Regulation of GnRH mRNA expression by the ω-3 polyunsaturated fatty acid docosahexaenoic acid and the saturated fatty acid palmitate in a GnRH-synthesizing neuronal cell model

by

Dean Tran

A thesis submitted in conformity with the requirements for the degree of Master of Science
Department of Physiology
University of Toronto

© Copyright by Dean Tran 2015
Regulation of *Gnrh* mRNA expression by the ω-3 polyunsaturated fatty acid docosahexaenoic acid and the saturated fatty acid palmitate in a GnRH-synthesizing neuronal cell model

Dean Tran

Master of Science

Department of Physiology
University of Toronto

2015

Abstract

Gonadotropin-releasing hormone (GnRH) neurons coordinate reproduction. Whether GnRH neurons directly sense free fatty acids (FFAs) is unknown. We investigated the individual effects of the FFAs palmitate, palmitoleate, oleate, and docosahexaenoic acid (DHA) (100 μM each) on *Gnrh* mRNA expression in the mHypoA-GnRH/GFP neuronal cell model. We report that 2 h exposure to palmitate or DHA increases *Gnrh* transcription. Using the inhibitors AH7614, K252c, U0126, wortmannin, and LY294002, we demonstrate that the effect of DHA is mediated through G protein-coupled receptor 120 to downstream PKC/MAPK and PI3K signaling. Moreover, we propose that the effect of palmitate may depend on palmitoyl-coA synthesis and PI3K signaling. Finally, we explored the effects of FFA combinations, and we report that oleate abolishes the effect of 2 h palmitate treatment on *Gnrh*. Overall, these studies advance our understanding of the cellular mechanisms underlying FFA sensing in a GnRH neuronal model and may relate to reproductive function.
Acknowledgements

Firstly, I would like to thank my advisor Dr. Denise Belsham for her support and guidance. Dr. Belsham has created a truly stimulating laboratory and learning environment for her students. My time in this laboratory has taught me invaluable lessons not only about science but also about myself. Here, I realized and learned to embrace our infinite ignorance about the universe, and here, I discovered my hunger for building knowledge—both old and new.

I also wish to thank my supervisory committee members, Dr. Theodore Brown and Dr. Ian Rogers, for their indispensable expertise and insight throughout this project.

Last but not least, I feel deep and earnest gratitude to my fellow labmates. In particular, I thank Alice with whom I have spent countless memorable hours in the lab. I thank Vicky, whose dedication, work-ethic, and generosity genuinely inspire me. And finally, I thank Leigh for her invaluable academic guidance and our engaging (and outlandish) discussions.
Table of Contents

Contents

Table of Contents ........................................................................................................................ iii
List of Figures and Tables ................................................................................................................ viii
Chapter 1 Introduction ...................................................................................................................... 1

1 Introduction ...................................................................................................................................... 1

1.1 Reproduction is coupled to nutritional status and energy balance ........................................... 1
1.2 Reproduction is coordinated by the HPG axis ............................................................................. 1
1.3 Fatty acid regulation of the HPG axis .......................................................................................... 3

1.3.1 Direct fatty acid regulation of anterior pituitary gonadotropes and gonadal cells ................... 3
1.3.2 Direct fatty acid regulation of gonadal cells ............................................................................ 3
1.3.3 Lines of evidence suggesting that fatty acids regulate GnRH neurons .............................. 3

1.4 GnRH neurons ............................................................................................................................ 4

1.4.1 Developmental origin and anatomical location ...................................................................... 4
1.4.2 GnRH neurons are indispensable to reproductive function ............................................... 5
1.4.3 Gnrh gene (summarized in Figure 1.2) ................................................................................ 5

1.5 Types of fatty acids .................................................................................................................... 7

1.5.1 Short-chain fatty acids (SCFAs) and medium-chain fatty acids (MCFAs) ....................... 7
1.5.2 Long-chain fatty acids (LCFAs) (Figure 1.3) ......................................................................... 9

1.6 LCFA signaling mechanisms ....................................................................................................... 11

1.6.1 Membrane LCFA receptors ................................................................................................. 11
1.6.2 Peroxisome proliferator-activated receptors (PPARs) ......................................................... 17
1.6.3 Bioactive lipid signaling ....................................................................................................... 18

1.7 Immortalized cell lines for the study of GnRH neurons ........................................................... 21

1.7.1 Previously described GnRH neuronal cell models ............................................................... 21
1.7.2 The novel mHypoA-GnRH/GFP neuronal cell line ........................................ 22

1.8 Study Rationale and Hypothesis ........................................................................ 25

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

2 Materials and Methods ......................................................................................... 27

2.1 Cell culture and reagents .................................................................................. 27

2.2 Fatty acid preparation ......................................................................................... 27

2.3 Quantitative RT-PCR ......................................................................................... 28

2.4 Immunoblotting ................................................................................................. 28

2.5 Statistical analysis ............................................................................................. 29

Chapter 3 Results .................................................................................................... 32

3 Results .................................................................................................................. 32

3.1 DHA and palmitate upregulate Gnrh gene expression .................................... 32

3.2 DHA-mediated increases in Gnrh mRNA expression depend on PKC/MAPK and PI3K signaling while palmitate-mediated increases in Gnrh mRNA expression partially depend on PI3K signaling ................................................................. 32

3.3 The effects of DHA, but not palmitate, depend on GPR120 signaling ........... 35

3.4 The effects of palmitate on Gnrh may not be mediated by TLR4. ................. 36

3.5 Neither activation nor inhibition of NF-κB affects Gnrh mRNA expression, and NF-κB signaling does not mediate the effect of palmitate on Gnrh mRNA expression .... 37

3.6 Palmitate-mediated increases in Gnrh mRNA expression require palmitoyl-coA synthesis ............................................................................................................ 37

3.7 Palmitate-mediated increases in Gnrh mRNA expression do not require de novo ceramide synthesis ............................................................................................. 40

3.8 The effect of palmitate on Gnrh does not depend on protein palmitoylation .... 40

3.9 DHA and palmitate increase Gnrh transcription. ............................................. 43

3.10 Unsaturated fatty acids modulate the ability of palmitate to increase Gnrh .... 43

3.11 Oleate may not require PI3K signaling nor GPR120 to block palmitate-mediated increases in Gnrh mRNA expression ......................................................... 45

3.12 PPAR-γ and PPAR-α upregulate Gnrh mRNA expression ............................ 45
3.13 The effect of DHA on Gnrh may not be mediated by PPAR-γ. ........................................ 45
3.14 Sodium nitroprusside (SNP) increases Gnrh mRNA expression. ............................... 50
3.15 In mHypoA-GnRH/GFP cells, Gnrh mRNA expression may oscillate. .................. 50

Chapter 4 Discussion ........................................................................................................ 53

4 Discussion ...................................................................................................................... 53

4.1 Previous reports of Gnrh regulation by FFAs .............................................................. 53

4.2 Regulation of Gnrh mRNA expression by docosahexaenoic acid (DHA) (summarized in Figure 4.1) .............................................................................................................. 53
  4.2.1 mHypoA-GnRH/GFP neurons express functional GPR120. ............................... 53
  4.2.2 DHA may differentially regulate Gnrh mRNA expression via divergent pathways downstream of GPR120. .............................................................. 56

4.3 Regulation of Gnrh mRNA expression by palmitate (summarized in Figure 4.2) ........ 58
  4.3.1 In mHypoA-GnRH/GFP neurons, palmitate increases both Gnrh mRNA expression and pro-inflammatory signaling independently of toll-like receptor 4 (TLR4) signaling .............................................................. 58
  4.3.2 In mHypoA-GnRH/GFP neurons, NF-κB signaling does not appear to regulate Gnrh mRNA expression. .............................................................. 61
  4.3.3 The role of palmitate metabolism in Gnrh regulation (summarized in Figure 4.4) .............................................................. 61
  4.3.4 Alternative mechanisms through which palmitate may regulate Gnrh mRNA expression .............................................................. 64

4.4 Regulation of Gnrh by peroxisome proliferator-activated receptors (PPARs) ............ 67

4.5 Transcriptional activation of Gnrh by DHA and palmitate may localize to the Gnrh proximal enhancer .............................................................. 70

4.6 Fatty acid interactions in Gnrh regulation .............................................................. 71
  4.6.1 The effects of DHA and palmitate on Gnrh are not additive. ............................... 71
  4.6.2 Oleate blocks the effect of palmitate on Gnrh mRNA expression. ....................... 71

4.7 The effect of LCFAs on oscillatory Gnrh mRNA expression ....................................... 73

4.8 Future studies .............................................................................................................. 73
4.8.1 FFA signaling may modulate sensitivity of mHypoA-GnRH/GFP neurons to other stimuli ................................................................. 73

4.8.2 Determine the effect of DHA and palmitate on GnRH peptide levels in mHypoA-GnRH/GFP neurons ............................................................. 74

4.8.3 Rationale for studying the effects of LCFAs on GnRH secretion in mHypoA-GnRH/GFP neurons ................................................................. 74

4.9 Study limitations ................................................................................................................................................. 76

4.10 Closing remark .................................................................................................................................................. 77

References .......................................................................................................................................................... 78
List of Figures and Tables

1 Introduction........................................................................................................................................2

Figure 1.1. Current understanding of how free fatty acids (FFAs) regulate the HPG axis. ...............2

Figure 1.2. Rat and mouse Gnrh regulatory elements. ................................................................8

Figure 1.3. Structures of the long-chain fatty acids (LCFAs) used in the present thesis..................10

Figure 1.4. GPR120 and GPR40 signaling pathways. .......................................................................14

Figure 1.5. TNFR, TLR4, and TLR2 signaling can activate the NF-κB pathway............................16

Figure 1.6. Example metabolic fates of LCFAs .................................................................................20

Figure 1.7. Generation of the mHypoA-GnRH/GFP cell line and relevant gene expression profile.................................................................23

Table 2.1. List of primers used for quantitative reverse transcription PCR ......................................29

Figure 3.1. DHA and palmitate upregulate Gnrh expression. ..............................................................33

Figure 3.2. DHA-mediated increases in Gnrh expression are dependent on both PKC/MAPK and PI3K signaling while palmitate-mediated increases in Gnrh expression are dependent on PI3K signaling ..............................................................34

Figure 3.3. The effect of DHA but not palmitate on Gnrh expression is mediated by GPR120. ....36

Figure 3.4. The effect of palmitate on Gnrh expression is not mediated by toll-like receptor 4 (TLR4) ........................................................................................................................................38

Figure 3.5. NF-κB signaling does not regulate Gnrh ........................................................................39

Figure 3.6. The effect of palmitate is dependent on palmitoyl-coA synthesis. However, it is not mediated by de novo ceramide synthesis .........................................................................................41

Figure 3.7. The effect of palmitate on Gnrh does not require protein palmitoylation .......................42

Figure 3.8. DHA and palmitate increase Gnrh transcription ..............................................................44

Figure 3.9. DHA and palmitate together increase Gnrh mRNA expression more than either DHA or palmitate alone .............................................................................................................46

Figure 3.10. Oleate does not require PI3K signaling to block palmitate-mediated increases in Gnrh expression ......................................................................................................................................47

Figure 3.11. PPAR-α and PPAR-γ agonists increased Gnrh expression ..............................................48

Figure 3.12. PPAR-γ may not mediate the effect of DHA on Gnrh ......................................................49
Figure 3.13. Sodium nitroprusside (SNP) increased Gnrh expression ........................................... 51
Figure 3.15. In mHypoA-GnRH/GFP cells, Gnrh expression appears to oscillate .................. 52
Figure 4.1. Proposed model for regulation of Gnrh transcription by DHA.............................. 54
Figure 4.2. PKC/MAPK and PI3K signaling tonically repress Gnrh mRNA expression......... 59
Figure 4.3. Proposed model for regulation of Gnrh transcription by palmitate. ................. 60
Figure 4.4. Schematic illustrating the metabolic fates of palmitate...................................... 63
Figure 4.5. Schematic illustrating potential nitric oxide signaling in mHypoA-GnRH/GFP neurons. ........................................................................................................................................... 65
Figure 4.6. Schematic illustrating alternatives pathways that palmitate may activate. ........... 68
Chapter 1
Introduction

1 Introduction

1.1 Reproduction is coupled to nutritional status and energy balance.

Reproduction is an energetically-expensive function. Pregnant women, for instance, must intake an extra 500 kcal/day in order to support pregnancy-induced metabolic demands (1). While reproduction secures the survival of a species, it is expendable for individual survival. Thus, reproductive programs must be tightly regulated so that they are only carried out under conditions of sufficient nutrition. In example, undernutrition represses reproductive function in mammals including rodents, sheep, and humans (2-4), and in children, malnutrition represses reproductive development (5-7). Conversely, overnutrition, which can lead to obesity, can also negatively impact reproductive function (2,8). Together, these observations reinforce the idea that reproduction and nutrition are closely coupled (9). Nonetheless, we do not fully understand the physiological mechanisms linking nutrition to the regulation of reproduction. Particularly, this thesis focuses on the effects of dietary fatty acids on the hypothalamic neurons controlling reproduction. Presently, we have not closely studied how free fatty acids affect the cell physiology of reproductive neurons.

1.2 Reproduction is coordinated by the HPG axis.

The reproductive system is coordinated by the hypothalamic-pituitary-gonadal (HPG) axis, which comprises three regulatory cores: the hypothalamic gonadotropin-releasing hormone (GnRH) neurons, the anterior pituitary gonadotropes, and the gonads. GnRH neurons synthesize and episodically secrete the decapeptide GnRH into the hypophyseal portal vessels for transport to the anterior pituitary gonadotropes (10,11). GnRH stimulates the gonadotropes to biosynthesize and release the gonadotropins luteinizing hormone (LH) and follicle-stimulating hormone (FSH) into circulation (10). These gonadotropin hormones signal to the gonads where they regulate follicle development and spermatogenesis (10). Moreover, the gonadotropins also stimulate the gonads (ovaries and testes) to produce sex steroids (androgens, estrogens,
FFAs regulate gonadotropin gene expression and secretion in anterior pituitary gonadotropes. Furthermore, they also regulate steroidogenesis in gonadal cells. It is currently unknown, however, if FFAs regulate GnRH gene expression or release in hypothalamic GnRH neurons, which are the central controllers of the HPG axis.
progesterone), which are released to complete a feedback loop to the hypothalamus and pituitary (12).

1.3 Fatty acid regulation of the HPG axis

1.3.1 Direct fatty acid regulation of anterior pituitary gonadotropes and gonadal cells

If and how the HPG axis detects nutrients such as fatty acids is a focus of ongoing research. Recent studies have demonstrated that FFAs directly regulate the HPG axis by modulating gonadotropin biosynthesis and secretion in anterior pituitary gonadotropes. In combination, saturated, monounsaturated, and polyunsaturated fatty acids upregulated Lhb expression while downregulating Fshb expression in the immortalized mouse gonadotrope LβT2 cell line (13). In addition, intracarotid infusion of intralipid, a lipid emulsion, increased LH secretion in rats. Moreover, oleic (C18:1n-9) and linoleic (C18:2n-6) acid increased LH secretion in rat anterior pituitary primary culture and LβT2 cells (14).

1.3.2 Direct fatty acid regulation of gonadal cells

In addition, multiple lines of evidence suggest that FFAs also directly and indirectly regulate gonadal function. For instance, it has also been reported that the FFAs palmitate, stearate, and oleate regulate cell proliferation and steroidogenesis in bovine ovarian granulosa cells (15). Moreover, ω-3 PUFAs increase progesterone production in sheep ovarian thecal cells (16), and in men, FFA infusion increases androgen precursor production (17). Thus, FFAs directly regulate the HPG axis at the level of the anterior pituitary gonadotropes and gonads. (overview in Figure 1.1)

1.3.3 Lines of evidence suggesting that fatty acids regulate GnRH neurons

Currently, however, it is unknown if GnRH neurons also directly sense FFAs. Previous in vivo studies report conflicting evidence. For example, it was recently reported that high-fat diet (HFD) decreased Gnrh expression in mice (18). Similarly, 24 wk HFD (35% kcal from fat) decreased Gnrh expression in female DBA/2J mice (19); however, this effect depended on mouse strain as HFD did not affect Gnrh expression in female C57BL/6J mice (19). On the other hand, intracarotid intralipid infusion modestly increased Gnrh expression in rats (14), and
increasing $\omega$-3:$\omega$-6 PUFA ratio in rat diets increased serum GnRH levels (20). Thus, it appears that FFAs at least indirectly regulate GnRH neurons and Gnrh expression; the mechanisms underlying this regulation, however, have not been described.

1.4 GnRH neurons

1.4.1 Developmental origin and anatomical location

GnRH neurons differentiate outside of the central nervous system and arise in the olfactory placode, which is the embryonic structure that develops into the olfactory epithelium (21-24). During embryonic development, GnRH neurons migrate caudally, traversing the nasal septum into the medial forebrain and ultimately terminating in the preoptic area (POA) of the hypothalamus (21-24). Olfactory placode GnRH neurons more weakly express and secrete GnRH than mature, post-migratory GnRH neurons (25,26). During migration, GnRH neurons develop pulsatile secretory patterns of functionally mature GnRH neurons (26), and furthermore, epigenetic modifications increase Gnrh expression during this maturation (25).

GnRH neurons number at approximately 800 in adult mice (27) and 2000 in primates (28), and this relatively small population of neurons is dispersedly distributed across the hypothalamic POA (29,30). Thus, it is difficult to study the molecular cell physiology of individual GnRH neurons in vivo. Further, dense projections from GnRH neurons terminate in the median eminence (ME) (31,32); specifically, it has been reported that 50% of GnRH neurons project to the ME. GnRH neurons secrete GnRH into the median eminence, where the peptide enters the hypophyseal portal system for transport to the anterior pituitary. Moreover, these projections possess both spike initiation sites and spines and thus function as axons and dendrites (33). These “dendrons” (portmanteau of axon and dendrite) may allow GnRH neurons to directly integrate information derived from peripheral circulation passing through the ME. The ME is vascularized by permeable fenestrated capillaries, which allow circulating peripheral factors to enter the ME, bypassing the blood brain barrier (BBB) (34,35). Furthermore, GnRH neurons also project highly-branched dendrites outside of the BBB to the organum vasculosum of the lamina terminalis (OVLT). GnRH neurons, these observations suggest, may sense and integrate signals from circulating FFAs regardless of the BBB.
1.4.2 GnRH neurons are indispensable to reproductive function.

