, Kaplan SL, Bristow JD, Grumbach MM** 1994 Nitric oxide synthesized by gonadotropin-releasing hormone neurons is a mediator of N-methyl-D-aspartate (NMDA)-induced GnRH secretion. Endocrinology 135:1709-1712

293. **Moretto M, Lopez FJ, Negro-Vilar A** 1993 Nitric oxide regulates luteinizing hormone-releasing hormone secretion. Endocrinology 133:2399-2402


304. **Sisk CL, Zehr JL** 2005 Pubertal hormones organize the adolescent brain and behavior. Front Neuroendocrinol 26:163-174


