Effects of the Saturated Fatty Acid Palmitate on Neuroinflammation, ER Stress, and Pomc mRNA Expression in Hypothalamic mHypoA-POMC/GFP Neurons

by

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

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University of Toronto

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Abstract

Dietary fats can modulate brain function. How free fatty acids (FFAs) alter hypothalamic pro-opiomelanocortin (POMC) neurons remain undefined. The saturated FFA, palmitate, increased neuroinflammatory and ER stress markers, as well as Pomc mRNA levels, but did not affect insulin signalling, in mHypoA-POMC/GFP-2 neurons. This effect was mediated through the MAPK JNK and ERK. Further, the increase in Pomc was dependent on palmitoyl-coA synthesis, but not de novo ceramide synthesis, as inhibition of SPT enhanced palmitate-induced Pomc expression, while methylpalmitate had no effect. While palmitate concomitantly induces neuroinflammation and ER stress, these effects were independent of changes in Pomc expression. Palmitate thus has direct acute effects on Pomc, which appears to be important for negative feedback, but not directly related to neuroinflammation. The monounsaturated FFA oleate completely blocked the palmitate-mediated increase in neuroinflammation, ER stress, and Pomc mRNAs. This study provides insight into the complex central metabolic regulation by FFAs.
Acknowledgments

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<table>
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<th>Description</th>
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<tbody>
<tr>
<td>ActD</td>
<td>actinolysin D</td>
</tr>
<tr>
<td>ACTH</td>
<td>adrenocorticotropic hormone</td>
</tr>
<tr>
<td>AgRP</td>
<td>agouti-related protein</td>
</tr>
<tr>
<td>ANOVA</td>
<td>analysis of variance</td>
</tr>
<tr>
<td>α-MSH</td>
<td>alpha-melanocyte stimulating hormone</td>
</tr>
<tr>
<td>cDNA</td>
<td>complementary DNA</td>
</tr>
<tr>
<td>ChIP</td>
<td>chromatin immunoprecipitation</td>
</tr>
<tr>
<td>CNTF</td>
<td>ciliary neurotrophic factor</td>
</tr>
<tr>
<td>CHOP</td>
<td>C/EBP-homologous protein</td>
</tr>
<tr>
<td>CPE</td>
<td>carboxypeptidase E</td>
</tr>
<tr>
<td>CPT1</td>
<td>carnitine palmitoyltransferase 1</td>
</tr>
<tr>
<td>FFAR1</td>
<td>free fatty acid receptor 1</td>
</tr>
<tr>
<td>DAG</td>
<td>diacylglycerol</td>
</tr>
<tr>
<td>DMEM</td>
<td>Dulbecco’s modified eagle medium</td>
</tr>
<tr>
<td>DMSO</td>
<td>dimethyl sulfoxide</td>
</tr>
<tr>
<td>DNA</td>
<td>deoxyribonucleic acid</td>
</tr>
<tr>
<td>DRB</td>
<td>5, 6-Dichloro-1-β-D-ribofuranosylbenzimidazole</td>
</tr>
<tr>
<td>ER</td>
<td>endoplasmic reticulum</td>
</tr>
<tr>
<td>ERK</td>
<td>extracellular signal-related kinase</td>
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<td>Abbreviation</td>
<td>Full Form</td>
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<tr>
<td>--------------</td>
<td>-----------------------------------------------</td>
</tr>
<tr>
<td>FACS</td>
<td>fluorescence-activated cell sorting</td>
</tr>
<tr>
<td>FBS</td>
<td>fetal bovine serum</td>
</tr>
<tr>
<td>FFA</td>
<td>free fatty acid</td>
</tr>
<tr>
<td>Foxo1</td>
<td>forkhead box protein O1</td>
</tr>
<tr>
<td>GFP</td>
<td>green fluorescence protein</td>
</tr>
<tr>
<td>GRP78</td>
<td>glucose regulated protein 78 kDa</td>
</tr>
<tr>
<td>GPCR</td>
<td>G protein-coupled receptor</td>
</tr>
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<td>GPR40</td>
<td>G protein-coupled receptor 40</td>
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<tr>
<td>IκBα</td>
<td>inhibitor of nuclear factor kappa B alpha</td>
</tr>
<tr>
<td>IKKβ</td>
<td>inhibitor of I kappa B kinase beta</td>
</tr>
<tr>
<td>IL-1β</td>
<td>interleukin-1 beta</td>
</tr>
<tr>
<td>IL-6</td>
<td>interleukin-6</td>
</tr>
<tr>
<td>IR</td>
<td>insulin receptor</td>
</tr>
<tr>
<td>IRS</td>
<td>insulin receptor substrate</td>
</tr>
<tr>
<td>JAK</td>
<td>janus kinase</td>
</tr>
<tr>
<td>JNK</td>
<td>c-Jun N-terminal kinase</td>
</tr>
<tr>
<td>LPS</td>
<td>lipopolysaccharide</td>
</tr>
<tr>
<td>MAPK</td>
<td>mitogen-activated protein kinase</td>
</tr>
<tr>
<td>MC3R</td>
<td>melanocortin 3 receptor</td>
</tr>
<tr>
<td>MC4R</td>
<td>melanocortin 4 receptor</td>
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x
<table>
<thead>
<tr>
<th>Abbreviation</th>
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</table>
mRNA | messenger RNA |
MTT | 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide |
N-AT | n-acetyltransferase |
NF-κB | nuclear factor kappa B |
NPY | neuropeptide Y |
PAM | peptidyl α-amidating mono-oxygenase |
PC1/3 | prohormone convertase 1/3 |
PC2 | prohormone convertase 2 |
PI3K | phosphoinositide 3-kinase |
PKA | protein kinase A |
PKC | protein kinase C |
POMC | pro-opiomelanocortin |
qRT-PCR | quantitative reverse transcriptase polymerase chain reaction |
RNA | ribonucleic acid |
ROS | reactive oxygen species |
SP1 | specificity protein 1 |
SPT | serine palmitoyltransferase |
STAT3 | signal transducer and activator of transcription 3 |
SV40 | simian virus 40 |
TAG | triacylglycerol |
<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>TAK1</td>
<td>transforming growth factor beta-activated kinase 1</td>
</tr>
<tr>
<td>TLR4</td>
<td>toll-like receptor 4</td>
</tr>
<tr>
<td>TNFα</td>
<td>tumour necrosis factor alpha</td>
</tr>
</tbody>
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Chapter 1
Introduction

1 Introduction

1.1 General introduction

Two thirds of North American adults are either overweight or obese, and this epidemic has been attributed to the increased consumption of a high-fat diet [1]. Consumption of high levels of dietary fat increases levels of the saturated free fatty acid (FFA) palmitate in the serum [2] and brain, specifically the hypothalamus [3]. Consequently, accumulation of this 16-carbon saturated FFA and its metabolites in the hypothalamus results in inflammation and endoplasmic reticulum (ER) stress, leading to a decrease in insulin signalling, weight gain, and metabolic dysfunction [3, 4].