Pulsatile secretion of GnRH is essential to reproductive function (36). For instance, in Kallman syndrome, failure of GnRH neurons to migrate into the brain and mature results in hypogonadotropic hypogonadism and infertility (36). Moreover, in hypothalamic amenorrhea, suppression of GnRH pulsatility abolishes the menstrual cycle (37). Hypothalamic amenorrhea-associated infertility can be rescued by pulsatile intravenous GnRH therapy, which restores menstrual cyclicity and induces ovulation (38,39). Similarly, hypothalamic lesion ablates endogenous GnRH production and pulsatility in rhesus monkeys; however, intermittently administering GnRH reestablishes normal ovulatory menstrual cycles (40).

In females, GnRH pulsatility regulates ovarian development. High frequency GnRH pulses favorably induce LH secretion while low frequency GnRH pulses induce FSH secretion (41). Through the follicular phase of the ovarian cycle, GnRH pulsatility increases in amplitude and frequency, driving LH production and secretion that ultimately culminates in the ovulation-inducing LH surge (42). The early follicular phase 90 min pulse period accelerates to 60 min/pulse at ovulation. And after, during the luteal phase of the ovarian cycle, GnRH pulsatility gradually slows to a 3-5 h period, which instead drives FSH synthesis and secretion (42). On the other hand, in males, GnRH regulates spermatogenesis and sex steroid synthesis, but GnRH pulsatility maintains a pulse period of approximately 2 h (43).

Pulsatile GnRH secretion also drives the activation of puberty. For instance, in prepubertal juvenile mammals, GnRH activity is suppressed (44). As juveniles develop, however, this suppression is released: near puberty, GnRH pulsatility exhibits increased frequency and nighttime amplitude spikes (45). In addition, episodically administering GnRH to prepubertal female rhesus monkeys induces ovulatory menstrual cycles that are extinguished by halting GnRH therapy (46). In summation, pulsatile GnRH secretion not only regulates adult reproductive function but also mediates pubertal onset.

1.4.3 Gnrh gene (summarized in Figure 1.2)

1.4.3.1 Gnrh upstream regulatory elements

The function of GnRH neurons is conferred by the expression of the Gnrh gene. GnRH expression, or biosynthesis, is transcriptionally regulated (47), and Gnrh transcription is
controlled by evolutionarily-conserved regulatory elements upstream of the \textit{Gnrh} transcription start site (TSS): a proximal promoter (GnRH-P; -173 to +1 bp in the rat \textit{Gnrh} gene) (48), a proximal enhancer (GnRH-E1; -1571 to -1863 bp in rat \textit{Gnrh}) (49), and two distal enhancers (GnRH-E2, -3135 to -2631 bp; GnRH-E3, -4199 to -3895 bp in rat \textit{Gnrh}) (50). Together, these regulatory elements drive basal, neuron-specific \textit{Gnrh} expression \textit{in vitro} and \textit{in vivo} (47,49-52).

The 5000-bp upstream region of \textit{Gnrh} is 97\% conserved between rat and mouse sequences (50). More specifically, the regulatory elements of \textit{Gnrh} are also highly conserved between rat and mouse (50,53). In the upstream region of mouse \textit{Gnrh}, the -278/-97 region contains GnRH-P; the -2404/-2100 region contains GnRH-E1; the -3622/-3100 contains GnRH-E2; and the -4688/-4385 contains GnRH-E3 (54).

Studies primarily in the immortalized, GnRH-secreting GT1-7 cell line have demonstrated that in the rat \textit{Gnrh} gene, these regulatory elements control \textit{Gnrh} transcription through interactions with transcriptional co-activators and co-repressors. For instance, the entire length of GnRH-P is bound by proteins (48), such as the homeodomain transcription factors Oct-1 (Pou2f1) (55), MSX and DSX (56), and OTX2 (57). Furthermore, GnRH-E1 interacts with Oct-1 (58,59), MSX and DLX (56), and the CCAAT-enhancer-binding protein \(\beta\) (C/EBP-\(\beta\)) transcription factor (59,60). Finally, GnRH-E2 and GnRH-E3 similarly confer \textit{Gnrh} expression through interactions with Oct-1, MSX1, and DLX2 (50).

In addition to transcription factor binding, epigenetic chromatin modifications also regulate \textit{Gnrh} transcription. Chromatin can adopt open or closed conformations that activate or silence genes, respectively, and these conformations can be induced by post-translational histone 3A (H3) modifications (61). H3 acetylation (H3Ac), in example, promotes open chromatin, and on the other hand, H3 Lysine 9 di-methylation (H3K9-Me2) promotes closed chromatin (54). Studies in GT1-7 cells have demonstrated that transcription of mouse \textit{Gnrh} is also epigenetically regulated through chromatin modifications at the regulatory elements of the mouse \textit{Gnrh} gene (54). To repress \textit{Gnrh} transcription, tetradecanoylphorbol-13-acetate (TPA) modifies chromatin at GnRH-E1 and GnRH-P. At GnRH-E1, TPA decreases H3Ac and increases H3K9-Me2 (54). And at GnRH-P, TPA induces the closing of chromatin (54).
1.4.3.2  *Gnrh* enhancer-derived RNAs (eRNAs)

Interestingly, it has been demonstrated that RNA polymerase II (RNA PII) occupies *Gnrh* enhancers and transcribes enhancer-derived RNAs (eRNAs) within GnRH-E1 (54). eRNA expression positively correlates with enhancer activity and nearby gene expression (62). And genetic knockdown of GnRH-E1-derived eRNA decreased *Gnrh* mRNA levels in GT1-7 cells (63), suggesting that this eRNA plays a role in *Gnrh* transcriptional activation.

The mechanisms of eRNA function are currently being investigated (64,65). It has been suggested that eRNAs contribute to enhancer function. Enhancers promote transcription by recruiting RNA PII and general transcription factors, forming the transcriptional pre-initiation complex (66). Further, although enhancers can be distally upstream of their target genes, chromatin looping places enhancers within close proximity to their target genes (66). This spatial organization brings the PIC to the core promoter and TSS (66). It has been suggested that eRNAs may promote transcription by stabilizing chromatin-looping-mediated enhancer-promoter interactions (67,68).

Over a decade ago, it was demonstrated that in the rat *Gnrh* gene, GnRH-P interacts with GnRH-E1 to increase *Gnrh* transcription (51). It was proposed that GnRH-P binds a homeodomain protein that in turn interacts with GnRH-E1-binding proteins such as Oct-1 (51). Recently, it was demonstrated that GnRH-E2 and GnRH-P of the mouse *Gnrh* gene interact via chromatin looping in resting GT1-7 cells (69). Moreover, kisspeptin increased GnRH-E2 and GnRH-P interactions, potentially by stabilizing this chromatin conformation (69). In GnRH neurons, eRNAs may play a role in stabilizing chromatin loops that promote transcription-activating enhancer-promoter interactions.

1.5  Types of fatty acids

1.5.1  Short-chain fatty acids (SCFAs) and medium-chain fatty acids (MCFAs)

Short-chain fatty acids (SCFAs) are carboxylic acids with six or fewer carbons (70). They are produced in the colon through bacterial fermentation of non-digestible carbohydrates and primarily function as energy sources (71). The SCFAs acetate (C2:0), propionate (C3:0), and butyrate (C4:0) comprise 95% of all SCFAs in the plasma (72), and together, SCFAs are present
**Gnrh1 (Rattus norvegicus)**
5’ flanking region

![Diagram of Gnrh1 regulatory elements in Rattus norvegicus.](image)

**Associated Transcription Factors**

<table>
<thead>
<tr>
<th>Oct-1</th>
<th>Oct-1</th>
<th>Oct-1</th>
<th>Oct-1</th>
</tr>
</thead>
<tbody>
<tr>
<td>MSX1</td>
<td>MSX1</td>
<td>MSX</td>
<td>MSX</td>
</tr>
<tr>
<td>DLX2</td>
<td>DLX2</td>
<td>DLX</td>
<td>DLX</td>
</tr>
<tr>
<td></td>
<td>C/EBP-β</td>
<td></td>
<td>OTX2</td>
</tr>
</tbody>
</table>

**Gnrh1 (Mus musculus)**
5’ flanking region

![Diagram of Gnrh1 regulatory elements in Mus musculus.](image)

Figure 1.2. **Rat and mouse Gnrh regulatory elements.** A proximal promoter (P), proximal enhancer (E1), and two distal enhancers (E2 and E3) control Gnrh transcription. These regulatory elements demonstrate open chromatin and interact with transcription factors such as those listed.
at approximately 80 μM in peripheral venous circulation (73). SCFAs are the endogenous ligands of the G protein-coupled receptors GPR43 (FFAR2) and GPR41 (FFAR3), which seem to mediate pro-inflammatory signaling (72,74).

Medium-chain fatty acids (MCFAs) are carboxylic acids with eight to twelve carbons (75). These FAs are derived from dietary sources such as coconut oil and butter (75). Recently, it has been reported that MCFAs are the endogenous ligands of the G protein-coupled receptor 84 (GPR84) (76,77), and GPR84 mediates MCFA-induced pro-inflammatory signaling (76).

Despite this signaling capability, however, the MCFA capric acid (C10:0) is undetectable in fasting plasma and present at less than 0.5 μM in non-fasting plasma (78).

1.5.2 Long-chain fatty acids (LCFAs) (Figure 1.3)

The majority of dietary fatty acids, however, are long-chain fatty acids (LCFAs) (79). Dietary LCFAs are even-chain carboxylic acids with 14 to 22 carbons that function as energy sources or signaling molecules (79-81). These LCFAs comprise structurally distinct saturated fatty acids (SFAs), monounsaturated fatty acids (MUFAs), and polyunsaturated fatty acids (PUFAs).

1.5.2.1 Palmitate, a saturated fatty acid (SFA)

SFAs do not have double bonds and include FAs such as stearate (C18:0), myristate (C14:0), and palmitate (C16:0). Palmitate, accounting for 28.3% of plasma free LCFAs, is the second most abundant LCFA and most abundant SFA in human plasma (82). Moreover, palmitate is the predominant SFA in animal-derived dietary fats such as butterfat (83), and it is also found in vegetable oils such as palm oil (80). Palmitate directly impairs insulin signaling, induces NPY gene expression, and regulates core clock gene expression in hypothalamic neuronal cell models (84-86). The direct effect of palmitate on hypothalamic GnRH neurons, however, is unknown.

1.5.2.2 Oleate and palmitoleate, monounsaturated fatty acids (MUFAs)

MUFAs have one carbon-carbon double bond. The most common dietary MUFA is the ω-9 MUFA oleate (C18:1n-9), which accounts for 92% of dietary MUFAs (79). Oleate is also the most abundant LCFA in human plasma, making up 32.7% of plasma free LCFAs (82). Other MUFAs such as palmitoleate can be obtained in minor amounts in our diets; palmitoleate, for instance, is found in macadamia nuts (87). While MUFAs such as oleate block the pro-
Figure 1.3. Structures of the long-chain fatty acids (LCFAs) used in the present thesis. In this thesis, we investigated the specific, individual effects of the saturated LCFA palmitate; the monounsaturated LCFAs palmitoleate and oleate; and the polyunsaturated LCFA docosahexaenoic acid (DHA) on Gnrh mRNA expression in mHypoA-GnRH/GFP neurons.
inflammatory effects of palmitate in neurons (88), the specific, direct effect of oleate on GnRH neurons is also currently unknown.

1.5.2.3 Docosahexaenoic acid, a polyunsaturated fatty acid (PUFA)
PUFAs are classified as ω-6 or ω-3 PUFAs. ω-6 PUFAs have more than one carbon-carbon double bond with one at the sixth carbon from the terminal methyl group (79). These include arachidonate (C20:4n-6), which is the metabolic precursor to lipid signaling mediators named eicosanoids (89). ω-3 PUFAs, on the other hand, have at least one double bond at the third carbon from the terminal methyl group (79), and these include FAs such as α-linolenic acid (C18:3n-3) and docosahexaenoic acid (DHA; C22:6n-3). DHA is found in algal and fish oil (79,80). DHA, similarly to palmitate, regulates core clock gene expression in hypothalamic neuronal cell models (84); moreover, in hypothalamic neurons, DHA also promotes anti-inflammatory gene expression (90). The effect of DHA in hypothalamic GnRH neurons, however, is unknown at present.

1.6 LCFA signaling mechanisms
1.6.1 Membrane LCFA receptors
1.6.1.1 G protein-coupled receptor 40 (GPR40) (overview in Figure 1.4)
The cell surface G protein-coupled receptor 40 (GPR40), or free fatty acid receptor 1 (FFAR1), is expressed in human and rodent pancreatic tissue, brain, monocytes, and gastrointestinal tract tissue (91-93). More specifically, in the brain, GPR40 is expressed by hypothalamic neurons but not astrocytes (94,95). Despite this, we currently do not know if GPR40 is expressed by reproductive neuroendocrine neurons such as GnRH neurons.

GPR40 is activated by LCFAs (91,93,96), and competitive radioactive ligand binding assays using high-affinity synthetic GPR40 ligands have demonstrated that LCFAs are the endogenous ligands of GPR40 (97,98). Activating GPR40 results in calcium influx. By using calcium reporters, GPR40 activation studies in transgenic Chinese hamster ovary (CHO) and HeLa-derived cells have suggested that unsaturated LCFAs more potently activate than saturated LCFAs (93,96); however, studies in transgenic human embryonic kidney 293 (HEK-293) cells suggested that both saturated and unsaturated LFCAs activate GPR40 with similarly potency (91). These discrepancies may be attributed to the cell models and calcium reporters used. In
addition, the therapeutic promise of GPR40 agonists in treating diabetes has led laboratories to study the molecular pharmacology of GPR40 (99). For example, a recent study identified that GPR40 has multiple—orthosteric and allosteric—binding sites (97). Moreover, allosteric interactions at GPR40 enhance ligand binding at other binding sites and also amplify ligand-induced insulin secretion in pancreatic islets (97). Interestingly, the investigators here proposed that LCFAs may bind GPR40 at multiple sites, and this may amplify signaling in vivo. Current research aims to further characterize the binding sites of GPR40; for instance, using the synthetic GPR40 ligand TAK-875, a recent study has established the crystal structure of human GPR40 (98).

GPR40 can signal via the Gaq/11, Gas, or Gai/o proteins in transgenic HEK-293 and CHO cells (92,93,96,100). The signaling pathway through which GPR40 signals in hypothalamic neurons, however, has not been studied. Gaq/11 proteins activate phospholipase C (PLC), which cleave phosphatidylinositol 4, 5-bisphosphate (PIP2) to generate inositol triphosphate (IP3) and diacylglycerol (DAG) (101). IP3 and DAG stimulate protein kinase C (PKC) activation (102). Protein kinase C (PKC) signals to the mitogen-activated protein kinase (MAPK) pathway: it phosphorylates and activates the Raf kinase-1 (Raf-1) (103). Raf-1 then activates MKK1/2, which subsequently phosphorylate ERK1/2. ERK1/2 is a protein kinase that can translocate to the nucleus and modulate transcription factor activity (104).

Gas proteins activate adenylyl cyclase (AC), which generates the secondary messenger cAMP, and cAMP signals to protein kinase A (PKA) to activate the CREB transcription factor (105). Furthermore, Gai/o proteins inhibit AC and can activate MAPK and PI3K signaling (105).

Physiologically, GPR40 signals to acutely stimulate insulin and glucagon secretion from pancreatic β and α cells, respectively (93,106). GPR40 also seems to regulate genes related to chemokines and the MAPK signaling pathway (107). Additionally, insulin secretion is reduced in mice lacking GPR40 (108). These GPR40 knockout mice, however, are fertile (108), but whether they demonstrate any other reproductive phenotypes has not be addressed.

1.6.1.2 G protein-coupled 120 (GPR120) (overview in Figure 1.4)

The cell surface G protein-coupled receptor 120 (GPR120, or free fatty acid receptor 4 (FFAR4), is expressed in lung cells, adipocytes, macrophages, and gastrointestinal tract tissue (109-113).
And recently, immunohistochemistry studies demonstrated that GPR120 is expressed by NPY-expressing cells in the arcuate nucleus of the hypothalamus (114). Moreover, our laboratory recently demonstrated that GPR120 is expressed by the rHypoE-7 hypothalamic NPY-expressing neurons (90). GPR120 is expressed in hypothalamic neuroendocrine NPY neurons; however, it is unknown if GPR120 is expressed by GnRH neurons.

Membrane GPR120 is internalized following exposure to LCFAs, indicating that LCFAs are also endogenous GPR120 ligands (109). GPR120 activation, similarly to GPR40 activation, also leads to calcium influx, and GPR120-mediated calcium influx is more potently activated by unsaturated LCFAs than saturated LCFAs (109). The molecular pharmacology of GPR120-ligand interactions has not been well-studied as that of GPR40. However, recent studies using the novel high-affinity, GPR120-selective agonist TUG-891 explored the pharmacology of GPR120 activation and signaling (115). Further, recent research investigated the structure-activity relationships of GPR120 agonists (116), and these studies may help to identify or synthesize high-affinity GPR120 ligands. Describing the crystal structures of ligand-bound GPR120 would teach us about how GPR120 functions.

GPR120 can signal via the Gaαq/11 protein or β-arrestin 2 (109,112). DHA, for instance, binds and activates GPR120, which signals to activate phosphoinositide 3-kinase (PI3K) and protein kinase C (PKC) (109,112). PKC subsequently activates the MAPK/ERK pathway (PKC/MAPK pathway), and PI3K signals to induce Akt phosphorylation (112). And similar to ERK1/2, Akt is a protein kinase that also modulates the activity of transcription factors (117,118). Moreover, it has also been demonstrated that in STC-1 mouse enteroendocrine cells expressing GPR120, α-linolenic acid, a GPR120 agonist, activates ERK, PI3K, p38 MAPK, and JNK signaling (119); however, whether this signaling is mediated by GPR120 has not been addressed.

In addition, GPR120 mediates distinct physiological functions in adipocytes, macrophages, stomach cells, hypothalamic neurons, and intestinal cells (90,109,112,120-122). In adipocytes, GPR120 signaling upregulates glucose intake (112). Further, in intestinal cells, GPR120 signaling induces GLP-1 secretion (109,122), and in gastric cells, it induces ghrelin secretion (120). In macrophages and hypothalamic neurons, GPR120 activation promotes anti-inflammatory signaling (90,112). Global deficiency or dysfunction in GPR120 leads to obesity in
**Figure 1.4. GPR120 and GPR40 signaling pathways.** GPR120 can signal through \(G_{\alpha q/11}\) proteins and \(\beta\)-arrestin-2 (\(\beta\)-arr-2). \(G_{\alpha q/11}\) signaling activates PKC and PI3K/Akt signaling. GPR40 can signal through \(G_{\alpha q/11}\), \(G_{\alpha i/o}\), and \(G_{\alpha s}\) proteins. \(G_{\alpha s}\) signaling activates PKA/CREB signaling. \(G_{\alpha i/o}\) signaling can inhibit cAMP-dependent signaling and activate PI3K signaling.
mice and humans (123); the reproductive phenotypes resulting from GPR120 dysfunction have not been studied, however.

1.6.1.3 Immune receptors: TLR4, TLR2, NOD1, NOD2 (Figure 1.5)

It has also been suggested that LCFAs activate immune pattern recognition receptors, namely the membrane-bound toll-like receptor 4 (TLR4) and toll-like receptor 2 (TLR2), and the cytosolic nucleotide-binding and oligomerization domain-containing protein 1 (NOD1) and NOD2 (124-128). These receptors are primarily expressed by myeloid cells (129-131). The TLRs, nonetheless, are also expressed in GI tract, lung, and adipose tissues (129). Moreover, TLR4 is expressed by mHypoE-43/5 (N43) and rHypoE-7 immortalized neurons derived from rodent hypothalamic culture (90,132). Likewise, TLR2 is expressed by native hypothalamic arcuate neurons and mHypoE-42 (N42) immortalized mouse hypothalamic neurons (133). On the other hand, while NOD1 and NOD2 are also expressed by epithelial and endothelial cells (134), it is currently unknown if these receptors are expressed in neurons such as GnRH neurons.

These receptors primarily detect molecules associated with pathogens. For instance, TLR4 binds bacterial lipopolysaccharide (LPS) (129,135), and TLR2 binds bacterial lipopeptides (130). Also, NOD1 and NOD2 primarily bind bacterial peptidoglycans (136). Multiple lines of evidence from transgenic knockdown of receptor activity have suggested that TLR4, TLR2, NOD1, and NOD2 are also activated by SFAs (126-128,137). These ligands activate receptor-mediated pro-inflammatory signaling. In brief, TLR2 and TLR4 transactivate the transforming growth factor β-activated kinase-1 (TAK1) complex. Moreover, TAK1 activation is mediated by TAK1 binding protein 1 (TAB1) (138), and TAB1 is sequestered by GPR120 signaling, consequently inhibiting TAK1 signaling (112). TAK1 signaling can activate p38 MAPK, JNK, and pro-inflammatory NF-κB signaling (130).

NF-κB is a transcription factor (139). In resting cells, however, it is sequestered in the cytoplasm due to the repressive activity of IκBα. The IκB kinase (IKK) enzymes phosphorylate IκBα, dissociating IκBα from NF-κB and targeting it for degradation (140). Subsequently, the activated NF-κB complex can translocate into the nucleus and bind to DNA to regulate gene transcription (139). NF-κB upregulates target genes such as Nfkb1 and Nfkbia (IκBα) (141,142). In addition, genetic overexpression over-activation of NF-κB signaling decreased Gnrh promoter-driven luciferase reporter activity in GT1-7 cells (143), suggesting that NF-κB signaling regulates Gnrh
TNFα, LPS, and lipopeptides (LP) are the respective endogenous ligands of TNFR, TLR4, and TLR2. These receptors can signal to the TAK1 complex, which mediates JNK, IKK, and p38 MAPK activation. IKK signaling activates NF-κB, which upregulates transcription of target genes such as *Nfkbia* and *Nfkb1*.
transcription. However, whether FFAs activate this signaling pathway in neurons, particularly GnRH neurons, is presently under-studied.

Further, p38 MAPK and JNK signaling can also regulate gene expression by activating the cAMP response element-binding protein (CREB) and AP-1 transcription factors, respectively (130). In addition, NOD1 and NOD2 can transactivate p38 MAPK, JNK, NF-κB, and ERK signaling (136). While these immune receptors seem to primarily activate pro-inflammatory signaling, they may mediate other physiological functions. For instance, it was recently reported that TLR2 and TLR4 mediate FFA-induced Lh expression in anterior pituitary gonadotropes (13).

1.6.2 Peroxisome proliferator-activated receptors (PPARs)

LCFA signaling is also mediated by peroxisome proliferator-activated receptors (PPARs). PPARs are nuclear receptors that function as ligand-dependent transcription factors (144); ligand binding activates PPARs, permitting them to interact with coactivator and corepressor proteins in order to modulate gene transcription (144). There are three PPAR isoforms: PPAR-α, PPAR-γ, and PPAR-δ. PPAR-α is primarily expressed by liver and skeletal muscle tissues, where it regulates genes promoting fatty acid oxidation (144). Moreover, PPAR-δ is ubiquitously expressed and also promotes fatty acid oxidation (144). In contrast, PPAR-γ, which is predominantly expressed in adipose tissue, regulates genes promoting fatty acid storage (144). While previous research has focused on PPAR activity in peripheral tissues, it has also been demonstrated that all three PPAR isoforms are expressed in the brain, for example by hypothalamic neurons (145-147). Neurons in the dorsomedial, ventromedial, paraventricular, and lateral hypothalamus express both PPAR-γ and PPAR-δ (146), and intrahypothalamic administration of a specific PPAR-α agonist induced PPAR-α target gene expression in ventral hypothalamic neurons (145), suggesting subpopulations hypothalamic neurons also express PPAR-α. Throughout the brain and in the hypothalamus, moreover, PPAR-δ is more highly expressed than PPAR-γ and PPAR-α (146). And PPAR-γ is more weakly expressed in neurons than in hepatocytes (147). However, we currently do not know the specific functions of PPARs in distinct subpopulations of hypothalamic neurons such as GnRH neurons.

The endogenous ligands of PPARs are LCFAs. PUFAs such as DHA seem to be the most potent ligands; while shorter chain LCFAs such as palmitate bind and activate PPARs, they demonstrate
 weaker affinity than PUFAs (148,149). In addition, PPARs also bind fatty acid derivatives (148,149); for instance, the PUFA arachidonic acid can be metabolized to form eicosanoids, and the eicosanoid 8-hydroxyeicosatetraenoic acid (8-HETE) binds and activates PPAR-α (149). Furthermore, PPARs also bind hypolipidemic and insulin-sensitizing drugs. Specifically, fibrates, which are hypolipidemic drugs, bind and activate PPAR-α to induce fatty acid oxidation, and thiazolidinediones (TZDs) such as rosiglitazone bind and activate PPAR-γ, which promotes insulin-sensitizing signaling (149).

1.6.3 Bioactive lipid signaling

LCFA are used in the de novo synthesis of lipid signaling mediators such as glycerolipids, glycerophospholipids, and sphingolipids (150). In addition, PUFAs are also precursors to lipid signaling mediators named eicosanoids (151,152). These LCFA derivatives can activate GPCRs, PPARs, or protein kinases to regulate cellular physiology (102,149,152,153).

1.6.3.1 LCFA metabolism

LCFAs can be metabolized into fatty acid derivatives that are secondary messengers that induce physiologically-relevant effects (81). However, carboxylic acids such as LCFAs are chemically unreactive (154). Thus, the synthesis of fatty acid derivatives is preceded by LCFA activation. In this step, a LCFA is esterified to coenzyme A to form a fatty acyl-coA thioester (154). For example, palmitate is activated to form palmitoyl-coA. Palmitoyl-coA can be β-oxidized to generate ATP, or it can be used to biosynthesize LCFA-derived esterification products, namely: glycerolipids, glycerophospholipids, and sphingolipids (150). In addition, unsaturated LCFA-derived fatty acyl-coAs can be used to synthesize eicosanoids, such as the DHA-derived resolvin D1 (150). These LCFAs function not only as structural molecules but also as second messengers (81).

1.6.3.2 LCFA derivatives: Glycerolipids

Fatty acyl-coAs can contribute to de novo synthesis of glycerolipids, namely diacylglycerol and triacylglycerol, via the glyceraldehyde 3-phosphate (G3P) pathway or the monoacylglycerol (MAG) pathway (150). In the G3P pathway, fatty acyl-coA is added to G3P to form lysophosphatidic acid (LPA) (150); after, another fatty acyl-coA is added to LPA to generate phosphatidic acid (PA). Lastly, the phosphate group of PA is removed, generating DAG, and
DAG can be converted into TAG or phospholipids. In the MAG pathway, fatty acyl-coA is added to MAG to generate DAG (150). DAG binds to and activates isozymes of the protein kinase C (PKC) family (102). To be activated, conventional PKC isozymes requires DAG, calcium, and the phospholipid phosphatidylserine (PS) (155). Activating novel PKC isozymes, on the other hand, only requires DAG and PS, and activating atypical PKC isozymes only requires PS (155). In addition, DAG-derived glycerophospholipids also act as secondary messengers.

1.6.3.3 LCFA derivatives: Glycerophospholipids

Glycerophospholipids include the DAG synthesis intermediates PA and LPA as well as DAG-derived phosphatidylcholine (PC), phosphatidylserine (PS), phosphatidylethanolamine (PE), phosphatidylinositol (PI), and phosphatidylglycerol (PG) (150). PA can bind and modulate the activity of the mammalian target of rapamycin (mTOR) and Raf kinases, which respectively regulate Akt and ERK signaling (156,157). In addition, LPA binds and activates G protein-coupled receptors (LPA1, LPA2, LPA3, LPA4, LPAR5) that can signal through Gαq/11, Gαs, and Gαi/o proteins (105). Furthermore, it has been suggested that other lysophospholipids, namely LPE, can also activate LPA1 (158).

1.6.3.4 LCFA derivatives: Sphingolipids

Palmitoyl-coA is used in de novo synthesis of the sphingolipid ceramide (81,159,160). In the first step, palmitoyl-coA and serine are condensed by serine palmitoyltransferase (SPT), forming 3-keto-sphinganine (160). After, 3-keto-sphinganine is converted into sphinganine. Then, ceramide synthase (CerS) condenses sphinganine and a fatty acyl-coA such as palmitoyl-coA to form dihydroceramide (160). Finally, dihydroceramide is converted into ceramide (160). Ceramides can activate protein phosphatase 2A (PP2A) (161). PP2A dephosphorylates and inactivates kinases such as MEK1, ERK1/2, and Akt (162). In addition, ceramides are intermediates in the biosynthesis of sphingosine 1-phosphate (S1P) (81). Similar to LPA, S1P can activate G protein-coupled receptors (S1PR1-5) that signal through Gαq/11, Gαs, and Gαi/o proteins (163).
Figure 1.6. Example metabolic fates of LCFAs. LCFAs are activated to form the precursor molecule LCFA-coA. LCFA-coAs are used to generate energy through β-oxidation, and they are also used in the de novo synthesis of glycerolipids, phospholipids, and sphingolipids. LCFA-coA with a palmitate-derived carbon chain (palmitoyl-coA) is used in protein palmitoylation. Further, PUFAs are used to synthesize lipid signaling mediators called eicosanoids.
1.6.3.5 LCFA derivatives: Eicosanoids

Unsaturated LCFA derivatives can be metabolized to synthesize bioactive lipid mediators named eicosanoids (89). Eicosanoids are primarily derived from arachidonic acid (AA; C20:4n-6) (89). In brief, cyclooxygenases (COX-1, COX-2) convert AA into prostanoids such as prostaglandins, and lipoxygenases convert AA into hydroxyeicosatetraenoic acids (HETEs) and lipoxins (89). Prostaglandins and lipoxins can activate G protein-coupled receptors (89), and HETEs can bind and activate PPARs (148).

In addition, eicosanoids can also be synthesized from the PUFA DHA (152). For instance, lipoxygenases and COX catalyze the conversion of DHA into docosatrienes, neuroprotectins, and resolvin Ds (152). Recent research suggests that resolvins seem to signal by activating G protein-coupled receptors (153). For instance, resolvin D1 activates GPR32, promoting anti-inflammatory signaling (153).

1.7 Immortalized cell lines for the study of GnRH neurons

1.7.1 Previously described GnRH neuronal cell models

Studying the direct effects of fatty acids on GnRH neurons is difficult in vivo: the hypothalamus is a complex, heterogeneous tissue, and GnRH neurons are sparsely distributed across the hypothalamus (29). Moreover, the GnRH neuron population is heterogeneous, containing neuronal subpopulations exhibiting distinct morphology and electrophysiological membrane properties (164, 165).

Previous studies of GnRH neurons have used immortalized GnRH neuronal cell models such as the GT1-7 and Gn11 cell lines. The GT1-7 cell line was isolated and sub-cloned from a Gnhr expressing hypothalamic tumor. This tumor was generated by genetically targeting an SV40 T antigen (TAg) transgene under the control of the rat Gnhr promoter to GnRH neurons in a female mouse (166). GT1-7 cells are immortalized, differentiated GnRH-secreting neurons (166). Gn11 cells, on the other hand, were isolated from an olfactory tumor (167, 168). This tumor was generated by genetic targeting of an SV40 TAg transgene to Gnhr expressing cells. Transgene expression led to the absence of GnRH neurons in the hypothalamus due to developmental migratory arrest of GnRH neurons (167).
GT1-7 and Gn11 cells have been indispensable in investigating the mechanisms regulating GnRH biosynthesis and secretion (59,169-176). These cell lines, however, comprise clonal cells representing a single type of GnRH neuron. In addition, the GnRH neurons composing the GT1-7 and Gn11 cell lines may not represent adult GnRH neurons.

1.7.2 The novel mHypoA-GnRH/GFP neuronal cell line

1.7.2.1 Generation of the mHypoA-GnRH/GFP cell line

Recently, our laboratory has established and described an adult mouse-derived, non-clonal GnRH-synthesizing and -secreting mHypoA-GnRH/GFP cell line (177,178). In brief, primary hypothalamic culture from a 2-month-old, female transgenic GnRH/GFP mouse (179) was immortalized through retroviral transfer of the simian virus 40 T antigen (SV40 TAg); the cells were then fluorescence-activated cell sorted to generate a non-clonal neuronal population of GnRH/GFP-expressing cells (178).

1.7.2.2 mHypoA-GnRH/GFP neurons express markers of GnRH neurons.

In addition to synthesizing and secreting GnRH, mHypoA-GnRH/GFP neurons express markers indicating their GnRH lineage. For instance, these neurons express Otx2, the gene encoding the orthodenticle homeobox 2 (OTX2) homeodomain transcription factor (178). OTX2 confers Gnrh expression to maturing GnRH neurons (57,180), and deletion of Otx2 in GnRH neurons of mice reduces GnRH neuron numbers in the hypothalamus and causes hypogonadotropic hypogonadism (181). In addition, mHypoA-GnRH/GFP cells express the melatonin receptor, androgen receptor, estrogen receptor α, estrogen receptor β, and G protein-coupled estrogen receptor (GPR30) (178). The hormone ligands for these receptors have been reported to regulate Gnrh expression, GnRH secretion, and GnRH neuronal activity (174,175,182-184).

1.7.2.3 In mHypoA-GnRH/GFP neurons and GT1-7 cells, PKC, PKA, and NO increase GnRH secretion.

Furthermore, mHypoA-GnRH/GFP neurons and GT1-7 cells respond similarly to the PKC activator 12-O-Tetradecanoylphorbol-13-acetate (TPA), the PKA activator forskolin, and the nitric oxide (NO) donor sodium nitroprusside (SNP). In both cell lines, TPA represses Gnrh
Figure 1.7. Generation of the mHypoA-GnRH/GFP cell line and relevant gene expression profile. In brief, primary hypothalamic culture from adult female transgenic GnRH/GFP mice was immortalized and then fluorescence-activated cell sorted (FACS) to isolate non-clonal mHypoA-GnRH/GFP cell line. qRT-PCR screening demonstrates that mHypoA-GnRH/GFP neurons express relevant fatty acid signaling receptors and machinery. (+) indicates that the gene is expression while (-) indicates absence or weak expression of a gene.
expression and stimulates GnRH secretion (178,185); forskolin does not affect \textit{Gnrh} expression (178,185); and SNP stimulates GnRH secretion (178,186).

1.7.2.4 mHypoA-GnRH/GFP neurons directly sense glucose.

In the past decade, transgenic GnRH/GFP mouse models have allowed laboratories to visualize GFP-labeled GnRH neurons and study the whole-cell electrophysiology of individual GnRH neurons (179,187,188). Using this technology, it was demonstrated that GnRH neurons are electrically excited by increasing extracellular glucose levels (188). Moreover, a subpopulation of GnRH neurons express glucokinase, a glucose metabolism enzyme and glucose sensor (188). Considering these findings, it was proposed that GnRH neurons directly sense environmental glucose (189).

Similarly, our laboratory recently demonstrated glucose responsiveness in mHypoA-GnRH/GFP neurons (178). In mHypoA-GnRH/GFP cells, high glucose levels (5 mM) increase \textit{Gnrh} expression, potentially by downregulating AMPK signaling (178). Further, in accordance with the finding that high glucose increases GnRH neuron firing rate, high glucose also increases GnRH secretion in mHypoA-GnRH/GFP neurons (178). Thus, it appears that mHypoA-GnRH/GFP neurons directly sense glucose.

1.7.2.5 \textit{Gnrh} expression in mHypoA-GnRH/GFP neurons is regulated by RF-amide-related peptide-3 [RFRP-3, or gonadotropin-inhibitory hormone (GnIH)] and kisspeptin-10.

Kisspeptins, neuropeptides encoded by the \textit{Kiss1} gene, play an indispensable role in reproduction (190). For instance, in humans, mutations in the gene encoding the kisspeptin receptor GPR54 result in hypogonadotropic hypogonadism (191,192). Kisspeptins govern the reproductive axis primarily by stimulating GnRH neurons (187,193,194). For example, kisspeptin-10 upregulates \textit{Gnrh} expression in GT1-7 cells (171). Similarly, our laboratory has recently reported that kisspeptin-10 also increases \textit{Gnrh} expression in mHypoA-GnRH/GFP neurons (177).