The arcuate nucleus (ARC) of the hypothalamus contains opposing orexigenic neuropeptide Y (NPY)/agouti-related peptide (AgRP) neurons and anorexigenic pro-opiomelanocortin (POMC) neurons that regulate food intake and energy expenditure by integrating signals of energy status from hormones and nutrients, including FFAs [5, 6]. Consumption of a high-fat diet or exposure to palmitate treatment directly alters hypothalamic neuropeptide expression. However, the direct effects of palmitate on POMC-expressing neurons has not been investigated. Therefore, this study examines the direct effects of palmitate on Pome, neuroinflammation, and ER stress in immortalized, non-clonal POMC-expressing cell lines.
1.2 Palmitate and palmitate signalling mechanism(s)

1.2.1 Palmitate general

Palmitate, a 16-carbon saturated fatty acid, is the most abundant saturated fatty acid found in animals and accounts for 27.3% of total circulating plasma FFAs in humans [7, 8]. Additionally, circulating levels of saturated FFAs were found to be strongly correlated with diet [7], as palmitate comprises the largest percentage of saturated FFAs in the diet [9]. Additionally, consumption of a high-carbohydrate diet increases plasma [10] and adipose [11] palmitate levels as a result of decreased fatty acid oxidation. Baseline palmitate levels are ~0.3-0.7 mM and levels rising above 1 mM after consumption of a high-fat meal [12-15] and these levels are further elevated in obese individuals compared to normal weight individuals [16, 17]. As a nutrient, palmitate has a role in energy homeostasis, however, high levels of palmitate, as seen with high-fat feeding, can induce lipotoxic effects in tissues and disrupt feeding circuits [18-30], a potential mechanism for diet-induced obesity. However, palmitate is not simply a signal of nutrient status, but has many biological and structural functions [31].

1.2.2 Palmitate in the brain

While the peripheral effects of palmitate have been well described, particularly in adipose tissue, β-cells, and other metabolic tissues, the effects of palmitate in the brain, and specifically the hypothalamus in terms of energy homeostasis is not fully understood. Increased serum levels of palmitate results in increased brain and hypothalamic palmitate levels [16, 32], as it can pass the blood brain barrier [33-35]. Palmitate is the most abundant saturated FFA in the brain [36, 37], with normal basal levels between 50-80 µmol/kg wet tissue [38-41], but also comprises the largest percent of total brain phospholipids [42] in rodents. Palmitate is rapidly taken up by cells
and ~30 percent is esterified into phospholipids [35] and ~50 percent is rapidly oxidized [43]. Similarly to increased plasma saturated FFA levels with high-fat diet, hypothalamic palmitoyl-coA levels are increased with high-fat diet [3].

### 1.2.3 Membrane receptor signalling

Palmitate is shown to activate a number of cell membrane receptors and resultant downstream signalling pathways. Palmitate can activate the long chain fatty acid receptor G protein-coupled receptor 40 (GPR40)/free fatty acid receptor 1 (FFAR1) [44-48], as well as the classical pro-inflammatory receptor toll-like receptor 4 (TLR4) [49-58].

TLR4 is a transmembrane pattern recognition receptor, which is highly expressed in the brain and hypothalamus [54, 55, 59-64]. Lipopolysaccharide (LPS) is the endogenous ligand for TLR4 (reviewed in [65]), however, palmitate is also shown to activate this TLR due the structural similarity to the LPS lipid-A tail [49]. Through activation of TLR4, palmitate activates the downstream inhibitor of I kappa B kinase beta (IKKβ)/ nuclear factor kappa B (NF-κB) and mitogen-activated protein kinase (MAPK) pathways to induce a sterile pro-inflammatory response and ER stress (reviewed in [66-68]). Activation of TLR4 results in the phosphorylation and activation of transforming growth factor beta-activated kinase 1 (TAK1). TAK1 phosphorylates IKKβ, which phosphorylates inhibitor of nuclear factor kappa B alpha (IκBα), IκBα then dissociates from NF-κB and is targeted for degradation. Following dissociation from IκBα, NF-κB is free to enter the nucleus where it acts as a pro-inflammatory transcription factor, primarily to increase the production of cytokines and chemokines. Additionally, TAK1 phosphorylates and activates the MAPKs c-Jun N-terminal kinase (JNK) and p38 MAPK resulting in an increase in pro-inflammatory cytokines and inducing ER stress. The actions of JNK and p38 MAPK are predominantly through the transcription factors c-Jun,
SP-1 and AP-1 transcription factor complexes [69-71]. Inhibition of TLR4 or other downstream components of these signaling cascades in the hypothalamus attenuates palmitate-induced inflammation and ER stress [3, 54], identifying these pathways in mediating some of the lipotoxic effects of palmitate.

In addition to signaling through TLR4, palmitate can activate the long chain fatty acid receptor GPR40 and downstream pathways. Activated GPR40 can signal through the G proteins Gαq/11, Gαs, or Gαi/o to increase diacylglycerol (DAG) levels and phosphoinositide 3-kinase (PI3K)/Akt, protein kinase C (PKC) and extracellular signal-related kinase (ERK) signalling cascades [72-75].

1.2.4 Lipid metabolite signaling

While palmitate can activate cell membrane receptors, it can also translocate across the membrane via fatty acid transport proteins and binding proteins [76-80]. Upon entry into the cell, acyl-coA synthetase promotes the activation of palmitate to palmitoyl-coA [81], and as a fatty acyl-coA ester is retained within the cell [82]. Palmitoyl-coA can serve as a substrate for lipid metabolite synthesis, including sphingolipid, glycerolipid, and phospholipid synthesis, or β-oxidation [83]. The metabolic fate of palmitate depends on the nutrient status of the tissue, as different processes may be favoured in times of energy deficit or energy excess [84-86]. In an energy deficient state where β-oxidation is favoured, palmitoyl-coA is shuttled through CPT1 to the mitochondria where successive reactions break down the long chain fatty acyl-coA to produce adenosine triphosphate (ATP) [85].

When the tissue is in a state of positive energy balance, fatty acid storage as glycerolipids and structural phospholipids is favoured. In the presence of glucose, fatty acid oxidation is inhibited
by CPT1 [84] and fatty acids increase the generation of phospholipids and glycerolipids [87, 88]. Specifically, there are increased DAG levels in neuronal cells exposed to palmitate [89, 90].

The palmitoyl-coA pool alternatively can act as a substrate for the sphingolipid pathway, which includes many bioactive lipid species with metabolic implications [91], notably ceramide. Serine palmitoyltransferase (SPT) is the rate-limiting step in ceramide generation and catalyzes de novo ceramide synthesis through the condensation of palmitoyl-coA and serine [92]. Ceramide is proposed to be a lipid metabolite that significantly contributes to the lipotoxic effects of palmitate as it has been shown to induce insulin resistance and apoptosis through inflammation and ER stress [93-99]. Specifically in the hypothalamus the generation of ceramide has lipotoxic effects and alters energy homeostasis and feeding behaviour [21, 22, 100, 101].