In the past decade, studies have uncovered the role of the novel hypothalamic neuropeptide gonadotropin-inhibitory hormone (GnIH) in regulating avian reproduction (195,196). Furthermore, more recently, it was reported that the mammalian GnIH orthologue RF-amide-related peptide-3 (RFRP-3) also regulates reproductive function in mice; namely, RFRP-3
suppresses the excitability of mouse GnRH neurons (197). In line with this, our laboratory recently demonstrated that RFRP-3 represses *Gnrh* expression in mHypoA-GnRH/GFP neurons (177). In conclusion, mHypoA-GnRH/GFP neurons express markers of GnRH neurons and are regulated similarly to native GnRH neurons and previously used GnRH neuronal cell models; thus, mHypoA-GnRH/GFP neurons may be an appropriate model for studying the direct regulation of GnRH neurons.

### 1.8 Study Rationale and Hypothesis

FFAs directly regulate the HPG axis at the level of the anterior pituitary gonadotropes (13,14) and the gonads (15-17). Moreover, it has been suggested that FFAs may at least indirectly regulate GnRH neurons, the central coordinators of the HPG axis. Specifically, it has been reported that high-fat diet modulates hypothalamic *Gnrh* expression in rodents (18,19). Currently, however, we do not know if FFAs directly regulate hypothalamic GnRH neurons. GnRH neurons extend dendrites to the OVLT and ME (33,198), which receive peripheral circulation. Further, GnRH neurons sense the nutrient glucose (178,188). Therefore, we hypothesized that GnRH neurons may also directly sense circulating FFAs. To investigate this general hypothesis, this project pursued three aims.

GnRH biosynthesis, the result of *Gnrh* expression, is key to GnRH neuron function (199,200). Furthermore, FFAs are structurally and functionally diverse. Hence, in **Aim 1**, we first determined the specific, individual effects of the SFA palmitate, the ω-9 MUFA oleate, the ω-7 MUFA palmitoleate, and the ω-3 PUFA DHA on *Gnrh* mRNA expression in the mHypoA-GnRH/GFP GnRH neuronal cell model. Here, we treated mHypoA-GnRH/GFP neurons with palmitate, oleate, palmitoleate, or DHA over a 24 h time course.

Additionally, as previously described in this introduction, FFAs can signal through diverse pathways. In **Aim 2**, we described the molecular mechanisms underlying the effects of DHA and palmitate on *Gnrh* expression. To achieve this, we first identified the FFA signaling machinery, namely GPR120 and TLR4, expressed by mHypoA-GnRH/GFP cells. Further, we investigated the roles of PKC/MAPK, PI3K/Akt, and NF-κB signaling in DHA- and palmitate-mediated *Gnrh* regulation. In these experiments, we used GPR120 and TLR4 antagonists to determine whether these membrane receptors mediate the effects of DHA and palmitate. Further, to study the roles of PKC/MAPK, PI3K/Akt, and NF-κB signaling, we used small molecule inhibitors of PKC,
MEK1/2, PI3K, and IKK. In addition, we used palmitate analogs and fatty acid metabolism inhibitors to investigate the role of palmitate metabolism in \textit{Gnrh} regulation.

Finally, because native GnRH neurons are exposed to FFA mixtures, in \textbf{Aim 3}, we began to explore the effects of FFA mixtures on \textit{Gnrh} expression in mHypoA-GnRH/GFP neurons. Particularly, we studied how combinations of DHA/palmitate and palmitate/oleate regulate \textit{Gnrh} expression.
Chapter 2
Materials and Methods

2 Materials and Methods

2.1 Cell culture and reagents

mHypoA-GnRH/GFP cells were cultured in DMEM (Sigma-Aldrich; Oakville, ON, Canada) containing 5.5 mM glucose and supplemented with 5% FBS (Gibco, Burlington, ON, Canada) and 1% penicillin-streptomycin (Gibco), as previously described (177,178,201). K252c, U0126, LY294002, Wortmannin, AH 7614, 5, 6-dichlorobenzimidazole riboside (DRB), TAK-242, fumonisin B1, PS1145, rosiglitazone, GW0742, Wy 14643, and T 0070907 were purchased from Tocris Bioscience; Ellisville, MO, USA, and reconstituted in dimethyl sulphoxide (DMSO; Sigma-Aldrich). Actinomycin D (ActD) and myriocin were purchased from Sigma-Aldrich and also reconstituted in DMSO. Inhibitor and agonist stock solutions were diluted in cell culture media for treatment. C16 ceramide (N-palmitoyl-D-erythro-sphingosine; Sigma-Aldrich) was prepared in 95% etOH at 60 °C. Sodium nitroprusside (SNP, Sigma-Aldrich) and tumor necrosis factor α (TNFα; Sigma-Aldrich) were reconstituted in molecular grade water at room temperature. Lipopolysaccharide (LPS; Sigma-Aldrich) was reconstituted in 1X phosphate-buffered saline (PBS).

2.2 Fatty acid preparation

Sodium palmitate, sodium oleate, and methyl palmitate (Sigma-Aldrich) stock solutions (100 mM) were prepared in molecular grade water (Thermo Scientific; Nepean, Ontario, Canada) by heating at 70 °C. In brief, sodium palmitate powder was weighed out into an Eppendorf tube; molecular grade water was added and the solution was heated using a block heater buffered with water and set at 70 °C. Docosahexaenoic acid (DHA; Sigma-Aldrich) and 2-bromopalmitate (2-BP; Sigma-Aldrich) stock solution (100 mM) was prepared in DMSO. Palmitoleic acid (Sigma-Aldrich) stock solution (200 mM) was prepared in 50% etOH at 60 °C. Fatty acid stock solutions were then diluted in cell culture media for treatment.
2.3 Quantitative RT-PCR

Total RNA was isolated using the guanidium isothiocynate-phenol-chloroform extraction method with Turbo DNase (Ambion; Streetsville, Ontario, Canada) treatment, as previously described (201), or the PureLink RNA Kit with on-column PureLink DNase (Ambion). cDNA was synthesized using the high-capacity cDNA reverse transcription kit (Applied Biosystems). 25 ng cDNA was amplified using a qRT-PCR master mix (Platinum SYBR Green qPCR SuperMix-UDG with ROX; Invitrogen), as previously described (84,177). Samples were loaded in triplicate and run in an Applied Biosystems Prism 7000 Sequence Detection System machine. qRT-PCR data was analyzed using the standard curve method and normalized to histone 3a as a reference gene. Primers have been validated and sequenced (90,178). qRT-PCR primers for GnRH-E1, GnRH-E2, and GnRH-E3 enhancer-derived RNAs (eRNAs) were designed and published by Iyer et al (50,54).

2.4 Immunoblotting

Cells were incubated in serum-free culture media for 4 h and subsequently challenged with DHA or palmitate. Protein was harvested using 1 x cell lysis buffer (Cell Signaling Technology; CST; Danvers, MA, USA) containing 1 mM PMSF, 1% phosphatase inhibitor cocktail 2 (Sigma-Aldrich), and 1% protease inhibitor. Lysates were centrifuged at 14,000 x g for 10 min, and supernatant was collected. Protein concentration was measured using BCA assay (Thermo Fisher). 15-25 μg protein was run on 10% SDS-PAGE gel and transferred to a 0.22 μM PVDF membrane (Bio-Rad, Mississauga, ON, Canada). Membranes were blocked at room temperature for 1 h in 5% nonfat dry milk-TBS-T (Tris-buffered saline with Tween-20). Blots were incubated with 1:1000 dilutions of phospho-Akt (S472; CST #9271), phospho-p44/42 MAPK (ERK1/2; Thr202/Tyr204; CST #9101), Akt (CST #9272), and p44/42 MAPK (ERK1/2; CST #4695) primary antibodies overnight at 4 °C. Membranes were then washed in 0.1% TBS-T and incubated with HRP-conjugated secondary anti-rabbit antibodies (1:7,500; CST) for 1 h at room temperature. Blots were visualized using Signal Fire ECL reagent (CST) and quantified using Kodak Image Station 2000R (Eastman Kodak Company, Rochester, NY, USA) and ImageJ (National Institute of Mental Health, Bethesda, MD, USA).
2.5 Statistical analysis

Data are expressed as mean ± standard error of the mean (SEM). A Student’s unpaired t test or one-way ANOVA with Tukey’s post test was used to analyze one-factor experiments. A multivariable ANOVA with Bonferroni’s post test was used to analyze multi-factor experiments. $\alpha = 0.05$. Data was analyzed using GraphPad Prism 6 (GraphPad Software; La Jolla, CA) or SigmaPlot 12.5 (Systat; San Jose, CA, USA).

<table>
<thead>
<tr>
<th>Gene Name</th>
<th>Primer Sequence (5’ $\rightarrow$ 3’)</th>
<th>Amplicon Size (bp)</th>
<th>Annealing Temp (°C)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Histone 3a (Hist)</td>
<td>F: CGC TTC CAG AGT GCA GCT ATT</td>
<td>72</td>
<td>60.7</td>
</tr>
<tr>
<td></td>
<td>R: ATC TTC AAA AAG GCC AAC CAG AT</td>
<td></td>
<td>58.5</td>
</tr>
<tr>
<td>Gonadotropin-releasing hormone (Gnrh)</td>
<td>F: CGT TCA CCC CTC AGG GAT CT</td>
<td>51</td>
<td>61.0</td>
</tr>
<tr>
<td></td>
<td>R: CTC TTC AAT CAG ACT TTC CAG AGC</td>
<td></td>
<td>59.4</td>
</tr>
<tr>
<td>GnRH-E1 enhancer-derived RNA (eRNA)</td>
<td>F: GCC AAA CAC CAC AGT CTT CTC TTG AGT</td>
<td>162</td>
<td>67.4</td>
</tr>
<tr>
<td></td>
<td>R: CTG GCA CAA AGA GCA AAA GAA CCT CCT</td>
<td></td>
<td>67.5</td>
</tr>
<tr>
<td>GnRH-E2 eRNA</td>
<td>F: CTA CAG GCT GGT CGG CTT GAG GCA GTG AAT C</td>
<td>153</td>
<td>71.5</td>
</tr>
<tr>
<td></td>
<td>R: TGC TCT CTC CTC CAT GTA AGC CCT TTA CTG TG</td>
<td></td>
<td>70.4</td>
</tr>
<tr>
<td>GnRH-E3 eRNA</td>
<td>F: TTC CCC TCA TTG GGA CTG TAA CAG AAG GAC</td>
<td>174</td>
<td>67.4</td>
</tr>
<tr>
<td></td>
<td>R: TTG AAC CAA GAT GGC ACT TCC ACA CAA TGC</td>
<td></td>
<td>68.1</td>
</tr>
<tr>
<td>Nuclear factor of kappa light polypeptide gene enhancer in B cells 1, p105 (NfkB1)</td>
<td>F: GGA TGA CAG AGG CGT GTA TTA G</td>
<td>114</td>
<td>58.4</td>
</tr>
<tr>
<td></td>
<td>R: CCT TCT CTC TGT CTG TGA GTT G</td>
<td></td>
<td>58.1</td>
</tr>
<tr>
<td>Gene Name</td>
<td>Forward Primer</td>
<td>Reverse Primer</td>
<td>Tm (°C)</td>
</tr>
<tr>
<td>-----------</td>
<td>----------------</td>
<td>----------------</td>
<td>---------</td>
</tr>
<tr>
<td>Nuclear factor of kappa light polypeptide gene enhancer in B cells inhibitor, α (IkBa) (Nfkbia)</td>
<td>F: TGC CTG GCC AGT GTA GCA GTC TT</td>
<td>R: CAA AGT CAC CAA TGT CTC CAC GAT</td>
<td>150</td>
</tr>
<tr>
<td>Nitric oxide synthase 2 (Nos2)</td>
<td>F: CTT GGA AGA GGA GCA ACT ACT G</td>
<td>R: CCT GAA GGT GTG GTT GAG TT</td>
<td>124</td>
</tr>
<tr>
<td>Aryl hydrocarbon receptor nuclear translocator-like (Bmal1)</td>
<td>F: GGG AGG CCC ACA GTC AGA TT</td>
<td>R: GTA CCA AAG AAG CCA ATT CAT CAA</td>
<td>78</td>
</tr>
<tr>
<td>Period circadian clock 2 (Per2)</td>
<td>F: TCA TCA TTG GGA GGC ACA AA</td>
<td>R: GCA TCA GTA GCC GGT GGA TT</td>
<td>135</td>
</tr>
<tr>
<td>G protein-coupled receptor 120/Free fatty acid receptor 4 (Gpr120 or Ffar4)</td>
<td>F: ACC ACC GTT CTG GGA CTC</td>
<td>R: TGA AGA GCA AAT CCG CGC AGA AGA</td>
<td>136</td>
</tr>
<tr>
<td>G protein-coupled receptor 40/Free fatty acid receptor 1 (Gpr40 or Ffar1)</td>
<td>F: CTG CCC GTC TCA GTT TCT CC</td>
<td>R: TGA GCT TCC GTT TGT GGC TC</td>
<td>120</td>
</tr>
<tr>
<td>Toll-like receptor 4 (Tlr4)</td>
<td>F: GCT TGA ATC CCT GCA TAG</td>
<td>R: GCT CAG ATC TAG GTT CTT GG</td>
<td>113</td>
</tr>
<tr>
<td>Peroxisome proliferator-</td>
<td>F: TCT CAG TCC ATC GGT GAG GA</td>
<td></td>
<td>113</td>
</tr>
<tr>
<td>Activated receptor α (Ppara)</td>
<td><strong>F:</strong> GCT GGA GAG AGG GTG TCT GT</td>
<td><strong>R:</strong> CTG TGT CAA CCA TGG TAA TTT CAG T</td>
<td>60.9</td>
</tr>
<tr>
<td>-----------------------------</td>
<td>----------------------------------</td>
<td>---------------------------------</td>
<td>------</td>
</tr>
<tr>
<td>Peroxisome proliferator-activated receptor γ (Pparg)</td>
<td><strong>F:</strong> ACG TTC TGA CAG GAC TGT GT</td>
<td><strong>R:</strong> CTG TGT CAA CCA TGG TAA TTT CAG T</td>
<td>116</td>
</tr>
<tr>
<td>Peroxisome proliferator-activated receptor δ (Ppard)</td>
<td><strong>F:</strong> CAA CGC ACC CTT TGT CAT CC</td>
<td><strong>R:</strong> ACG TGC ACA CTG ATC TCG TT</td>
<td>114</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>
Chapter 3
Results

3 Results

3.1 DHA and palmitate upregulate Gnrh gene expression.

To investigate the specific, individual effects of LCFAs on Gnrh mRNA expression, mHypoA-GnRH/GFP cells were treated with 100 μM DHA, 100 μM palmitate, 100 μM palmitoleate, 100 μM oleate, or vehicle over 24 h. After exposure for 2 or 8 h, DHA treatment increased Gnrh mRNA levels (Figure 3.1A). On the other hand, 1, 2, 4, 8, and 24 h palmitate treatment increased Gnrh mRNA levels (Figure 3.1Bi). Both DHA and palmitate time-dependently increased Gnrh mRNA expression. In addition, the increase in Gnrh mRNA expression 2 h after palmitate exposure was a threshold response (Figure 3.1Bii). In contrast, neither palmitoleate (Figure 3.1C) nor oleate (Figure 3.1D) altered Gnrh mRNA expression. In short, DHA and palmitate upregulate Gnrh mRNA expression in mHypoA-GnRH/GFP cells.

3.2 DHA-mediated increases in Gnrh mRNA expression depend on PKC/MAPK and PI3K signaling while palmitate-mediated increases in Gnrh mRNA expression partially depend on PI3K signaling.

Observing that both DHA and palmitate increase Gnrh mRNA expression, we next inquired into the mechanisms underlying these effects. To test whether DHA activated PKC/MAPK and PI3K/Akt signaling, we incubated mHypoA-GnRH/GFP neurons in serum-free culture media for 4 h and then challenged the cells with 100 μM DHA for 5 min. DHA increased phosphorylation of both Akt and ERk1/2 (Figure 3.2A). In addition, to evaluate if DHA and palmitate require PKC/MAPK signaling to increase Gnrh mRNA expression, we pretreated mHypoA-GnRH/GFP cells with the conventional PKC inhibitor K252c (5 μM) or the MKK1/2 inhibitor U0126 (25 μM) for 1 h, and subsequently co-incubated the cells with 100 μM DHA or palmitate for 2 h. When PKC/MAPK signaling was inhibited, DHA failed to increase and instead decreased Gnrh mRNA expression (Figure 3.2Bi). On the other hand, despite PKC/MAPK inhibition, palmitate still increased Gnrh mRNA expression (Figure 3.2Ci). Of note, inhibiting either PKC or MKK1/2 alone increased Gnrh mRNA expression (Figure 3.2Bi and 3.2Ci).
Figure 3.1. DHA and palmitate upregulate Gnrh expression. mHypoA-GnRH/GFP cells were treated with 100 μM DHA (A), 100 μM palmitate (Bi), 100 μM palmitoleic acid (C), 100 μM oleate (D), or vehicle for 1, 2, 4, 8, and 24 h, or increasing doses of palmitate (Bii) for 2 h. Gnrh expression was determined by qRT-PCR. Results are expressed as mean ± SEM. n ≥ 4. A, Bi, C, D, two-way ANOVA; Bonferroni’s post test indicated the following significant interactions: Palmitate x time, DHA x times; *, P < 0.05, ****, P < 0.0001, H2O versus palmitate, time matched. Bii, one-way ANOVA; Tukey’s post test; means with different letters are significantly different from each other.
Figure 3.2. DHA-mediated increases in Gnrh expression are dependent on both PKC/MAPK and PI3K signaling while palmitate-mediated increases in Gnrh expression are dependent on PI3K signaling. A, mHypoA-GnRH/GFP cells were incubated in serum-free culture media for 4 hours and then challenged with 100 μM DHA for 5 minutes. Akt and ERK1/2 phosphorylation were assessed by immunoblotting. Bi, Ci, mHypoA-GnRH/GFP cells were pretreated with the PKC inhibitor K252c (5 μM), MEK1/2 inhibitor U0126 (25 μM), or vehicle (DMSO) for 1 h, and subsequently co-incubated with 100 μM DHA (Bi), 100 μM palmitate (Ci), or vehicle (DMSO and H2O, respectively) for 2 h. Bi, Cii, Cells were also pretreated with PI3K inhibitors LY294002 (50 μM), wortmannin (1 μM), or vehicle (DMSO) for 1h, and then co-incubated with 100 μM DHA (Bi), 100 μM palmitate (Cii), or vehicle (DMSO and H2O, respectively) for 2 h. Gnrh expression was determined by qRT-PCR. Results are expressed as mean ± SEM. n ≥ 4. A, unpaired Student’s t-test; **, P < 0.01, ***, P < 0.001. B, C, Two-way ANOVA (unique analysis for each inhibitor) indicated the following significant interactions: DHA x K252c, DHA x U0126, DHA x LY, DHA x Wort, Pal x Wort; no interaction: Pal x K252c, Pal x U0126; +, P < 0.05, ++++, P < 0.0001. Bonferroni’s post test; *, P < 0.05, **, P < 0.01, ****, P < 0.0001, vehicle versus fatty acid; #, P < 0.05, ##, P < 0.01, ###, P < 0.001, ####, P < 0.0001, no inhibitor control versus +inhibitor.
To test if DHA- and palmitate-mediated increases in \textit{Gnrh} mRNA expression depend on PI3K signaling, we pretreated mHypoA-GnRH/GFP cells with the PI3K inhibitors LY294002 (50 μM) or wortmannin (1 μM) for 1 h, and subsequently co-incubated the cells with 100 μM DHA or palmitate for 2 h. When we inhibited PI3K signaling, DHA did not significantly increase \textit{Gnrh} mRNA expression (Figure 3.2Bii). However, inhibiting PI3K signaling attenuated the effect of palmitate on \textit{Gnrh} mRNA expression (Figure 3.2Cii). In the palmitate/wortmannin experiments (Figure 3.2Cii), inhibiting PI3K, similarly to inhibiting PKC or MKK1/2, slightly increased \textit{Gnrh} mRNA expression.

Taken together, these results suggest that DHA requires both PKC/MAPK and PI3K signaling to increase \textit{Gnrh} mRNA expression, and because it decreased \textit{Gnrh} mRNA expression when PKC/MAPK signaling was inhibited, DHA may regulate \textit{Gnrh} mRNA expression through an alternate pathway. Additionally, palmitate may increase \textit{Gnrh} mRNA expression through a partially PI3K-dependent mechanism.

### 3.3 The effects of DHA, but not palmitate, depend on GPR120 signaling.

To detail how DHA and palmitate activate PKC/MAPK and/or PI3K signaling, we next investigated whether membrane receptors mediate this signaling. Using qRT-PCR, we found that mHypoA-GnRH/GFP cells express the free fatty acid receptor \textit{Gpr120} but not \textit{Gpr40} (data not shown; cycle threshold (CT) of \textit{Gpr120} was 27 whereas CT of \textit{Gpr40} was 33 for 25 ng of total cDNA); hypothalamic tissue harvested from C57BL/6 mice expressed \textit{Gpr40} (CT 29 for 12.5 ng total cDNA). To assess whether DHA and palmitate increase \textit{Gnrh} mRNA expression through GPR120, we pretreated mHypoA-GnRH/GFP cells with the GPR120-specific antagonist AH 7614 for 1 h, and then co-incubated cells with 100 μM DHA or palmitate for 2 h. Antagonizing GPR120 abolished the effect of DHA on \textit{Gnrh} mRNA expression (Figure 3.3A) but did not affect the ability of palmitate to increase \textit{Gnrh} mRNA expression (Figure 3.3B). In contrast to DHA, palmitate may not increase \textit{Gnrh} mRNA expression through GPR120 signaling.
3.4 The effects of palmitate on *Gnrh* may not be mediated by TLR4.

Palmitate, however, also transactivates the toll-like receptor 4 (TLR4) (202-204). TLR4 primarily detects bacterial lipopolysaccharide (LPS) (135), and TLR4 signaling activates the NF-

![Diagram](image)

**Figure 3.3.** The effect of DHA but not palmitate on *Gnrh* expression is mediated by GPR120. mHypoA-GnRH/GFP cells were pretreated with the GPR120 antagonist AH 7614 (100 μM) or vehicle (DMSO) for 1 h, and subsequently co-incubated with 100 μM DHA (A), 100 μM palmitate (B), or vehicle (DMSO and H2O, respectively) for 2 h. *Gnrh* expression was determined by qRT-PCR. Results are expressed as mean ± SEM. n ≥ 4. Two-way ANOVA indicated the following significant interaction: DHA x AH 7614; no interaction: Pal x AH 7614; ++++, P < 0.0001. Bonferroni’s post test: *, P < 0.05, **** P < 0.0001.
κB transcription factor, which upregulates target genes such as Nfkb1 and Nfkbia (IkBα) (141, 142). To test if palmitate increases Gnrh mRNA expression through TLR4 signaling, we pretreated mHypoA-GnRH/GFP cells with the TLR4 antagonist TAK-242 (10 μM) for 1 h, and then co-incubated cells with 100 ng/mL LPS or 100 μM palmitate for 2 h. Antagonizing TLR4 abolished LPS-mediated increases in Nfkb1 expression (Figure 3.4A), but it did not block palmitate-mediated increases in Gnrh mRNA expression (Figure 3.4Bi). Interestingly, antagonizing TLR4 signaling did not abolish palmitate-induced Nfkbia expression (Figure 3.4Bii). Nonetheless, palmitate may not increase Gnrh mRNA expression through TLR4 signaling.

3.5 Neither activation nor inhibition of NF-κB affects Gnrh mRNA expression, and NF-κB signaling does not mediate the effect of palmitate on Gnrh mRNA expression.

We also investigated the role of NF-κB signaling in Gnrh regulation. We treated mHypoA-GnRH/GFP cells with the NF-κB-transactivating cytokine TNFα (205) (10 ng/ml) for 1, 2, 4, 8, and 24 h. While TNFα increased expression of the NF-κB target gene Nfkbia (Figure 3.5Ai), it did not affect Gnrh mRNA expression (Figure 3.5Aii). Additionally, we assessed whether NF-κB mediates the effect of palmitate on Gnrh. The IκB kinase (IKK) signals to activate NF-κB (140). To inhibit NF-κB signaling, we pretreated mHypoA-GnRH/GFP cells with the IKK inhibitor PS1145 (25 μM) for 1 h, and then co-incubated cells with 100 μM palmitate for 2 h. Inhibiting IKK decreased basal Nfkbia expression (Figure 3.5Bi) but did not change basal Gnrh mRNA expression (Figure 3.5Bii). Further, when we inhibited IKK, palmitate failed to increase Nfkbia expression (Figure 3.5Bi) but still increased Gnrh mRNA expression (Figure 3.5Bii). In mHypoA-GnRH/GFP cells, it seems that NF-κB signaling neither regulates Gnrh nor mediate the effects of palmitate on Gnrh mRNA expression.

3.6 Palmitate-mediated increases in Gnrh mRNA expression require palmitoyl-coA synthesis.

While the effect of palmitate on Gnrh mRNA expression is not mediated by GPR120 or TLR4, it may depend on the metabolism of palmitate into other lipid signaling metabolites. Palmitate is esterified to coenzyme A to form palmitoyl-coA, the precursor to all palmitate metabolites. To study the role of palmitate metabolism in regulating Gnrh mRNA expression, we used methyl
Figure 3.4. The effect of palmitate on GnRH expression is not mediated by toll-like receptor 4 (TLR4). mHypoA-GnRH/GFP cells were pretreated with the TLR4 antagonist TAK-242 (1 μM) or vehicle (DMSO) for 1 h, and subsequently co-incubated with 100 ng/mL LPS or vehicle (PBS) for 2 h (A), or 100 μM palmitate (B) or vehicle (H2O) for 24 h. Nfkb1 (A), Gnrh (Bi), and Nfkbia (Bi) expression were determined by qRT-PCR. Results are expressed as mean ± SEM. n ≥ 3. Two-way ANOVA indicated the following significant interaction: LPS x TAK-242; no interaction: Pal x TAK-242; +, P < 0.05. Bonferroni’s post test, *, P < 0.05, **, P < 0.01, ****, P < 0.001.
Figure 3.5. NF-κB signaling does not regulate Gnrh. A, mHypoA-GnRH/GFP cells were treated with 10 ng/ml TNFα or vehicle (H2O) for 1, 2, 4, 8, and 24 h. C, Cells were pretreated with the IKK inhibitor PS1145 (25 μM) or vehicle (DMSO) for 1 h, and subsequently co-incubated with 100 μM palmitate or vehicle (H2O) for 2 h. Nfkbia (Ai, Bi) and Gnrh (Aii, Bii) expression were determined by qRT-PCR. Results are expressed as mean ± SEM. n ≥ 3 except in A, n=2 for t = 4, 8, 24 h. Two-way ANOVAs indicated the following significant interaction: PS1145 x Palmitate (C, for Nfkbia); no interaction: PS1145 x Palmitate (D, for Gnrh); +, P < 0.05; Bonferroni’s post test, *, P < 0.05, **, P < 0.01, ****, P < 0.0001, vehicle versus TNFα/palmitate.
palmitate, a palmitic acid methyl ester that cannot be esterified to coenzyme A (206-208). We treated mHypoA-GnRH/GFP cells with 100 μM methyl palmitate over 24 h. Methyl palmitate had no effect on Gnrh mRNA expression (Figure 3.6A). Thus, to increase Gnrh mRNA expression, palmitate may require palmitoyl-coA synthesis.

3.7 Palmitate-mediated increases in Gnrh mRNA expression do not require de novo ceramide synthesis

Palmitoyl-coA is used in the de novo synthesis of ceramides. These sphingolipids can regulate gene expression (81,209) and are key intermediates in the biosynthesis of other bioactive sphingolipids. We investigated whether ceramides regulate Gnrh by inhibiting de novo ceramide synthesis. To this end, we pretreated mHypoA-GnRH/GFP cells with the serine palmitoyltransferase (SPT) inhibitor myriocin (100 μM) or the ceramide synthase (CerS) inhibitor fumonisin B₁ (25 μM) for 1 h, and after, co-incubated cells with 100 μM palmitate for 2 and 24 h. Inhibiting SPT potentiated the effect of 24 h palmitate treatment on Gnrh mRNA expression (Figure 3.6Bii); the effect of 2 h palmitate treatment, however, did not change (Figure 3.6Bi). In contrast to inhibiting SPT, inhibiting CerS had no effect (Figure 3.6C). We also studied if exogenous ceramides affect Gnrh mRNA expression. We treated mHypoA-GnRH/GFP cells with 1, 10, and 100 μM C16 ceramide (N-palmitoyl-D-erythro-sphingosine) for 1 h. C16 ceramide treatment did not change Gnrh mRNA expression (Figure 3.6D). In summary, palmitate-mediated changes in Gnrh mRNA expression are independent of ceramide synthesis and may rely on the generation of an unknown palmitate metabolite.

3.8 The effect of palmitate on Gnrh does not depend on protein palmitoylation.

Palmitoyl-coA can be covalently added to cysteine residues of proteins by palmitoyl acyltransferases (PATs) in a process called protein palmitoylation (210). To determine if the effect of palmitate on Gnrh mRNA expression was dependent on palmitoylation, we treated mHypoA-GnRH/GFP cells for 24 h with increasing doses of 2-bromopalmitate (2-BP), a palmitate analog that cannot be used by PATs for palmitoylation. 24 h 2-BP treatment increased Gnrh mRNA expression (Figure 3.7), similarly to palmitate, suggesting that the effect of palmitate on Gnrh mRNA expression does not require protein palmitoylation.
Figure 3.6. The effect of palmitate is dependent on palmitoyl-coA synthesis. However, it is not mediated by de novo ceramide synthesis. A, mHypoA-GnRH/GFP cells were treated with 100 μM palmitate, 100 μM methyl palmitate, or vehicle (H₂O) for 1, 2, 4, 8, and 24 h. B-C, mHypoA-GnRH/GFP cells were pretreated with the serine palmitoyltransferase inhibitor myriocin (B, C, 100 μM), the ceramide synthase inhibitor fumonisin B₁ (D, 25 μM), or vehicle (DMSO) for 1 h, and subsequently co-incubated with 100 μM palmitate or vehicle (H₂O) for 2 h (Bi) or 24 h (Bii, C). D, mHypoA-GnRH/GFP cells were treated with increasing doses of C₁₆ ceramide (N-palmitoyl-D-erythro-sphingosine) or vehicle (0.1% EtOH) for 1 h. Gnrh expression was determined by qRT-PCR. Results are expressed as mean ± SEM. n ≥ 3. B, Three-way ANOVA indicated the following significant interaction: Pal x Myr x Time (B). A, C, Two-way ANOVA indicated the following significant interaction: Pal x Time (A); no interaction: Pal x FB₁ (C); ++, P < 0.01. Bonferroni’s post test; *, P < 0.05, **, P < 0.01, ****, P < 0.0001, H₂O versus palmitate; ####, P < 0.0001, no inhibitor control versus +inhibitor. D, One-way ANOVA; Dunnett’s post test indicated no significant differences.
Figure 3.7. The effect of palmitate on *Gnrh* does not require protein palmitoylation. mHypoA-GnRH/GFP cells were treated with increasing doses of the protein palmitoylation-inhibiting palmitate analog 2-bromopalmitate (2-BP) for 24 h. *Gnrh* expression was determined by qRT-PCR. Results are expressed as mean ± SEM. n ≥ 4. One-way ANOVA; Tukey’s post test, means with different letters are significantly different from each other.
3.9 DHA and palmitate increase *Gnrh* transcription.

To effect changes in *Gnrh* mRNA expression, DHA and palmitate may modulate *Gnrh* mRNA stability or transcription. To examine whether DHA and palmitate increase *Gnrh* transcription, we pretreated mHypoA-GnRH/GFP cells with the transcriptional inhibitors 5, 6-Dichloro-1-β-D-ribofuranosylbenzimidazole (DRB, 50 μM) or actinomycin D (ActD, 50 μM) for 1 h, and then co-incubated the cells with 100 μM DHA or palmitate for 2 h. Inhibiting transcription abolished the effect of both DHA (Figure 3.8A) and palmitate (Figure 3.8C) on *Gnrh* mRNA expression. Both DRB and ActD alone, however, increased *Gnrh* mRNA expression. DHA and palmitates appear to increase *Gnrh* transcription, and we next investigated the involvement of *Gnrh* regulatory elements.

Next, we determined whether DHA and palmitate stimulate transcription of *Gnrh* enhancers. To achieve this, we treated mHypoA-GnRH/GFP cells with 100 μM DHA or palmitate for 2 h and assessed enhancer-derived RNA (eRNA) levels using qRT-PCR and specific primers for the GnRH-E1, -E2, and -E3 enhancer regions (50). Both DHA (Figure 3.8B) and palmitate (Figure 3.8D) increased GnRH-E1 eRNA levels while neither changed GnRH-E2 or -E3 eRNA levels. In brief, DHA and palmitate, in addition to increasing *Gnrh* transcription, also increase expression of an eRNA derived from the GnRH-E1 enhancer.

3.10 Unsaturated fatty acids modulate the ability of palmitate to increase *Gnrh*.

Because *in vivo* GnRH neurons are likely exposed to a diverse fatty acid profile, we began to explore how co-treatment with two distinct fatty acids affects *Gnrh* mRNA expression. mHypoA-GnRH/GFP cells were co-incubated for 2 h with equimolar fatty acid concentrations: 100 μM DHA and palmitate; palmitate and oleate; or palmitate and palmitoleic acid. The effects of DHA and palmitate on *Gnrh* mRNA levels were not completely additive (Figure 3.9A). In contrast, when co-incubated with oleate, palmitate failed to increase *Gnrh* mRNA expression (Figure 3.9B). Similarly, palmitoleic acid modestly attenuated the ability of palmitate to increase *Gnrh* mRNA expression (Figure 3.9C). In brief, the monounsaturated fatty acids oleate and palmitoleic acid differentially attenuate the effect of palmitate on *Gnrh* while DHA and palmitate have a modestly additive effect on *Gnrh*. 
Figure 3.8. DHA and palmitate increase Gnrh transcription. A, C, mHypoA-GnRH/GFP cells were pretreated with the transcriptional inhibitors DRB (50 μM) or actinomycin D (50 μM), or vehicle (DMSO) for 1 h, and subsequently co-incubated with 100 μM DHA (A), 100 μM palmitate (B), or vehicle (DMSO or H2O, respectively) for 2 h. Gnrh expression was determined by qRT-PCR. B, D, mHypoA-GnRH/GFP cells were treated with 100 μM DHA (A), 100 μM palmitate (B), or vehicle (DMSO and H2O, respectively) for 2 h. Enhancer RNA (eRNA) expression was determined by qRT-PCR. Results are expressed as mean ± SEM. n ≥ 3. A, C, Two-way ANOVA (unique analysis for each inhibitor) indicated the following significant interactions: DHA x DRB, DHA x ActD, Pal x DRB, Pal x ActD; +, P < 0.05, ++, P < 0.01, ++++, P < 0.0001. Bonferroni’s post test; ****, P < 0.0001, vehicle versus fatty acid; ###, P < 0.001, ####, P < 0.0001, no inhibitor control versus +inhibitor; B, D, Student’s t-test; ***, P < 0.001.
3.11 Oleate may not require PI3K signaling nor GPR120 to block palmitate-mediated increases in Gnrh mRNA expression.

We investigated whether inhibiting PI3K would restore the effect of palmitate on Gnrh mRNA expression. mHypoA-GnRH/GFP cells were pretreated with the PI3K inhibitor wortmannin (1 μM) for 1 h, and subsequently co-incubated with 100 μM palmitate and oleate for 2 h. As shown previously, inhibiting PI3K attenuated the effect of palmitate alone on Gnrh mRNA expression (Figure 3.10Aii). However, when we inhibited PI3K, oleate still blocked the ability of palmitate to increase Gnrh mRNA expression (Figure 3.10Aii). Additionally, oleate can bind to GPR120 (109). To determine if oleate signals through GPR120 to block the effect of palmitate, we pretreated mHypoA-GnRH/GFP cells with the GPR120 antagonist AH 7614 for 1 h, and then co-incubated cells with 100 μM palmitate and oleate for 2 h. Although we only performed two replicates of the experiment, it appears that antagonizing GPR120 does not abolish the effect of palmitate (Figure 3.10Bii). To summarize, oleate abolishes the effect of palmitate on Gnrh, and this abolishment is independent of PI3K signaling and possibly GPR120.