1.3 Hypothalamic neuropeptides

1.3.1 The hypothalamus

The hypothalamus is a region of the brain containing many nuclei that regulate key processes including body temperature [102, 103], circadian rhythms [104], and feeding [105, 106]. As a major regulator of metabolic processes, the hypothalamus connects the nervous system and the endocrine system. The mediobasal hypothalamus contains the arcuate nucleus (ARC), which is adjacent to the third ventricle and the median eminence, allowing the neurons and glia to easily access circulating factors due to the incomplete blood brain barrier in this region [107, 108]. The median eminence is composed of fenestrated capillaries permeable to circulating hormones, nutrients, and drugs, allowing the neurons of this region of the hypothalamus to integrate all factors [109, 110]. Additionally, it is reported that ARC neurons send projections into median eminence to sense circulating factors [111]. The ARC contains many neuroendocrine neurons,
of which there are two subsets of neurons that produce feeding-related neuropeptides; NPY/AgRP- and POMC/CART-expressing neurons.

1.3.2 The central melanocortin system

The central melanocortin system, comprised of NPY/AgRP-expressing, POMC-expressing neurons, and neurons expressing the G protein-coupled melanocortin-3 receptor (MC3R) or MC4R, is a neuronal circuit that integrates hormonal, nutrient, and neural inputs of energy status to regulate whole body energy homeostasis [112-115], summarized in Figure 1.1. Here the coordinated action of NPY and POMC neurons regulates feeding, as NPY potently increases feeding and decreases energy expenditure, while POMC acts to decrease feeding and increase energy expenditure [5, 6, 105, 114, 116] and the balance of these neuropeptides is regulated by energy status. Given the importance of the central melanocortin system in overall energy homeostasis, it is a promising target for anti-obesity and anti-diabetic drugs, highlighting the importance of understanding the complexity of this circuit. α-MSH derived from pro-opiomelanocortin, acts as an agonist for the MC3R and MC4R, while AgRP antagonizes both receptors [117, 118]. Mutations in a part of the central melanocortin system is the most common cause of monogenic obesity [119, 120].

The majority of central POMC-expressing neurons originate in the ARC, where there are ~3000 POMC-expressing neurons as determined in mice expressing GFP under the control of the POMC promoter [121], while ~300 POMC-expressing neurons originate in the brainstem [122]. Many of these ARC POMC neurons send projections to the parventricular nucleus (PVN) of the hypothalamus [114] and interestingly, NPY/AgRP neurons send dense projections to the PVN [123]. In addition to sending projections to second-order neurons to antagonize the MC3R and MC4R, NPY/AgRP neurons send projections to directly inhibit POMC neurons [124].
Figure 1.1 The central melanocortin system. In the central melanocortin system, hormones and nutrients act on NPY/AgRP and POMC neurons of the ARC. Activation of POMC neurons leads to the release of α-MSH which acts via MC4R on second order neurons, primarily neurons in the PVN to decrease feeding. Conversely, activation of NPY/AgRP neurons leads to the release of AgRP which antagonizes the MC4R, as well as NPY/AgRP neurons sending inhibitory projects directly to POMC neurons.
1.3.3 Pro-opiomelanocortin (POMC)

Pro-opiomelanocortin is a prohormone that is sequentially enzymatically cleaved to produce a number of bioactive neuropeptides. Post-translational processing of POMC is tissue-specific, with hypothalamic POMC importantly producing α-MSH which decreases feeding and increases energy expenditure through its action as the endogenous ligand for the MC3R [125] and MC4R [115, 126], and therefore has major implications in feeding regulation and energy homeostasis. POMC post-translational processing and peptides are shown in Figure 1.2. To generate α-MSH, 32 kilodalton (kDa) POMC is first cleaved by the serine protease, prohormone convertase 1 (PC1), to pro-adrenocorticotropic hormone (ACTH) and β-lipotrophin. Pro-ACTH is further cleaved by PC1 to ACTH, which is then cleaved by prohormone convertase 2 (PC2) to generate ACTH1-17. ACTH1-17 is then further processed by the enzymes carboxypeptidase E (CPE), peptidyl α-amidating mono-oxygenase (PAM), and N-acetyltransferase (N-AT) to produce the mature α-MSH peptide (reviewed in [127]).

1.3.4 Regulation of POMC

1.3.4.1 Hormonal and nutrient regulation of POMC

POMC is increased in energy excess and decreased in energy deficit and it was found that fasting decreased Pomc mRNA levels by 20-50% [128]. The adipostatic hormone leptin, through the leptin receptor, activates POMC neurons as determined by c-Fos expression as a marker of neuronal activation [121, 129]. Leptin binding the leptin receptor activates the JAK/STAT pathway and increases translocation of the transcription factor STAT3 into the nucleus to increase Pomc transcription [130, 131]. These studies identify leptin as a key hormonal regulator of Pomc mRNA expression.
Figure 1.2 Pro-opiomelanocortin (POMC) post-translational processing. PC1 cleaves POMC to pro-ACTH and β-lipotrophin. Pro-ACTH is further cleaved by PC1 to ACTH, which is then cleaved by PC2 to generate ACTH1-17. ACTH1-17 is then further processed by the enzymes carboxypeptidase E (CPE), peptidyl α-amidating mono-oxygenase (PAM), and n-acetyltransferase (N-AT) to produce the mature α-MSH peptide. The melanocortin peptides are coloured in grey.
In addition to leptin, insulin administration increases Pomc mRNA in the hypothalamus [132]. Activation of the insulin receptor (IR) and downstream PI3K/Akt signalling is necessary for this anorexigenic effect of insulin [133]. Insulin activation of the PI3K/Akt pathway through the IR increases Akt-induced phospho-Foxo1, removing Foxo1 repression of Pomc transcription [134-136].

Similarly to hormonal cues of energy status, nutrients themselves can alter feeding signals in the hypothalamus. For example, glucose increases Pomc mRNA expression [137], as well as increasing POMC neuron activation [135, 138].

1.3.4.2 High-fat diet and palmitate regulation of POMC

High-fat diet fed animals have increased Pomc mRNA expression [139, 140]. Specifically looking at increased levels of FFA with high-fat diet, β-oxidation of long chain fatty acids through CPT1 activates POMC neurons [141], presenting a possible mechanism for FA-induced changes in POMC. Central administration of palmitate metabolites increases Pomc expression [22, 101]. However, sustained nutrient excess from prolonged high-fat diet decreases Pomc and α-MSH-inhibited feeding resulting in increased food intake and increased body weight [140, 142, 143].