3.12 PPAR-γ and PPAR-α upregulate Gnrh mRNA expression.

We next investigated the function of peroxisome proliferator-activated receptors in mHypoA-GnRH/GFP neurons. Here, we determined the effects of specific PPAR-γ, -α, or –δ agonists on Gnrh mRNA expression. mHypoA-GnRH/GFP cells were treated with the PPAR-γ agonist rosiglitazone (10 μM), PPAR-α agonist Wy 14643 (5 μM), or PPAR–δ agonist GW0742 (100 nM) for 1, 2, 4, 8, and 24 h. After 1 h, agonizing either PPAR-γ (Figure 3.11A) or PPAR-α (Figure 3.11B) increased Gnrh mRNA expression. PPAR-δ agonism, in contrast, had no effect on Gnrh (Figure 3.11C). In brief, both PPAR-γ and PPAR-α upregulate Gnrh mRNA expression.

3.13 The effect of DHA on Gnrh may not be mediated by PPAR-γ.

Because PPAR-γ agonism increased Gnrh and DHA binds and activates PPAR-γ (151), we tested whether PPAR-γ mediates the effect of DHA on Gnrh mRNA expression. We pretreated mHypoA-GnRH/GFP cells with the PPAR-γ antagonist T0070907 (100 nM) for 1 h, and subsequently co-incubated cells with 100 μM DHA for 2 h. When PPAR-γ signaling was
Figure 3.9. DHA and palmitate together increase GnRH mRNA expression more than either DHA or palmitate alone. Oleate blocks the effect of palmitate on GnRH mRNA expression. mHypoA-GnRH/GFP cells were co-incubated for 2 h with 100 μM DHA and palmitate (A); palmitate and oleate (B); or palmitate and palmitoleic acid (C). GnRH expression was determined by qRT-PCR. Results are expressed as mean ± SEM. n ≥ 4. Two-way ANOVA (unique analysis for each co-incubation) indicated the following significant interactions: DHA x Pal, DHA x Oleate; Bonferroni’s post test; **, P < 0.01, ***, P < 0.001, ****, P < 0.0001, H2O versus palmitate; ##, P < 0.01, ####, P < 0.0001, no DHA versus +DHA.
Figure 3.10. Oleate does not require PI3K signaling to block palmitate-mediated increases in GnRH expression. A, mHypoA-GnRH/GFP cells were pretreated with the PI3K inhibitor wortmannin (Aii, 1 μM) or vehicle (Ai, DMSO) (A) for 1 h, and then co-incubated with 100 μM palmitate and oleate for 2 h. GnRh expression was determined by qRT-PCR. Results are expressed as mean ± SEM. n = 8. Three-way ANOVA revealed there was no Wort x Pal x Ole interaction; Pal x Ole interactions were significant as indicated. B, mHypoA-GnRH/GFP cells were pretreated with the GPR120 antagonist AH 7614 (Bii, 100 μM) or vehicle (Bi, DMSO) for 1 h, and then co-incubated with 100 μM palmitate and oleate for 2 h. Gnrh expression was determined by qRT-PCR. Results are expressed as mean ± SD. n = 2.
Figure 3.11. PPAR-α and PPAR-γ agonists increased Gnrh expression. mHypoA-GnRH/GFP cells were treated with the PPAR-γ rosiglitazone (A; 10 μM), PPAR-α agonist Wy 14643 (B; 15 μM), or PPAR-δ agonist GW0742 (C; 100 nM), for 1, 2, 4, 8, and 24 h. Gnrh expression was determined by qRT-PCR. Results are expressed as mean ± SEM. n ≥ 3. Two-way ANOVA; Bonferroni’s post test, ***, P < 0.001, ****, P < 0.0001.
Figure 3.12. PPAR-γ may not mediate the effect of DHA on *Gnrb*. mHypoa-GnRH/GFP cells were pre-incubated with the PPAR-γ antagonist T0070907 (100 nM) for 1h, and subsequently co-incubated with 100 μM DHA for 2 h. *Gnrb* expression was determined by qRT-PCR. Results are expressed as mean ± SEM. n ≥ 7. Two-way ANOVA indicated no significant interaction; Bonferroni’s post test, *, P < 0.05, **, P < 0.01, DMSO versus DHA.
inhibited, DHA still increased Gnrh mRNA expression (Figure 3.12). DHA, then, may not require PPAR-γ to upregulate Gnrh mRNA expression.

3.14 Sodium nitroprusside (SNP) increases Gnrh mRNA expression.

Palmitate upregulates Nitric oxide synthase 2 (Nos2) expression, resulting in increased cellular nitric oxide (NO) levels (211,212), and although palmitate increases Gnrh mRNA expression in mHypoA-GnRH/GFP cells, whether nitric oxide increases Gnrh mRNA expression is unknown. To investigate this, we treated mHypoA-GnRH/GFP cells with the nitric oxide donor sodium nitroprusside (SNP) (100 μM) for 2 and 24 h. Both 2 and 24 h SNP treatment increased Gnrh mRNA expression (Figure 3.13A). Interestingly, 2 h of TNFα exposure induced Nos2 expression (Figure 3.13B). In mHypoA-GnRH/GFP cells, thus, nitric oxide seem to upregulate Gnrh mRNA expression. Whether nitric oxide mediates the effect of palmitate on Gnrh is undetermined, however.

3.15 In mHypoA-GnRH/GFP cells, Gnrh mRNA expression may oscillate.

In the previously described time-course experiments, we observed that basal Gnrh mRNA expression temporally fluctuates in mHypoA-GnRH/GFP cells; however, we did not know if this time-dependence is programmed. To demonstrate that Gnrh mRNA expression oscillates in mHypoA-GnRH/GFP cells, we investigated the effect of cell cycle arrest-start synchronization on the expression of Gnrh and the core clock genes Bmal1 and Per2. Cells were serum starved for 16 h to induce cell cycle arrest, and subsequently subjected to 30 min 30% (v/v) serum shock to restart the cell cycle. Following serum shock, cells were incubated in fresh culture media supplemented with 5% (v/v) serum for up to 36 h. Without serum shock synchronization, Gnrh (Figure 3.15Ai), Bmal1 (Figure 3.15Aii), and Per2 (Figure 3.15Aiii) do not demonstrate noticeable oscillations. However, when cells were synchronized with serum shock, Gnrh oscillated with a seemingly ultradian period (Figure 3.14Bi), and Bmal1 (Figure 3.14Bii) and Per2 (Figure 3.14Biii) oscillated with approximately circadian periods and antiphase with respect to each other. In brief, mHypoA-GnRH/GFP cells express oscillating circadian genes and also express Gnrh in a time-dependent manner.
Figure 3.13. Sodium nitroprusside (SNP) increased Gnrh expression. (A) mHypoA-GnRH/GFP cells were treated with 100 μM SNP for 2 and 24 h. (B) Cells were treated with 10 or 50 ng/ml TNFα for 2 h. Gnrh (A), Nfkbia (Bi), and Nos2 (Bi) expression were determined by qRT-PCR. Results are expressed as mean ± SEM. n =3. (A) Two-way ANOVA; Bonferroni’s post test, **, P < 0.01, ***, P < 0.001. (B) One-way ANOVA; Dunnett’s post test, *, P < 0.05, **, P < 0.01.
Figure 3.15. In mHypoA-GnRH/GFP cells, *Gnrh* expression appears to oscillate. mHypoA-GnRH/GFP cells were incubated in serum-free culture media for 16 h. Subsequently, cells were subjected to 30% serum (v/v) culture media (B) or serum-free culture media (A) for 30 min. Following serum shock, cells were incubated in fresh culture media supplemented with 5% serum (v/v). *Gnrh* (Ai, Bi), *Bmal1* (Aii, Bii), and *Per2* (Aiii, Biii) expression were determined by qRT-PCR. n=1.
Chapter 4
Discussion

4 Discussion

4.1 Previous reports of Gn rh regulation by FFAs

Hypothalamic neurons can sense FFAs (213-216), and GnRH neurons can detect peripherally circulating factors through dendrites extending outside the blood-brain barrier (198). GnRH neurons, these observations suggest, may sense and integrate signals from circulating FFAs. Previous in vivo studies report conflicting evidence: high-fat diet decreased Gn rh mRNA expression in mice (18); however, this effect depended on mouse strain (19). On the other hand, intracarotid intralipid infusion modestly increased Gn rh mRNA expression in rats (14), and increasing ω-3:ω-6 PUFA ratio in rats’ diets increased serum GnRH levels (20). FFAs, then, at least indirectly regulate GnRH neurons and Gn rh mRNA expression. We report here, however, that the PUFA DHA and the SFA palmitate directly upregulate Gn rh mRNA expression in mHypoA-GnRH/GFP cells.

4.2 Regulation of Gn rh mRNA expression by docosahexaenoic acid (DHA) (summarized in Figure 4.1)

4.2.1 mHypoA-GnRH/GFP neurons express functional GPR120.

In the present paper, we describe for the first time functional GPR120 in a GnRH neuronal cell model. LCFAs bind and activate the free fatty acid receptors GPR40 (FFAR1) and GPR120 (FFAR4) to evoke diverse responses in pancreatic, adipose, and muscle tissues (93,109,112,217-219). Both receptors are expressed in the mouse hypothalamus in vivo (95,114); however, we report that mHypoA-GnRH/GFP neurons express Gpr120 but not Gpr40. GPR120 binds LCFAs such as DHA, palmitate, palmitoleate, and oleate (109). These LCFAs activate GPR120 and induce calcium influx (109); however, DHA (pEC50 = 5.41) and palmitoleate (pEC50 = 5.49) activate GPR120 more potently than palmitate (pEC50 = 4.28) and oleate (pEC50 = 4.51) (109). We report that among these LCFAs, only DHA signals through GPR120 to regulate Gn rh mRNA expression. Thus, we demonstrate here that mHypoA-GnRH/GFP neurons can directly sense LCFAs by expressing functional GPR120.
Figure 4.1. Proposed model for regulation of *Gnrh* transcription by DHA. DHA activates GPR120, which stimulates PKC/MEK/ERK1/2 and PI3K/Akt signaling. ERK1/2 and Akt may transactivate the GnRH-E1 enhancer region, stimulating the expression of GnRH-E1 eRNA. This eRNA promotes the transcription of Gnrh, thus increasing Gnrh mRNA levels. Moreover, DHA also appears to activate an alternate pathway downstream of GPR120 that decreases Gnrh mRNA expression.
4.2.1.1 Biased agonism may occur at GPR120.

G protein-coupled receptors such as GPR120 can signal through multiple pathways (220,221). Agonists may not uniformly activate the signaling pathways coupled to a receptor. Instead, they stabilize unique receptor conformations that selectively activate signaling. This concept is biased agonism (220). That not all GPR120 agonists regulate Gnrh suggests that functional biased agonism occurs at GPR120. Specifically, DHA signals through GPR120 and then PKC/MAPK and PI3K signaling to increase Gnrh mRNA expression. Although palmitoleate and oleate are also GPR120 agonists (109), they do not affect Gnrh mRNA expression, suggesting that they may activate different downstream signaling than DHA does. Future studies should profile the timing and intensity of how different LCFAs activate GPR120 and downstream signaling pathways. It has been suggested, for instance, that ω-3 PUFAs cause faster and more potent GPR120-mediated ERK1/2 phosphorylation than ω-6 PUFAs.

4.2.1.2 Biased antagonism may occur at GPR120.

The GRP120 antagonist AH 7614 blocks the effect of DHA but not palmitate on Gnrh mRNA expression. Biased antagonism by AH 7614 at GPR120 may also be occurring in our studies. AH 7614 blocks synthetic GPR120 agonist- and linoleic acid-induced increases in intracellular Ca\(^{2+}\) in U2OS cells expressing GPR120 (222); however, because AH 7614 was only recently identified, we do not understand how it interacts with GPR120. AH 7614 may antagonize GPR120 signaling by competing with orthosteric agonists or by allosterically modulating the receptor. Furthermore, AH 7614 may be a biased allosteric modulator of GPR120. Biased allosteric modulation occurs at cannabinoid receptor type 1 (CB₁R), which is a GPCR activated by endogenous and exogenous cannabinoids (223). An allosteric modulator of CB₁R, Org27569, decreases binding of the exogenous cannabinoid SR141716A but has no effect on binding of other exogenous and endogenous-like cannabinoids (223). Moreover, while Org27569 blocks all cannabinoid-induced decreases in cAMP, it differentially modulates cannabinoid-induced ERK1/2 phosphorylation: Org27569 abolishes pERK1/2 increases in response to some cannabinoids but not others (223). AH 7614, then, may inhibit the binding of DHA but not palmitate to GPR120. Alternatively, AH 7614 may block Gnrh-regulating signaling pathways depending on the GPR120 agonist. RNA interference experiments to knockdown GPR120 in mHypoA-GnRH/GFP cells would further clarify whether palmitate regulates Gnrh through
GPR120. Regardless, the molecular pharmacology and binding dynamics between GPR120 and fatty acids requires further attention.

4.2.2 DHA may differentially regulate \textit{Gnrh} mRNA expression via divergent pathways downstream of GPR120.

4.2.2.1 DHA may increase \textit{Gnrh} mRNA expression via \textit{GPR120}-mediated \textit{G}_{\alpha q/11} signaling.

Observing that the effect of DHA was dependent on GPR120, we further characterized how DHA regulates \textit{Gnrh}. GPR120 is coupled to heterotrimeric G proteins composed of \textit{G}_{\beta\gamma} and \textit{G}_{\alpha q/11} subunits (109,112), and these G proteins transmit signals through the PKC/MAPK and PI3K pathways (90,112,119). Specifically, \textit{G}_{\alpha q/11} proteins activate phospholipase C (PLC), which cleaves phosphatidylinositol 4, 5-bisphosphate (PIP$_2$) to release inositol 1, 4, 5-triphosphate (IP$_3$) and diacylglycerol (DAG) (224). IP$_3$ increases cytosolic Ca$^{2+}$ levels, and DAG activates protein kinase C (PKC). PKC can signal to the MAPK pathway, causing MEK1/2 and subsequent ERK1/2 phosphorylation (103). Furthermore, PKC and \textit{G}_{\beta\gamma} can activate PI3K, which transduces events leading to Akt phosphorylation (225). GPR120-\textit{G}_{\alpha q/11} signaling can be activated by DHA, increasing glucose uptake in adipocytes (112). In mHypoA-GnRH/GFP cells, DHA increases ERK1/2 and Akt phosphorylation, indicating activation of the PKC/MAPK and PI3K signaling pathways. Moreover, inhibiting PKC/MAPK signaling reversed the effect of DHA, and inhibiting PI3K signaling only attenuated DHA-mediated increases in \textit{Gnrh} mRNA expression. Thus, in addition to being dependent on GPR120, DHA-mediated increases in \textit{Gnrh} seem to be absolutely dependent on PKC/MAPK signaling and partially dependent on PI3K signaling. Together, these findings also suggest that DHA-induced increases in \textit{Gnrh} mRNA expression may be mediated by \textit{G}_{\alpha q/11}. In the future, we may use RNA interference to knockdown \textit{G}_{\alpha q/11} protein levels, which has been established in RAW 264.7 macrophages (112), to test this hypothesis.

4.2.2.2 DHA may signal through an alternate pathway to downregulate \textit{Gnrh} mRNA expression.

Interestingly, in the absence of PKC/MAPK signaling, DHA decreases \textit{Gnrh} mRNA expression, and this effect also seems to be mediated by GPR120. Here, it appears that in mHypoA-GnRH/GFP neurons, DHA can activate divergent signaling pathways downstream of GPR120.
PKC/MAPK signaling downstream of GPR120 increases *Gnrh* mRNA levels, but an alternate signaling pathway downstream of GPR120 decreases *Gnrh* mRNA levels.

In addition to Gq/11, GPR120 can also signal through the scaffolding protein β-arrestin 2, which can recruit and signal through pathways such as the p38 MAPK, CREB, and NF-κB pathway (226-229). DHA can also activate GPR120-β-arrestin 2 signaling: in rat hypothalamic neurons and macrophages, DHA activates GPR120-β-arrestin 2 signaling to induce anti-inflammatory signaling (90,112). RNA interference experiments to knockdown Gq/11 and β-arrestin 2 could further dissect the distinct mechanisms through which DHA regulates *Gnrh*; Gq/11 and β-arrestin 2 knockdown has been established in RAW 264.7 macrophages (112). It would also be pertinent to investigate how synthetic GPR120 agonists (222,230) affect *Gnrh* mRNA expression considering the potential use of such agonists in clinical studies (231).

### 4.2.2.3 PKC/MAPK signaling differentially regulates *Gnrh* mRNA expression.

Acutely activating PKC/MAPK signaling with DHA increases *Gnrh* mRNA expression. Despite this, inhibiting PKC/MAPK independently increases *Gnrh* mRNA expression, suggesting that basal PKC/MAPK activity represses basal *Gnrh* mRNA expression. Previous studies suggest that whether PKC/MAPK signaling upregulates or downregulates *Gnrh* depends on the original stimulus. For instance, kisspeptin-10 activates PI3K and MAPK signaling to increase *Gnrh* mRNA expression in GT1-7 and Gn11 GnRH cell models (171). Moreover, in *Gnrh*-expressing NLT cells derived from the same culture as Gn11 cells, insulin-like growth factor 1 (IGF-1) activates MAPK signaling to increase *Gnrh* mRNA expression (232), and similarly, in Gn11 and GT1-7 cells, insulin also activates MAPK signaling to increase *Gnrh* mRNA expression (233). Contrarily, in GT1-7 cells, melatonin functions through MAPK signaling to decrease *Gnrh* mRNA expression (175), and the phorbol ester TPA, a PKC activator, repressed *Gnrh* mRNA expression (185); however, whether this repression occurred because of PKC activation or degradation is contested (234). This ultimately effect of PKC/MAPK signaling on Gnrh may depend on the subcellular localization of signaling components. For example, in rat pheochromocytoma PC12 cells, epidermal growth factor activates cytosolic MAPK signaling, which induces cell proliferation; in contrast, nerve growth factor activates nuclear MAPK signaling, which instead induces cell differentiation (235,236). In mHypoA-GnRH/GFP neurons, basal PKC/MAPK signaling that represses *Gnrh* may occur in a different cellular compartment.
than acute GPR120-mediated PKC/MAPK signaling, and future studies may address this by assessing nuclear and cytosolic protein fractions for pERK1/2.

4.3 Regulation of Gn rh mRNA expression by palmitate (summarized in Figure 4.2)

Similarly to DHA, in mHypoA-GnRH/GFP cells, palmitate increases Gn rh mRNA expression, and this effect was at least partially dependent on PI3K signaling. However, palmitate seems to increase Gn rh mRNA expression independently of GPR120 signaling. To explore how palmitate regulates Gn rh, we investigated the role of another palmitate-activated receptor, toll-like receptor 4 (TLR4).

4.3.1 In mHypoA-GnRH/GFP neurons, palmitate increases both Gn rh mRNA expression and pro-inflammatory signaling independently of toll-like receptor 4 (TLR4) signaling.

Palmitate transactivates toll-like receptor 4 (TLR4), inducing NF-κB signaling in beta cells, adipocytes, myocytes, and macrophages (202-204,237,238). Moreover, FFAs signal through TLR4 to regulate Lhb mRNA expression in gonadotropes (13). Observing that mHypoA-GnRH/GFP neurons express Tlr4, we explored the function of TLR4 signaling in these cells. In mHypoA-GnRH/GFP neurons, the endogenous TLR4 ligand lipopolysaccharide (LPS) increases NF-κB target gene expression, demonstrating functional TLR4 signaling in mHypoA-GnRH/GFP neurons. Furthermore, inhibiting TLR4 blocked LPS-induced NF-κB signaling but did not block palmitate-mediated increases in Gn rh mRNA expression. Thus, in mHypoA-GnRH/GFP neurons, palmitate appears to increase Gn rh mRNA expression independently of TLR4 signaling. Interestingly, inhibiting TLR4 also did not abolish the ability of palmitate to induce NF-κB signaling. In hypothalamic neurons, it seems that SFAs such as palmitate may not require TLR4 to activate NF-κB signaling, as has been reported in muscle cells (239). This finding further demonstrates the diversity of fatty acid signaling, and investigating how palmitate activates NF-κB signaling may give insight into how palmitate increases Gn rh mRNA expression. (summary in Figure 4.3)
Figure 4.2. PKC/MAPK and PI3K signaling tonically repress Gnrh mRNA expression. Inhibition of PKC, MKK1/2, and PI3K increased basal Gnrh mRNA expression, suggesting that these signaling pathways basally repress Gnrh mRNA expression.
Figure 4.3. Proposed model for regulation of Gnrh transcription by palmitate. Palmitate is activated and converted into palmitoyl-coA (pal-coA). Palmitoyl-coA may then transactivate the GnRH-E1 enhancer region, leading to expression of GnRH-E1 eRNA, and this eRNA may transcriptionally activate Gnrh, thus increasing Gnrh mRNA levels. To increase Gnrh mRNA expression, palmitate requires PI3K signaling; however, it remains to be determined whether PI3K signaling occurs upstream or downstream of palmitoyl-coA synthesis.
4.3.2 In mHypoA-GnRH/GFP neurons, NF-κB signaling does not appear to regulate \textit{Gnrh} mRNA expression.

In mHypoA-GnRH/GFP cells, TNFα stimulates NF-κB signaling but does not affect \textit{Gnrh} mRNA expression, and inhibiting basal NF-κB signaling with the IKK inhibitor PS1145 did not increase basal \textit{Gnrh} mRNA expression. Furthermore, inhibiting NF-κB did not block the ability of palmitate to increase \textit{Gnrh} mRNA expression. Hence, NF-κB signaling does not appear to regulate \textit{Gnrh} in mHypoA-GnRH/GFP neurons. In GT1-7 cells, on the other hand, over-activating NF-κB with constitutively active IKK-β decreased \textit{Gnrh} promoter-driven luciferase reporter activity, and repressing basal NF-κB activity with dominant-negative IκBα increased \textit{Gnrh} promoter activity (143). The promoter construct used in these studies, however, did not contain two distal \textit{Gnrh} enhancers (50), and because the reporter was randomly incorporated into the genome, it may have been subject to different epigenetic regulation than the endogenous \textit{Gnrh} gene (240). (summarized in Figure 4.3)

4.3.3 The role of palmitate metabolism in \textit{Gnrh} regulation (summarized in Figure 4.4)

We have thus far reported that the effect of palmitate on \textit{Gnrh} mRNA expression may not be mediated by the membrane receptors GPR120 or TLR4. LCFAs may not require membrane receptors to signal intracellularly. Lipids are bioactive: that is, changes in lipid levels elicit physiologically-relevant functional consequences (81). In the cell, palmitate can be activated to form palmitoyl-coA (150). We report that palmitate increases \textit{Gnrh} mRNA expression via palmitoyl-coA synthesis. Palmitoyl-coA is the precursor to glycerolipids, glycerophospholipids, and sphingolipids, which can function as secondary messengers (150). The sphingolipids ceramides, for instance, mediate SFA-driven pro-inflammatory and insulin resistance-promoting signaling in the periphery (203,209,241). Palmitoyl-coA directly contributes to the de novo synthesis of ceramides. To review, in the first step of de novo ceramide synthesis, serine palmitoyltransferase (SPT) condenses palmitoyl-coA and L-serine to form 3-keto-sphinganine, which is converted into sphinganine. Next, ceramide synthase (CerS) adds a fatty acyl-coA (e.g., palmitoyl-coA) to sphinganine to form a ceramide molecule (159). To determine if palmitate-mediated increases in \textit{Gnrh} required de novo ceramide synthesis, we inhibited either SPT or CerS. Interestingly, inhibiting CerS had no effect, but inhibiting SPT enhanced the ability of palmitate to increase \textit{Gnrh} mRNA expression, suggesting that perhaps a different palmitoyl-coA-
Figure 4.3. Schematic illustrating the function of NF-κB signaling in mHypoA-GnRH/GFP neurons. Lipopolysaccharide (LPS) binds and activates toll-like receptor 4 (TLR4), which transactivates IKK. Similarly, tumor necrosis factor α (TNFα) activates its receptor (TNFR) to also activate IKK. IKK signaling leads to activation of NF-κB, which translocates into the nucleus to regulate transcription. For instance, NF-κB upregulates Nfkbia transcription. In mHypoA-GnRH/GFP neurons, palmitate transactivates NF-κB signaling independently of TLR4. NF-κB signaling, however, does not appear to regulate Gnrh mRNA expression in mHypoA-GnRH/GFP neurons.
Figure 4.4. **Schematic illustrating the metabolic fates of palmitate.** In the cell, long-chain fatty acyl-coA synthetase (LCFACS) activates palmitate, converting it into palmitoyl-coA (pal-coA). Pal-coA can be shuttled to the mitochondria by carnitine palmitoyltransferase 1 (CPT1) for β-oxidation. Pal-coA can also contribute to de novo ceramide synthesis, which is catalyzed by enzymes such as serine palmitoyltransferase (SPT) and ceramide synthase (CerS). Pal-coA can also contribute to protein palmitoylation. Furthermore, pal-coA can be used in the de novo synthesis of glycerolipids or phospholipids. The glycerolipid diacylglycerol (DAG) can also activate novel protein kinase C (PKC) isoforms.
derived lipid mediates the effect. It is possible that inhibiting CerS still permits palmitoyl-coA to commit to the *de novo* ceramide synthesis pathway, and on the other hand, inhibiting SPT may allow channeling of more palmitoyl-coA to other metabolic pathways. Furthermore, adding palmitoyl-coA to sphinganine would generate C16 ceramide, and considering palmitate treatment may provide palmitoyl-coA in excess, we treated with exogenous C16 ceramide to determine if ceramides affect *Gnrh* mRNA expression. Overall, however, we report that ceramides do not seem to mediate the effect of palmitate on *Gnrh* mRNA expression.

Instead, data suggest a potential role for the glycerolipid DAG. In our experiments, palmitate induced NF-κB activity independent of TLR4. NF-κB can be activated by novel PKCs (242), which are activated by DAG. Palmitate treatment potentially increases DAG levels through *de novo* synthesis, and DAG may signal to increase *Gnrh* mRNA expression. Intermediates of *de novo* DAG synthesis—for example the glycerophospholipid lysophosphatidic acid, which can activate PI3K and ERK signaling (243)—may also mediate the effect of palmitate on *Gnrh* mRNA expression. Future studies using exogenous lipids or enzyme inhibitors can identify the functional palmitoyl-coA-derived lipid that may mediate the effect of palmitate on *Gnrh*.

### 4.3.4 Alternative mechanisms through which palmitate may regulate *Gnrh* mRNA expression

#### 4.3.4.1 Regulation of *Gnrh* by nitric oxide (summarized in Figure 4.5)

Palmitate can stimulate nitric oxide (NO) production in cardiomyocytes, and NO functions as a second messenger (211). Previous *in vivo* and *in vitro* studies demonstrate that NO stimulates GnRH secretion (186,244,245), and a role for NO in regulating *Gnrh* mRNA expression has also been reported. For instance, microinjection of nitric oxide synthase (NOS) inhibitors into the lateral ventricle of rats decreased *Gnrh* mRNA expression, and N-methyl-D-aspartate (NMDA), which induces NO synthesis (246), increased *Gnrh* mRNA expression 1 h after jugular injection in rats (247). It has also been demonstrated that nitric oxide paired with Ca\(^{2+}\) influx represses *Gnrh* transcription in GT1-7 cells (173). Similar to previous reports, nitric oxide stimulates GnRH secretion in mHypoA-GnRH/GFP cells (178). Here, to explore the potential role of NO in mediating palmitate-induced *Gnrh* mRNA expression, we investigated the effect of NO on *Gnrh* mRNA expression in mHypoA-GnRH/GFP neurons. Our findings suggest that NO may independently increase *Gnrh* mRNA expression. Our findings contrast the previous findings in
Figure 4.5. Schematic illustrating potential nitric oxide signaling in mHypoA-GnRH/GFP neurons. The nitric oxide (NO) donor sodium nitroprusside (SNP) increases Gnrh mRNA expression. TNFα increases Nitric oxide 2 (Nos2) mRNA expression in mHypoA-GnRH/GFP neurons. However, it is unknown if NOS2 protein levels increase in response to TNFα. Furthermore, palmitate may increase Nos2 expression in mHypoA-GnRH/GFP neurons. It is unknown if TNFα or palmitate increase NO levels in mHypoA-GnRH/GFP neurons.
GT1-7 cells (173). This may be because we used SNP alone while SNP and a calcium ionophore were previously used together in GT1-7 cells (173). Also, in GT1-7 cells, NO decreases GnRH mRNA expression via cGMP signaling (173); on the other hand, to increase Gnrh mRNA expression in mHypoA-GnRH/GFP neurons, NO may signal through a cGMP-independent pathway, for example through nitrosylation of transcription factors (248). Because palmitate and NO both increase Gnrh mRNA expression, we can hypothesize that palmitate increases the production of NO, which increases Gnrh mRNA expression.

Palmitate and cytokines such as tumor necrosis factor α (TNFα) increase NO production by stimulating Nitric oxide synthase 2 (Nos2; inducible NOS) transcription (211,212,249). We demonstrate Nos2 expression in mHypoA-GnRH/GFP neurons contrary to previous studies in GT1-7 cells observing only Nos1 and Nos3 expression (173). In mHypoA-GnRH/GFP cells, TNFα induced Nos2 expression, potentially increasing intracellular [NO], but did not change Gnrh mRNA expression at time points coinciding with increased Nos2 expression or after. Here, TNFα may not have sufficiently increased nitric oxide production to affect Gnrh mRNA expression. Moreover, because qRT-PCR did not sufficiently amplify Nos2 mRNA, palmitate may not increase Nos2 expression. It would be beneficial to measure changes in nitric oxide levels following TNFα and palmitate treatment to further understand the role of NO signaling in Gnrh regulation.

4.3.4.2 Reactive oxygen species (ROS) (summarized in Figure 4.6)

Palmitate can also activate NADPH oxidases (NOX) in adipocytes, myocytes, and pancreatic β-cells (250,251), and NOX transfers electrons to oxygen to generate superoxide, a reactive oxygen species (ROS) (252). Moreover, the mitochondrial β-oxidation of palmitate can also generate ROS (253). ROS function as secondary messengers; for instance, in LβT2 gonadotropes, NOX-generated ROS mediate the stimulation and nuclear translocation of MAPK signaling by GnRH (254). Further, ROS also mediate the activation of PI3K signaling by the cytokine interleukin-7 (255). The mechanisms through which ROS modulate kinase activity are not fully understood; however, it has been reported that ROS directly alter the catalytic cysteine of JNK-inactivating phosphatases, inhibiting them and allowing sustained JNK activation (256). In mHypoA-GnRH/GFP cells, palmitate may increase ROS production via NOX or mitochondrial β-oxidation, and ROS may stimulate PI3K signaling, which can mediate transcriptional activation
of *Gnrh*. Nonetheless, although previous reports suggest that NOX is expressed in the hypothalamus (257), we have yet to screen mHypoA-GnRH/GFP neurons for NOX expression. Additionally, future studies should measure ROS levels after palmitate treatment, and to determine whether ROS affect *Gnrh* mRNA expression at all, mHypoA-GnRH/GFP cells could be treated with hydrogen peroxide, a superoxide-derived ROS.

### 4.3.4.3 The novel LPS receptor, caspase-11 (summarized in Figure 4.6)

Palmitate may regulate *Gnrh* mRNA expression by binding to a novel LPS receptor. Recent studies report that murine caspase-11 functions as a cytosolic LPS receptor (258,259). It has been suggested that caspase-11 recognizes the lipid A moiety of LPS (258). Lipid A is composed of two phosphorylated glucosamines with six fatty-acyl chains (260). Currently, however, it is unknown if caspase-11 also detects the acyl chains of SFAs; fatty acid binding to caspase-11 may require an adaptor protein. Furthermore, we do not know if mHypoA-GnRH/GFP neurons express caspase-11, but if they do, caspase-11 may mediate the effect of palmitate on *Gnrh* mRNA expression.

### 4.3.4.4 Other potential palmitate-activated receptors (summarized in Figure 4.6)

The effect of palmitate on *Gnrh* may be mediated by other receptors such as the peroxisome proliferator-activated receptors (PPARs, discussed later) or TLR2. Palmitate and other SFAs can also activate the toll-like receptor 2 (TLR2) (125-127,261,262). Similarly to TLR4, TLR2 signals via the MyD88 adaptor protein to activate canonical NF-κB and JNK signaling (263). In addition, palmitate stimulates the recruitment of NOX2 to TLR2 (126). As previously discussed, ROS may play a role in *Gnrh* regulation. Further studies using TLR2 antagonists or genetic Tlr2 knockdown are required to determine the function of TLR2 signaling in mHypoA-GnRH/GFP neurons.

### 4.4 Regulation of *Gnrh* by peroxisome proliferator-activated receptors (PPARs)

To further describe how LCFAs may regulate *Gnrh* mRNA expression, we investigated the function of the LCFA-binding peroxisome proliferator-activated receptors (PPARs). The PPAR-α agonist Wy 14643 and PPAR-γ agonist rosiglitazone both acutely increase *Gnrh* mRNA expression.
Figure 4.6. Schematic illustrating alternatives pathways that palmitate may activate. Palmitate may activate the toll-like receptor 2 (TLR2), which signals similarly to TLR4. Moreover, palmitate may also induce the generation of reactive oxygen species (ROS) through NADPH oxidases (NOX) or fatty acid β-oxidation. ROS can activate PI3K signaling. Palmitate may also activate the novel cytosolic LPS receptor caspase-11. Additionally, palmitate may bind and activate the peroxisome proliferator-activated receptors (PPARs).
expression. Thus, PPAR-α and PPAR-γ appear to regulate Gnrh in mHypoA-GnRH/GFP neurons. To select PPAR agonist doses, we considered previous studies (148) and the reported EC50 concentrations for activation of each PPAR isoform. Notwithstanding, future studies need to further characterize the dose-dependence of the effects seen, and PPAR antagonists should be used with agonists to demonstrate truly isoform-specific effects. In addition, it is curious that rosiglitazone- and Wy 14643-induced increases in Gnrh mRNA expression subside as early as 2 h after exposure. We would expect sustained increases considering the Gnrh mRNA half-life is 20 to 30 h (264,265). However, the mRNA degradation rate may be counter-upregulated in response to increased Gnrh mRNA levels; it has been suggested, for instance, that transcription and mRNA degradation are coupled (266,267).

Observing that PPAR-γ may regulate Gnrh, we investigated whether PPAR-γ may mediate the effect of DHA on Gnrh mRNA expression. DHA is an endogenous PPAR-γ ligand (149). Presently, however, we report that PPAR-γ may not mediate the effect of DHA on Gnrh; however, PPAR-α may mediate the effect of DHA. Moreover, we have yet to study whether PPAR-γ or PPAR-α mediate the effect of palmitate on Gnrh. Further studies using isoform-specific PPAR antagonists can address these questions.

We cannot exclude the possibility that rosiglitazone or Wy 14643 may also be non-specifically agonizing another receptor such as GPR120. Rosiglitazone and other thiazolidinediones such as troglitazone also activate GPR120 and GPR40 (96,221,268). This non-specific agonism, however, may play an important role in the overall effects of PPAR agonists. For instance, rosiglitazone synergistically activates GPR40 and PPAR-γ, enhancing PPAR-γ-dependent transcription (269). In addition, PPAR-α and PPAR-γ agonists are used clinically to treat dyslipidemia and diabetes, respectively (270). Moreover, these agonists, such as rosiglitazone and the PPAR-α agonist fenofibrate, can cross the blood-brain barrier and accumulate in the brain (271-273). In the brain, rosiglitazone demonstrates an elimination half-life of approximately 3 h (272). Therefore, further analysis of how PPAR agonists affect GnRH neurons may be especially relevant.
4.5 Transcriptional activation of Gn rh by DHA and palmitate may localize to the Gn rh proximal enhancer.