1.4 Cell model for investigation

1.4.1 Experimental model rationale

The ARC of the hypothalamus contains opposing orexigenic NPY/AgRP neurons and anorexigenic POMC neurons that regulate food intake and energy expenditure by integrating signals of energy status from hormones and nutrients, including FFAs [5, 6]. Consumption of a
high-fat diet or exposure to palmitate treatment directly alters hypothalamic neuropeptide expression. The effects of palmitate are well documented in the hypothalamus and in hypothalamic NPY-expressing neurons, however, the direct effects of palmitate on POMC-expressing neurons has not been investigated. Further, the response to high-fat feeding differs among populations of hypothalamic neurons. For instance, it is reported that POMC neurons are selectively targeted for apoptosis compared to NPY neurons [55] suggesting that distinct neuronal populations differ in their sensitivity and response to FFAs.

Due to the architectural complexity and heterogeneous nature of the hypothalamus, it is necessary to employ the use of cell lines to investigate specific mechanisms involved in nutrient sensing. Therefore, to investigate the effects of palmitate directly on POMC neurons immortalized, non-clonal POMC-expressing mHypoA-POMC/GFP-1-4 cell lines were used in this study.

1.4.2 Generation of cell lines

The mHypoA-POMC/GFP-1, -2, -3, and -4 cell lines were previously generated and characterized by our laboratory [144, 145]. In brief, hypothalamic tissue was extracted from 8-week-old male transgenic mice with green fluorescence protein (GFP) under the control of the promoter for the Pomc gene. The hypothalamic tissue was dispersed into culture and treated with ciliary neurotrophic factor (CNTF) to induce neuroproliferation prior to immortalization by the retroviral transfer of simian virus 40 (SV40) T-antigen. Neurons were then selected for geneticin resistance and fluorescence-activated cell (FAC)-sorted to produce a non-clonal, mixed population of POMC-expressing neurons [144].
1.4.3 mHypoA-POMC/GFP-1-4

The mHypoA-POMC/GFP-1-4 cell lines were previously characterized and shown to express Pomc, processing enzymes, as well as a host of inflammatory receptors, mediators, and cytokines [145]. Functional validation of the mHypoA-POMC/GFP-1 cell line found that there was an increase in α-MSH secretion when stimulated with CNTF, insulin, or potassium chloride (KCl) [145]. Further investigations showed that insulin treatment increases Pomc mRNA expression in the mHypoA-POMC/GFP-1 cells and induces insulin resistance in all four mHypoA-POMC/GFP cell lines [145]. In mHypoA-POMC/GFP-2 cells, sodium nitroprusside (SNP) increases Pomc mRNA expression and α-MSH protein through decreased sirtuin 1 activity and increased Foxo1 phosphorylation [146]. These studies show that the mHypoA-POMC/GFP cell lines are valid models to investigate the molecular mechanisms of palmitate directly on POMC neurons.

1.5 Research hypothesis and aims

Accumulation of palmitate and its metabolites in the hypothalamus results in inflammation and ER stress, leading to a decrease in insulin signalling, weight gain, and metabolic dysfunction [3, 4]. Consumption of a high-fat diet or exposure to palmitate treatment directly alters hypothalamic neuropeptide expression. Exposure of cultured NPY neurons to palmitate increases Npy mRNA expression and this effect is blocked with pre-treatment with anti-inflammatory sodium salicylate [147] or the IKKβ inhibitor PS-1145 [148], implicating FFA-induced inflammation as a mediator for altered Npy mRNA expression. In the whole hypothalamus, early stages of high-fat diet-induced obesity are accompanied by an increase in Pomc mRNA expression [140], whereas Pomc levels are decreased with prolonged diet [143]. However, the direct effects of palmitate on POMC-expressing neurons have not been
investigated. Therefore, the **purpose** of this thesis project is to elucidate the mechanisms of palmitate action in the POMC-expressing cell models, mHypoA-POMC/GFP-1, -2, -3, and -4. It is **hypothesized** that palmitate will induce cellular neuroinflammation and ER stress in mHypoA-POMC/GFP neurons, increasing *Pomc* expression. This project was divided into two specific aims to investigate the hypothesis.

As palmitate has been shown to alter *Pomc* mRNA expression and induce inflammation, ER stress, and insulin resistance *in vivo*, therefore the first aim of this project was to characterize the direct effect of palmitate in POMC neurons. To address this question the mRNA levels of *Pomc*, and cellular markers of neuroinflammation and ER stress were measured in mHypoA-POMC/GFP-1-4 cells following palmitate treatment. As well, this aim investigated the effect of palmitate exposure on insulin signaling in mHypoA-POMC/GFP cells.

It is proposed that the lipotoxic effects of palmitate are mediated through IKKβ/NF-κB and MAPK signaling and through the actions of the palmitate metabolite ceramide. Therefore, aim 2 of this project investigates the role of these signaling pathways in mediating inflammation, ER stress, and *Pomc* mRNA expression. This was done by using specific inhibitors for TLR4, IKKβ, and the MAPKs JNK, p38, or ERK and measuring *Pomc* mRNA levels following palmitate treatment. Additionally, *Pomc* mRNA expression was measured following treatment with a palmitate metabolite, a structural analog, and following inhibition of palmitate metabolism through the sphingolipid pathway to determine the role of palmitate metabolism on *Pomc* mRNA expression.
Chapter 2

Materials and Methods

2 Materials and Methods

2.1 Cell culture and reagents

mHypoA-POMC/GFP neurons were cultured in DMEM (Sigma-Aldrich; St. Louis, MO, USA) containing 5.5 mM glucose and supplemented with 2% fetal bovine serum (Gibco; Grand Island, NY, USA) and 1% penicillin-streptomycin (Gibco). Sodium palmitate, sodium oleate, methylpalmitate, C16-ceramide, myriocin, and L-cycloserine were purchased from Sigma. TAK-242 was purchased from Calbiochem (San Diego, CA, USA). PS-1145, SP 600125, SB 202190, and PD 0325901 were purchased from Tocris Bioscience (Ellisville, MO, USA). Insulin was gifted by Novo Nordisk (Mississauga, ON, CAN). Sodium palmitate, methylpalmitate, and sodium oleate were dissolved in molecular grade water (Thermo Scientific; Nepean, ON, CAN) and heated to 70°C to dissolve, as described previously [147]. L-cycloserine was dissolved in sterile water (Thermo Scientific). C16-ceramide was dissolved in ethanol with 2% dodecane (Sigma). TAK-242, PS-1145, SP 600125, SB 202190, PD 0325901, and myriocin were dissolved in dimethyl sulfoxide (DMSO); final treatment containing 0.1% DMSO.

2.2 MTT assay

Cell viability after palmitate treatment was assessed by MTT 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide assay (Vybrant® MTT Cell Proliferation Assay Kit V-13154; Life Technologies; Eugene, OR, USA). Briefly, cells were grown on 96-well plates and treated with
10 µM, 50 µM, or 100 µM palmitate. After 24 hr, treatment was removed and cells were incubated in 1.1 mM MTT for 2 hr. Formazan was then solubilized by DMSO and concentration determined by optical density at 570 nm.

### 2.3 Quantitative RT-PCR

Total RNA was isolated using the PureLink RNA Mini Kit with on-column PureLink DNase (Ambion, Streetsville, ON, CAN). cDNA was synthesized with 1000 ng RNA using the SuperScript VILO cDNA synthesis kit (Invitrogen, Carlsbad, CA, USA). 12.5 ng cDNA was amplified using qRT-PCR master mix (Platinum SYBR Green qPCR SuperMix-UDG with ROX; Invitrogen) with gene specific primers (Table 2.1) on an Applied Biosystem Prism 7000 machine. qRT-PCR quantities were determined by a standard curve and normalized to the reference gene, histone 3a.

### 2.4 Western blotting

Total protein was harvested using 1X lysis buffer (Cell Signalling; Danvers, MA, USA) with 1 mM PMSF, 1% phosphatase inhibitor, and 1% protease inhibitor and supernatant isolated after centrifugation at 14000 rpm for 10 min at 4°C. Protein concentration was determined using the BCA protein assay kit (Thermo Scientific). 20-25 µg protein was run on a 10% SDS-PAGE gel and transferred to a PVDF membrane (Biorad). Membranes were blocked for 1 hr in 5% milk in tris-buffered saline with tween (TBS-T), then incubated in primary antibody (1:1000) at 4°C overnight. Primary antibodies were purchased from Cell Signalling, and include phospho-Akt (Ser473), Akt, phospho-SAPK/JNK (Thr183/Tyr185), and SAPK/JNK. Membranes were washed in TBS-T and incubated in secondary anti-rabbit antibody (1:7500, Cell Signalling) for 1 hr, then imaged using the Signal Fire ECL Reagent (Cell Signalling) and the Kodak Image Station
Densitometry was performed using ImageJ software (National Institute of Mental Health; Bethesda, MD, USA).

### 2.5 Statistical analysis

Data were analyzed using GraphPad Prism 6 (GraphPad Software, San Diego, CA, USA) and presented as mean ± SEM. One- or two-way ANOVA were used with Bonferroni post hoc test when appropriate. Unpaired t-test was used to analyze single factor experiments. Statistical significance is *$P < 0.05$, **$P < 0.01$, ***$P < 0.001$, ****$P < 0.0001$. 
### Table 2.1 List of primers for qRT-PCR

<table>
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<th>Gene name</th>
<th>Primer sequence (5’ → 3’)</th>
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<tr>
<td><strong>BAX</strong></td>
<td>F: GCT GAC ATG TTT GCT GAT GG</td>
</tr>
<tr>
<td></td>
<td>R: GAT CAG CTC GGG CAC TTT A</td>
</tr>
<tr>
<td><strong>BCL2</strong></td>
<td>F: GGA GGA TTG TGG CCT TCT TT</td>
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<tr>
<td></td>
<td>R: GTT CAG GTA CTC AGT CAT CCA C</td>
</tr>
<tr>
<td><strong>CHOP</strong></td>
<td>F: TAT GAG GAT CTG CAG GAG</td>
</tr>
<tr>
<td></td>
<td>R: CAG GGT CAA GAG TAG TGA AG</td>
</tr>
<tr>
<td><strong>GRP78</strong></td>
<td>F: GCG ACA AGC AAC CAA AGA TG</td>
</tr>
<tr>
<td></td>
<td>R: TTC TTC TCT CCC TCT CTC TTA TCC</td>
</tr>
<tr>
<td><strong>Histone 3a</strong></td>
<td>F: CGC TTC CAG AGT GCA GCT ATT</td>
</tr>
<tr>
<td></td>
<td>R: ATC TTC AAA AAG GCC AAC CAG AT</td>
</tr>
<tr>
<td><strong>IκBα</strong></td>
<td>F: TGC CTG GCG AGT GTA GCA GTC TT</td>
</tr>
<tr>
<td></td>
<td>R: CAA AGT CAC CAA GTG CTC CAC GAT</td>
</tr>
<tr>
<td><strong>IL-1β</strong></td>
<td>F: GCT AGT GTG TGA TGT TCC</td>
</tr>
<tr>
<td></td>
<td>R: GTT CTG TCC ATT GAG GTG</td>
</tr>
<tr>
<td><strong>IL-6</strong></td>
<td>F: GTG GCT AAG GAC CAA GAC CA</td>
</tr>
<tr>
<td></td>
<td>R: GGT TTG CCG ACT AGA CCT CA</td>
</tr>
<tr>
<td>Gene</td>
<td>Forward Strand</td>
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<tr>
<td>--------</td>
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</tr>
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<td>PC1/3</td>
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<tr>
<td>TNFα</td>
<td>F: CTC CTG GTA TGA GAT AGC</td>
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Chapter 3

Results

3 Results

3.1 Palmitate increases Il6 and Chop mRNA expression in mHypoA-POMC/GFP-1-4 and Pomc mRNA expression in mHypoA-POMC/GFP-1 and -2 cells

Exposure to the saturated FFA palmitate is shown to significantly alter food intake and body weight and dysregulate feeding-related neuropeptides [54, 148]. However, the effects of palmitate specifically on POMC neurons and the mechanisms mediating these effects have not been described. Therefore, to investigate the specific effects of palmitate in POMC neurons, mHypoA-POMC/GFP-1-4 cells were treated with palmitate. Treatment with 50 µM palmitate for 8 hr significantly increased Pomc mRNA expression in the mHypoA-POMC/GFP-1 and -2 cell lines, but had no effect on Pomc mRNA expression in the mHypoA-POMC/GFP-3 or 4 cell lines (Figure 3.1A). Palmitate induces inflammation and ER stress in the hypothalamus, and this effect is demonstrated in POMC neurons as palmitate increases Il6 (Figure 3.1B) and Chop (Figure 3.1C) mRNA expression in all four mHypoA-POMC/GFP cell lines. As each cell line represents all of the hypothalamic POMC-expressing cells from a mouse, the different results demonstrate the heterogeneity of responses between individuals.
Figure 3.1 Effects of palmitate on Pomc, Il6, and Chop mRNA expression in mHypoA-POMC/GFP-1-4 cells. mHypoA-POMC/GFP-1, -2, -3, and -4 cells were treated with 50 µM palmitate or water control for 8 hr and Pomc (A), Il6 (B), and Chop (C) mRNA expression was assessed by qRT-PCR; n = 4. Data are expressed as mean ± SEM; **P < 0.01, ****P < 0.0001. Statistical analysis was performed by unpaired t-test.
3.2 The effect of palmitate in mHypoA-POMC/GFP-2 cells is independent of changes in cell viability

First, the mHypoA-POMC/GFP-2 cell line was chosen for further investigation of the mechanism of palmitate action in POMC neurons as it had the most robust response to palmitate when looking at Pomc mRNA expression. Please refer to Appendix I for experiments carried out in mHypoA-POMC/GFP-1 cells.