Changes in mRNA expression may result from changes in mRNA stability or de novo transcription. To investigate the role of transcription in palmitate- and DHA-induced increases in Gn rh mRNA levels, we used the transcriptional inhibitors actinomycin D (ActD) and 5,6-Dichloro-1-β-D-ribofuranosylbenzimidazole (DRB). ActD intercalates to DNA and immobilizes the transcriptional complex, thus impeding the elongation of RNA in transcription (274). Moreover, DRB inhibits the positive transcription elongation factor (P-TEFb) by targeting the cyclin dependent kinase (CDK9) subunit (275), and this results in premature termination of transcription (276). In the presence of DRB or ActD, neither DHA nor palmitate affected Gn rh mRNA levels. Here, we report that DHA and palmitate may increase Gn rh mRNA expression through transcriptional activation. However, to further demonstrate that DHA and palmitate upregulate Gn rh transcription, we may use nuclear run-on assays to determine if DHA and palmitate initiate de novo transcription at Gn rh (277). Moreover, we can investigate changes in stability of Gn rh mRNA by measuring changes in poly (A) tail length, for example by RNase H assay (278). We next began to study the mechanisms through which DHA and palmitate regulate Gn rh transcription. Gn rh transcription is regulated by chromatin remodeling and transcription factor interactions at the evolutionarily conserved upstream Gn rh promoter, proximal enhancer (GnRH-E1), and distal enhancers (GnRH-E2 and GnRH-E3) (47-50,54,58,59,264). Recent studies report that RNA polymerase II (RNA PII) binds at these three enhancers and transcribes non-coding, enhancer-derived RNAs (eRNAs) at GnRH-E1 (54). siRNA-induced knockdown of GnRH-E1 eRNA decreased Gn rh mRNA expression in GT1-7 cells (63), suggesting that GnRH-E1 eRNA may function to facilitate transcriptional activation of Gn rh. Presently, we demonstrate that mHypoA-GnRH/GFP neurons, similar to GT1-7 cells, also express GnRH-E1 eRNA, indicating this enhancer region is active in mHypoA-GnRH/GFP neurons. Moreover, in mHypoA-GnRH/GFP neurons, DHA and palmitate also increase GnRH-E1 eRNA expression, and these eRNAs may promote Gn rh transcription. Whether increased eRNA expression mediates Gn rh transcriptional activation here could be assessed by RNA interference targeted degradation of GnRH-E1 eRNAs. We may also explore the role of interactions between GnRH-E1 and GnRH-P via chromatin looping by using chromatin conformation assays. In addition, we have yet to describe how DHA and palmitate increase GnRH-E1 eRNA expression. In the rat
\textit{Gnrh} gene, GnRH-E1 enhancer, which is highly conserved between rat and mouse \((53)\), interacts with transcription factors such as the CCAAT/enhancer-binding protein \(\beta\) (C/EBP-\(\beta\)), which mediates the regulation of \textit{Gnrh} by nitric oxide \((59)\). C/EBP-\(\beta\)-dependent gene expression can be activated by ERK1/2 and PI3K signaling \((279,280)\). Because the effects of DHA and palmitate depend on these pathways, C/EBP-\(\beta\) may transactivate GnRH-E1 eRNA transcription, leading to \textit{Gnrh} transcriptional activation. To address this possibility, in the future, we could use RNA interference to knockdown C/EBP-\(\beta\) or perform chromatin immunoprecipitation (ChIP) assays to investigate the role of C/EBP-\(\beta\) in the regulation of \textit{Gnrh} by DHA and palmitate.

\subsection*{4.6 Fatty acid interactions in \textit{Gnrh} regulation}

\subsection*{4.6.1 The effects of DHA and palmitate on \textit{Gnrh} are not additive.}

The effects of DHA and palmitate on \textit{Gnrh} mRNA expression are not additive, suggesting that DHA and palmitate share a common mechanism to regulate \textit{Gnrh}. Our findings demonstrate, for instance, that DHA and palmitate both increase expression of GnRH-E1-derived eRNA; DHA- and palmitate-mediated signals may converge on an effector that promotes GnRH-E1 enhancer activity, leading to \textit{Gnrh} transcriptional activation. In addition, the FFA concentrations used in these preliminary experiments may not represent the physiological proportion of free DHA and palmitate in plasma. On average in healthy men and women, palmitate and DHA account for 28.3\% and 0.4\% of total plasma FFAs, respectively. Dietary DHA supplementation, however, can increase plasma DHA levels \((281,282)\). While these considerations may provide modest rationale for human relevance, we cannot comment on whether these FFA levels are physiologically relevant to mouse cells and specifically, mouse GnRH neurons. Thus, it is important to characterize the dose-dependence of the effects of FFAs. The dose-dependence of the interaction between DHA and palmitate in \textit{Gnrh} regulation should be addressed in the future.

\subsection*{4.6.2 Oleate blocks the effect of palmitate on \textit{Gnrh} mRNA expression.}

In contrast, oleate blocked the effect of palmitate on \textit{Gnrh} mRNA expression. As oleate accounts for 32.7\% of plasma FFAs \((82)\), we may consider the equimolar proportion of palmitate to oleate used in our experiments to be near-physiological. However, the dose-dependence of this interaction should also be addressed in the future.
We next began to investigate the mechanism through which oleate blocks the effect of palmitate. Oleate protects against palmitate-induced insulin resistance and pro-inflammatory signaling in rat L6 myotubes (283) and mouse neuroblastoma Neuro-2a cells (88). In L6 myotubes, the ability of oleate to protect against palmitate required PI3K signaling. We report, however, that oleate blocks the effect of palmitate on Gnrh mRNA expression independently of PI3K signaling in mHypoA-GnRH/GFP neurons. In Neuro-2a cells, the protective effects of oleate are mediated by protein kinase A (PKA) signaling. We can determine if the effect of oleate in mHypoA-GnRH/GFP cells requires PKA signaling by using the PKA inhibitor H89 (284). Furthermore, in Neuro-2a cells, oleate enhanced palmitate-induced TAG synthesis and decreased palmitate-induced DAG synthesis. In mHypoA-GnRH/GFP cells, considering that the effect of palmitate appears to be metabolism-dependent, oleate may block the ability of palmitate to increase Gnrh mRNA expression by modulating the incorporation of palmitate into cellular DAG.

In mHypoA-GnRH/GFP neurons, oleate may block the effect of palmitate on Gnrh mRNA expression by modulating the β-oxidation of palmitoyl-coA. In addition to being used to form esterified lipids such as DAG, palmitoyl-coA is also used to generate energy in mitochondrial β-oxidation (285). In the rate-limiting step of mitochondrial β-oxidation, the enzyme carnitine palmitoyltransferase I (CPTI) facilitates the transport of palmitoyl-coA into the mitochondria. Activating CPTI using C75 (286) in primary hypothalamic culture decreased palmitate-induced accumulation of palmitoyl-coA-derived esterification products such as ceramides, acylglycerols, and cholesterol esters (287). Moreover, increasing FFA β-oxidation also protected against palmitate-induced Tnfa expression (287). In skeletal muscle cells, oleate can activate CPTI through an AMPK-mediated pathway, increasing the β-oxidation of palmitate (288). In mHypoA-GnRH/GFP cells, oleate may block the effect of palmitate on Gnrh by diverting palmitoyl-coA from the esterification pathway to the β-oxidation pathway. To test this hypothesis, we could co-incubate mHypoA-GnRH/GFP neurons with oleate and palmitate while inhibiting CPTI and β-oxidation with etomoxir (289).

In addition, palmitoleate seems to modestly block the effect of palmitate on Gnrh. It has been reported that palmitoleate protects against palmitate-induced pro-inflammatory signaling via AMPK signaling in macrophages (212). Because oleate and palmitoleate are both MUFAs, how they signal intracellularly may be similar. Further studies using activators and inhibitors of
AMPK, such as AICAR (290) and compound C (291), are required to determine whether the inhibitory effects of oleate and palmitoleate are mediated by AMPK signaling.

In summary, we report here that the structurally distinct LCFA s DHA, palmitate, palmitoleate, and oleate differentially affect Gnrh mRNA expression. Moreover, our preliminary co-incubation experiments demonstrate that distinct LCFA combinations also differentially affect Gnrh mRNA expression. Together, our findings further highlight the complexity of LCFA signaling and physiological function.

4.7 The effect of LCFA s on oscillatory Gnrh mRNA expression

Our preliminary studies suggest that mHypoA-GnRH/GFP neurons express Gnrh and the core molecular clock genes Bmal1 and Per2 in an oscillatory manner. And these oscillations do not appear to be a function of Histone 3a mRNA level fluctuations; His mRNA expression did not drastically change across time. These findings are consistent with previous findings. For instance, our laboratory previously demonstrated that synchronized GT1-7 cells express Gnrh and the clock genes Bmal1, Per1, Per2, Cry1, Cry2, and Clock, in a circadian-like rhythm (176). Furthermore, a recent study reports that single GnRH neurons demonstrate ultradian rhythms of Gnrh transcription (292). The molecular clock and Gnrh oscillation requires further characterization in mHypoA-GnRH/GFP cells. Particularly, the oscillatory period and amplitude of Gnrh mRNA expression should be determined and compared to previous findings about Gnrh mRNA expression rhythms. In addition, our laboratory recently demonstrated that palmitate and DHA modulate the circadian transcriptional patterns of molecular clock genes in hypothalamic neurons (84). Future studies can address whether LCFA s affect the molecular clock or oscillatory Gnrh mRNA expression in mHypoA-GnRH/GFP neurons.

4.8 Future studies

4.8.1 FFA signaling may modulate sensitivity of mHypoA-GnRH/GFP neurons to other stimuli

In gonadotropes, FFAs regulate gonadotropin biosynthesis and secretion through MAPK signaling (13,14). Interestingly, FFAs also negate the response of gonadotropes to GnRH; specifically, pretreatment of gonadotropes with the PUFA linoleate prevented GnRH from stimulating calcium influx and LH secretion (14). The GnRH receptor (GnRHR) is coupled to
Gaq/11 and signals through the MAPK pathway (293); FFAs may alter responses to GnRH by modulating the MAPK activity in the cell. Kisspeptin also signals through a Gαq/11-coupled receptor (GPR54) (294). And in mHypoA-GnRH/GFP neurons, kisspeptin-10 increases Gnrh mRNA expression (177). Considering the critical role of kisspeptin in regulating GnRH neurons, it would be insightful to test if FFAs modulate the response of mHypoA-GnRH/GFP neurons to kisspeptin stimulation. To investigate this, we can pretreat mHypoA-GnRH/GFP neurons with DHA or palmitate; after, we would wash the neurons to remove LCFAs and then treat with kisspeptin-10. We could use qRT-PCR to determine if DHA or palmitate altered the effect of kisspeptin-10 on Gnrh mRNA expression, and we could use immunoblotting to determine if DHA or palmitate modulate kisspeptin-10-induced protein signaling.

4.8.2 Determine the effect of DHA and palmitate on GnRH peptide levels in mHypoA-GnRH/GFP neurons.

Presently, we demonstrate that the FFAs DHA and palmitate upregulate Gnrh transcription, thus potentially increasing GnRH biosynthesis. We cannot measure intracellular GnRH peptide levels using standard immunoblotting because of the lack of an immunoblotting-grade GnRH antibody. Instead, to assess changes in GnRH levels, we can measure released GnRH after a potent depolarizing stimulus, which “unloads” GnRH from neurons. mHypoA-GnRH/GFP cells will be pre-incubated with DHA or palmitate, and then challenged with a secretion-stimulating depolarizing agents such as KCl (178) or veratridine (295). The culture media will be collected, and secreted GnRH levels will be measured using commercially-available enzyme-linked immunosorbent assay (ELISA) kits. Alternatively, to quantify and image intracellular GnRH, we may use immunocytochemistry (ICC) as ICC-validated GnRH antibodies are commercially available.

4.8.3 Rationale for studying the effects of LCFAs on GnRH secretion in mHypoA-GnRH/GFP neurons

4.8.3.1 Palmitate modulates the expression and activity of synaptosomal-associated protein 25 (SNAP-25), which regulates secretion.

GnRH secretion is the ultimate GnRH neuronal output, and it is essential to reproductive function (296). Previous studies suggest that palmitate may regulate cellular secretion machinery
and thus may also regulate GnRH secretion. For instance, in the MIN6 pancreatic β-cell line, palmitate increases synaptosomal-associated protein 25 (SNAP-25) gene expression (297). SNAP-25 is an essential component of the neuronal synaptic secretion machinery (298); specifically, it targets vesicle fusion to the plasma membrane. Furthermore, SNAP-25 is highly present in GT1-7 cells (166); whether it is expressed in mHypoA-GnRH/GFP neurons, however, is unknown. Palmitate also regulates the subcellular localization of SNAP-25: palmitoylation of SNAP-25 is required to recruit it to the plasma membrane (299). In addition to its role in vesicle fusion, SNAP-25 also negatively regulates voltage-gated calcium channels (VGCC) in neurons, reducing neuronal excitability (300). Hence, as a component of the neurosecretory machinery, SNAP-25 regulates basal secretion, and as a VGCC-modulator, SNAP-25 may also regulate stimulus-dependent secretion. mHypoA-GnRH/GFP cells may express SNAP-25 considering their neuronal phenotype and ability to secrete GnRH (177,178). Thus, because palmitate modulates SNAP-25 expression and subcellular localization, it may be reasonable to investigate whether palmitate changes spontaneous and/or stimulated GnRH secretion in mHypoA-GnRH/GFP neurons.

4.8.3.2 LCFAs induce calcium influx, which may modulate secretion.

Additionally, GnRH secretion depends on calcium influx. In primary hypothalamic cultures and GT1-1 cells (derived from the same culture as GT1-7 cells), calcium channel blockers and chelators repress spontaneous and depolarization-stimulated GnRH release (301,302). DHA, palmitate, and other LCFAs such as oleate and palmitoleate increase intracellular calcium levels via GPR120 activation in HEK 293 cells (109). Currently, it is unknown if LCFAs stimulate calcium influx in Gpr120-expressing mHypoA-GnRH/GFP cells. In future studies, we can measure acute changes in intracellular calcium in response to short-term LCFA treatment using calcium fluorometric assay kits. Acute calcium influx, which can stimulate secretion, may provide further rationale to study the regulation of GnRH secretion by LCFAs. Furthermore, we can determine if LCFAs acutely stimulate GnRH secretion using ELISAs. And if LCFAs stimulate GnRH secretion in mHypoA-GnRH/GFP cells, we can further investigate if calcium signaling and GPR120 mediate changes in secretion by using calcium channel blockers and RNA interference to knockdown GPR120.
4.8.3.3 The role of palmitate in glucose uptake and glucose-stimulated GnRH secretion

Our laboratory has previously demonstrated that glucose stimulates GnRH secretion in mHypoA-GnRH/GFP cells (178). Similarly, in primary hypothalamic cultures, depolarization-stimulated GnRH release and spontaneous GnRH release frequency depend on glucose availability (301). Glucose uptake in myocytes is acutely induced by palmitate (303); palmitate rapidly stimulates translocation of the glucose transporter GLUT4 to the plasma membrane, leading to increased intracellular glucose. Future studies can investigate the effect of palmitate on glucose uptake and potentially glucose-mediated GnRH secretion in mHypoA-GnRH/GFP cells.

4.8.3.4 The role of prostaglandin E2 (PGE2) in GnRH secretion

Palmitate could possibly cause a delayed increase in GnRH secretion. GnRH secretion is stimulated by prostaglandin E2 (PGE2) (304-307), which is an arachidonate-derived eicosanoid synthesized by cyclooxygenase-2 (COX2). In mHypoA-GnRH/GFP cells, palmitate increases Cox2 mRNA expression, and this may lead to increased COX2 levels and therefore PGE2 production. Whether mHypoA-GnRH/GFP cells release PGE2 and express the PGE2 receptor (EP2) remains to be determined. However, if we determine that mHypoA-GnRH/GFP neurons do express EP2, this may provide further rationale to study the effects of LCFAs, namely palmitate in this case, on GnRH secretion in mHypoA-GnRH/GFP neurons.

4.9 Study limitations

While using the mHypoA-GnRH/GFP cell line allows us to study the molecular mechanisms underlying Gnrh regulation, the use of an immortalized cell line inherently limits the conclusions we can draw from our findings. For instance, while FFAs seem to directly regulate Gnrh mRNA expression GnRH neurons, we cannot comment on the net effect of FFAs on Gnrh in vivo. GnRH neurons are complexly regulated by afferent signals such as (but not limited to) sex steroid hormones, kisspeptin, and neuropeptide Y (NPY) (171,174,308-312). FFAs increase the production of androgen sex steroid precursors in vivo (17). Furthermore, preliminary studies in our laboratory demonstrate that FFAs also regulate Kiss expression in kisspeptin neuronal cell models (unpublished data). Thus, in vivo, FFAs may modulate signals that converge onto GnRH neurons and determine the net effect of FFAs on Gnrh mRNA expression.
In addition, we used the mHypoA-GnRH/GFP cell line in part because as a non-clonal population of GnRH neurons, the cell line may represent a broader complement of phenotypically different GnRH neurons (164). However, passaging of these cells may eventually bias the population to one cell type, as one type of cell may divide faster than another (313). For instance, it has been widely reported that cross contaminating cell lines with the aggressively dividing immortal HeLa cell line results in HeLa cells dominating and replacing the original cell line (314,315). In the future, it may be beneficial to subclone and characterize the phenotypically different cells comprising the mHypoA-GnRH/GFP cell line.

The mHypoA-GnRH/GFP cell line was immortalized through retroviral transfer of the SV-40 T antigen (TAg) (178). TAg, however, can interact with transcription factors such as p53 and activate genes not expressed in native GnRH neurons. For example, the TAg-p53 complex can transcriptionally activate the IGF-I promoter (316). Furthermore, our laboratory has observed that the SV-40 TAg seems to induce ectopic Agrp and Oxytocin expression, and it also seem to increase basal phosphorylation of signaling phosphoproteins such as AMPK (Belsham et al. unpublished). Despite this, to minimize the confounding effect of this increased basal signaling when we assess phosphoprotein levels, we first incubate the cells in serum-free culture media, which reduces resting state signaling.

4.10 Closing remark

Overall, through demonstrating that the fatty acids DHA and palmitate regulate Gnrh mRNA expression, we highlight functional LCFA sensing in GnRH neurons. These findings deepen our understanding of how GnRH neurons integrate nutrient and metabolic signals to coordinate the reproductive axis, and may provide insight into the molecular links between nutrition/energy balance and reproductive function.
References


38. Leyendecker, G., Struve, T., and Plotz, E. J. (1980) Induction of ovulation with chronic intermittent (pulsatile) administration of LH-RH in women with hypothalamic and hyperprolactinemic amenorrhea. *Archives of gynecology* 229, 177-190


immortalized, hypothalamic neurons. *Biochemical and biophysical research communications* 413, 414-419