For the purpose of this project, it was important to identify a mechanism for this response while limiting potential confounding factors, which include changes in cell viability. Therefore, an MTT assay was performed to assess cell viability. The changes seen were not attributed to decreased cell viability as determined by an MTT assay, in which 50 µM palmitate over 24 hr did not significantly decrease cell viability compared to control (Figure 3.2).

3.3 Palmitate increases cellular markers of neuroinflammation and ER stress

As previously mentioned, palmitate can alter neuropeptide expression and this dysregulation has been proposed to be a result of neuroinflammation [3, 149]. Analysis of a host of pro-inflammatory markers found that palmitate increased the mRNA levels of pro-inflammatory cytokines Tnfa, and Il1β, the classical inflammatory receptor Tlr4, and the pro-inflammatory transcription factor Nfkβ (Figure 3.3A) in addition to the increase in Il6 mRNA expression (Figure 3.1B). No change was seen in an inhibitor of NF-κB, IκBa, mRNA expression. Palmitate also increased the mRNA expression of the ER stress markers Grp78, and the Bax/Bcl2 ratio (Figure 3.3B) in addition to the increase in Chop mRNA expression (Figure 3.1C), similar to the effect of palmitate on ER stress in the whole brain [150]. Thus, palmitate
Figure 3.2 Effects of palmitate on cell viability in mHypoA-POMC/GFP-2 cells. mHypoA-POMC/GFP-2 cells were treated with 10 µM, 50 µM, or 100 µM palmitate or water control for 24 hr and cell viability was assessed by MTT assay; n = 4. Data are expressed as mean ± SEM; **P < 0.01. Statistical analysis was performed by one-way ANOVA with Bonferroni post hoc test.
concomitantly increases *Pomc*, as well as, cellular neuroinflammation and ER stress markers, in the mHypoA-POMC/GFP-2 cells.

### 3.4 Palmitate exerts an acute effect in mHypoA-POMC/GFP-2 cells

The lipotoxic effects of palmitate in the hypothalamus are a result of prolonged exposure [3]. However, here we investigated the effect of a single palmitate treatment on *Pomc* mRNA expression and cellular stress markers. mHypoA-POMC/GFP-2 cells were treated with 50 µM palmitate for 4, 8, or 24 hr. After 24 hr palmitate exposure, *Il6* (**Figure 3.4B**) and *Chop* (**Figure 3.4C**) mRNA expression returns to near basal levels indicating an acute response to palmitate that is resolved after 24 hr.

### 3.5 Palmitate pre-treatment does not alter insulin signaling

Palmitate-mediated inflammation and ER stress are reported to contribute to cellular insulin resistance. It was previously found that immortalized hypothalamic NPY neurons exposed to palmitate have decreased insulin signalling illustrated by reduced insulin-stimulated phospho-Akt [4, 151]. As palmitate induces both markers of neuroinflammation and ER stress in the mHypoA-POMC/GFP-2 cells, we investigated the effect of palmitate on insulin signalling. mHypoA-POMC/GFP-2 cells were pre-treated with 50 µM palmitate for 24 hr, then challenged with 10 nM insulin for 15 min. Insulin stimulated an increase in phospho-Akt levels as expected, however, the insulin-stimulated Akt phosphorylation was unchanged with palmitate pre-exposure (**Figure 3.5A**), indicating normal insulin signalling, unlike that of NPY neurons that become resistant upon palmitate exposure. Interestingly, normal insulin signalling is maintained upon palmitate exposure in POMC neurons despite increased JNK phosphorylation (**Figure 3.5B**).
Figure 3.3 Palmitate increases cellular markers of neuroinflammation and ER stress in mHypoA-POMC/GFP-2 cells. mHypoA-POMC/GFP-2 cells were treated with 50 µM palmitate or water control for 8 hr and the mRNA expression of markers of cellular neuroinflammation (A), and markers of ER stress (B) were assessed by qRT-PCR; n = 4-5. Data are expressed as mean ± SEM; **P < 0.01, ***P < 0.001, palmitate to water control normalized to one. Statistical analysis was performed by unpaired t-test.
Figure 3.4 In mHypoA-POMC/GFP-2 cells the effects of a single palmitate treatment are acute. mHypoA-POMC/GFP-2 cells were treated with 50 µM palmitate or water control for 4, 8, or 24 hr and *Pomc* (A), *Il6* (B), and *Chop* (C) mRNA expression was assessed by qRT-PCR; n = 4. Data are expressed as mean ± SEM; *P < 0.05, **P < 0.01, ****P < 0.0001. Statistical analysis was performed by two-way ANOVA with Bonferroni post hoc test.
Figure 3.5 Palmitate does not alter insulin signaling in mHypoA-POMC/GFP-2 cells. mHypoA-POMC/GFP-2 cells were pre-treated with 50 µM palmitate or water control for 24 hr, serum starved for 1 hr, then challenged with 10 nM insulin or water control for 15 min. Relative Akt (A) and JNK (B) protein phosphorylation was assessed by western blot; n = 3. Data are expressed as mean ± SEM, water vehicle control normalized to one (A); **P < 0.01, ***P < 0.001. Statistical analysis was performed by two-way ANOVA with Bonferroni post hoc test.
3.6 Palmitate increases Pc1/3 mRNA expression in mHypoA-POMC/GFP-2 cells

To further characterize the effects of palmitate in POMC-expressing neurons, changes in the mRNA expression of the pro-hormone cleavage enzyme, PC1/3 was determined. PC1/3 is the first step in the post-translational cleavage of POMC to the mature α-MSH peptide. In the mHypoA-POMC/GFP-2 cells there was a significant increase in Pc1/3 mRNA expression after 8 hr 50 µM palmitate treatment (Figure 3.6). Considering palmitate increases the mRNA expression of both Pomc and Pc1/3, it suggests that palmitate would increase α-MSH levels.

3.7 TLR4 and IKKβ do not mediate the palmitate-induced increased in Pomc, while they do in part mediate the palmitate-induced increase in neuroinflammation

Palmitate can activate TLR4 in the hypothalamus to induce inflammation [52], but whether this can occur in POMC neurons is not known. Therefore, mHypoA-POMC/GFP-2 cells were pre-treated with the small molecule inhibitor for TLR4, TAK-242 (10 µM), for 1 hr, then treated with 50 µM palmitate for 8 hr. The palmitate-mediated increase in Pomc mRNA expression was unchanged with TAK-242 pre-treatment (Figure 3.7A). Inhibition of TLR4 attenuated the palmitate-mediated increase in Il6 mRNA expression (Figure 3.7B) and enhanced the Chop induction (Figure 3.7C). These data suggest that signalling through TLR4, at least in part, mediates the pro-inflammatory effects of palmitate, but not the increase in Pomc mRNA expression. In addition, the role of IKKβ in mediating the effects of palmitate was assessed, as inhibition of IKKβ in NPY neurons blocks the palmitate-mediated increase in Npy mRNA expression [148]. mHypoA-POMC/GFP-2 cells were pre-treated with PS-1145 (10 µM) for 1 hr, then treated with 50 µM palmitate for 8 hr. Pre-treatment with PS-1145 decreased palmitate-
Figure 3.6 Palmitate increases *Pc1/3* mRNA expression in mHypoA-POMC/GFP-2 cells. mHypoA-POMC/GFP-2 cells were treated with 50 µM palmitate or water control for 8 hr and *Pc1/3* mRNA expression was assessed by qRT-PCR; n = 5. Data are expressed as mean ± SEM; **P < 0.01. Statistical analysis was performed by unpaired t-test.
mediated *Il6* mRNA expression (*Figure 3.7E*), but no effect on *Pomc* or *Chop* mRNA expression (*Figure 3.7D and F*).

### 3.8 The MAP kinases JNK and ERK mediate the palmitate-induced increase in *Pomc* mRNA expression

MAPK signalling has been shown to play a role in palmitate-induced inflammation, ER stress, insulin resistance, and apoptosis [151-154]. To determine if the palmitate-mediated increase in *Pomc* mRNA expression is dependent on MAPK signalling, mHypoA-POMC/GFP-2 cells were pre-treated with the inhibitors for JNK (SP 600125), p38 MAPK (SB 202190), or MEK1/2 (ERK1/2; PD 0325901) for 1 hr, then treated with 50 µM palmitate for 8 hr. Pre-treatment with 50 µM SP 600125 or 10 µM PD 0325901 attenuated the palmitate-mediated increase in *Pomc* mRNA expression (*Figure 3.8A*), while 10 µM SB 202190 had no effect on palmitate-induced *Pomc* mRNA expression (*Figure 3.8A*). At the same time, SP 600125 and SB 202190 enhanced palmitate-induced *Il6* mRNA expression (*Figure 3.8B*).

Inhibition of JNK, p38 MAPK, or ERK1/2 each attenuated the palmitate-mediated increase in *Chop* mRNA expression (*Figure 3.8C*). Therefore, JNK and ERK signalling may be required for palmitate to increase *Pomc* mRNA expression, and this effect is independent of palmitate-mediated neuroinflammation or ER stress.

### 3.9 The palmitate-induced increase in *Pomc* mRNA expression is dependent on palmitoyl-coA synthesis

The palmitate-mediated increase in *Pomc* mRNA expression is not TLR4 dependent, thus may alternatively require the intracellular metabolism of palmitate. To determine if a palmitate metabolite is required to increase *Pomc* mRNA expression, mHypoA-POMC/GFP-2 cells were
Figure 3.7 Neither TLR4 nor IKKβ mediate the palmitate-mediated increase in Pomc mRNA expression. mHypoA-POMC/GFP-2 cells were pre-treated with 10 µM TAK-242 (TLR4 inhibitor; A-C), 20 µM PS-1145 (D-F) or DMSO control for 1 hr, then treated with 50 µM palmitate or water control for 8 hr. The mRNA expression of Pomc (A and D), Il6 (B and E), and Chop (C and F) were assessed by qRT-PCR; n = 3 - 4. Data are expressed as mean ± SEM, water vehicle control normalized to one; *P < 0.05, **P < 0.01, ****P < 0.0001. Statistical analysis was performed by two-way ANOVA with Bonferroni post hoc test.
The palmitate-mediated increase in *Pomc* mRNA expression is dependent on JNK and ERK signalling. mHypoA-POMC/GFP-2 cells were pre-treated with 50 µM SP 600125 (JNK inhibitor), 10 µM SB 202190 (p38 MAPK inhibitor), 10 µM PD 0325901 [MEK1/2 (ERK1/2) inhibitor], or DMSO control for 1 hr, then treated with 50 µM palmitate or water control for 8 hr. The mRNA expression of *Pomc* (A), *Il6* (B), and *Chop* (C) were assessed by qRT-PCR; n = 5. Data are expressed as mean ± SEM, water vehicle control normalized to one; *P < 0.05, **P < 0.01, ***P < 0.001, ****P < 0.0001. Statistical analysis was performed by two-way ANOVA (unique analysis for each inhibitor) with Bonferroni post hoc test.
treated with palmitate or non-metabolizable methylpalmitate for 8 hr. 50 µM methylpalmitate did not alter Pomc mRNA expression (Figure 3.9A). Thus, the esterification of palmitate to palmitoyl-coA appears to be required for palmitate to increase Pomc mRNA expression.

3.10 Ceramide increases Pomc mRNA expression, but de novo ceramide synthesis is not required for palmitate to increase Pomc mRNA expression

Ceramide, a palmitate metabolite, has been implicated in fatty acid-induced inflammation and insulin resistance [93, 155]. Through a series of enzymatic reactions, palmitoyl-coA can result in an increase in intracellular ceramide levels [156], but the effect of ceramide production on Pomc mRNA expression is unknown. Treatment of mHypoA-POMC/GFP-2 cells with 1 µM C16-ceramide for 8 hr increased Pomc mRNA expression (Figure 3.9B), illustrating the potential of exogenous ceramide to increase Pomc mRNA levels.

While we show that ceramide itself can increase Pomc mRNA expression, whether de novo ceramide synthesis is required for the palmitate-mediated increase in Pomc mRNA expression is not known. Therefore, mHypoA-POMC/GFP-2 cells were pre-treated separately with two inhibitors of serine palmitoyltransferase (SPT), the rate-limiting step in de novo ceramide synthesis. Pre-treatment with 100 µM myriocin or 50 µM L-cycloserine for 1 hr, followed by treatment with 50 µM palmitate for 8 hr enhanced the palmitate-mediated increase in Pomc mRNA expression, compared to the no inhibitor control (Figure 3.9Ci, ii). These data suggest that while ceramide can increase Pomc mRNA expression, de novo ceramide synthesis is not required for palmitate to increase Pomc mRNA expression, as inhibiting this process further enhanced the increase in Pomc.
Figure 3.9 The palmitate-mediated increase in *Pomc* mRNA expression is dependent on palmitoyl-coA synthesis, but not de novo ceramide synthesis. mHypoA-POMC/GFP-2 cells were treated with 50 µM palmitate, 50 µM methylpalmitate, or water control (A) or 1 µM or 10 µM C16-ceramide or ethanol/dodecane control (B) for 8 hr. mHypoA-POMC/GFP-2 cells were pre-treated with the SPT inhibitors myriocin (100 µM) or DMSO control (Ci) or L-cycloserine (50 µM) or water control (Cii) for 1 hr, then treated with 50 µM palmitate or water control for 8 hr. *Pomc* mRNA expression was assessed by qRT-PCR; n = 3 - 6. Data are expressed as mean ± SEM, water vehicle control normalized to one (Ci, ii); *P < 0.05, **P < 0.01, ***P < 0.001, ****P < 0.0001. Statistical analysis was performed by one-way ANOVA with Bonferroni post hoc test (A, B) or two-way ANOVA with Bonferroni post hoc test (Ci, ii).
3.11 Oleate blocks the effects of palmitate in POMC neurons

The monounsaturated FFA oleate has been reported to be protective against palmitate-mediated inflammation and insulin resistance in Neuro-2a and primary rat cortical neurons [89], but whether oleate has any effects against palmitate in POMC neurons is unknown. To determine the effect of oleate on palmitate-mediated changes in POMC neurons, mHypoA-POMC/GFP-2 cells were treated with 50 µM palmitate, 50 µM oleate, or co-treated with 50 µM palmitate and 50 µM oleate for 8 hr. The increase in Pomc mRNA expression by palmitate was blocked with oleate co-treatment (Figure 3.10A). Remarkably, oleate co-treatment also abolished the increase in Il6 (Figure 3.10B) and Chop (Figure 3.10C) mRNA expression seen with palmitate exposure. Thus, oleate is anti-inflammatory and neuroprotective, while also blocking the effects of palmitate on Pomc mRNA expression in mHypoA-POMC/GFP-2 neurons.
Figure 3.10 Oleate blocks the effects of palmitate on the mRNA expression of Pomc and markers of cellular neuroinflammation and ER stress. mHypoA-POMC/GFP-2 cells were treated with 50 μM palmitate, 50 μM oleate, 50 μM palmitate + 50 μM oleate, or water control for 8 hr. The mRNA expression of Pomc (A), Il6 (B), and Chop (C) were assessed by qRT-PCR; n = 4 - 5. Data are expressed as mean ± SEM; *P < 0.05, ****P < 0.0001. Statistical analysis was performed by one-way ANOVA with Bonferroni post hoc test.
Chapter 4
Discussion

4  Discussion

4.1 Previous reports of high-fat and palmitate on POMC

Consumption of high levels of dietary fat increases palmitate levels in the whole brain [34] and in
the hypothalamus [3], causing metabolic dysregulation of feeding-related neuropeptides.
Specifically, in the whole hypothalamus, early stages of high-fat diet-induced obesity are
accompanied by an increase in Pmc mRNA expression [139, 140, 157], whereas Pmc levels
are decreased with prolonged diet [143, 157, 158]. Despite data supporting the role of high-fat
and palmitate on altered Pmc levels, the direct effect of palmitate on POMC-expressing neurons
has not been previously investigated. Here, we provide evidence that POMC neurons respond
directly to an acute exposure to palmitate resulting in increased Pmc mRNA expression.

4.2 Regulation of mHypoA-POMC/GFP-2 cells by palmitate
(summarized in Figure 4.1)

4.2.1 Palmitate increases Pmc mRNA expression through metabolism to
palmitoyl-coA

The increase in Pmc mRNA expression by palmitate is dependent on intracellular metabolism
to palmitoyl-coA, as non-metabolizable methylpalmitate did not affect Pmc mRNA expression.
Upon esterification to palmitoyl-coA there are several pathways upon which the molecule can
act. Palmitoyl-coA can act as a precursor molecule for the sphingolipid pathway, a common
Figure 4.1 Summary of the proposed mechanism of action of palmitate in mHypoA-POMC/GFP-2 cells. Palmitate is converted to palmitoyl-coA within the cell and increases Pomc mRNA expression through JNK- and ERK-dependent signalling pathways. Inhibition of the de novo ceramide synthesis through SPT enhances Pomc mRNA expression. Concomitantly, palmitate increases cellular neuroinflammation through mechanisms mediated, in part, through TLR4/IKKβ/NF-κB signalling. Additionally, oleate blocks the effects of palmitate on neuroinflammation and Pomc through yet an unknown mechanism(s).
outcome of which is increased ceramide synthesis [93, 159]. It is proposed that palmitate incurs many of its deleterious effects, including inducing insulin resistance and apoptosis through an increase in ceramide species [22, 28, 93, 95, 153, 160-163]. In mHypoA-POMC/GFP-2 cells, treatment with C16-ceramide increased Pomc mRNA expression. However, inhibition of SPT, and consequently de novo ceramide synthesis by myriocin or L-cycloserine further enhanced the palmitate-induced increase in Pomc mRNA expression, suggesting that de novo ceramide synthesis is not the main mechanism for the palmitate-induced increase in Pomc mRNA expression. De novo ceramide synthesis from palmitate is only one mechanism for ceramide generation, as increased ceramide can also occur through the salvage pathway or through the hydrolysis of sphingomyelin [100, 155]. As ceramide was found to increase Pomc mRNA expression, yet the inhibition of its de novo synthesis from palmitate enhanced this effect, it is likely that palmitate does not act to increase Pomc mRNA expression through metabolism to ceramide, but potentially through one of the other mechanisms for ceramide synthesis. Further support for the idea that ceramide does not mediate the palmitate-induced increase in Pomc mRNA expression is that treatment of C16-ceramide had no effect on Pomc mRNA expression in the mHypoA-POMC/GFP-1 cell line as is seen by C16-ceramide in the mHypoA-POMC/GFP-2 cells, or by palmitate in the mHypoA-POMC/GFP-1 and -2 cells.

Alternative to action through the sphingolipid pathway, the increased palmitoyl-coA pool may instead be directed through carnitine palmitoyltransferase 1 (CPT1), an enzyme that integrates nutritional and hormonal cues for feeding regulation [164]. Shuttling of palmitate through CPT1 for β-oxidation, increases lipid metabolism as an energy source, and may act as a signal to increase Pomc mRNA expression. This idea is in line with reports showing that activation of CPT1 has hypophagic effects in hypothalamic neurons [165, 166]. Further, this group found that increasing palmitate metabolism through CPT1 increases reactive oxygen species (ROS) [166]
and generation of ROS increase POMC neuron activation and decreases feeding [167]. It could be that in mHypoA-POMC/GFP neurons, an increase in ROS increases Pomc mRNA expression through a JNK-dependent mechanism, as ROS were previously shown to increase JNK activation in hypothalamic neurons [168]. Additionally, the palmitoyl-coA pool may be directed to increase lipid metabolites including DAG and TAG, which themselves may affect Pomc mRNA expression [169].

To determine specific palmitate metabolites that can affect Pomc mRNA expression we can inhibit β-oxidation with etomoxir, an inhibitor of CPT1 [170], which is shown to alter food intake when administered directly into the hypothalamus [171, 172]. Alternatively, a specific DAG kinase inhibitor can be used to determine a role in FFA regulation of Pomc mRNA expression. DAG kinase is important in the regulation of DAG and TAG levels, and the activity of this kinase is altered by high-fat diet [173]. Hypothalamic DAG kinase levels are increased with high-fat exposure; however, levels are decreased in obese states [173].

4.2.2 The palmitate-induced increase in Pomc mRNA expression is JNK- and ERK-dependent

We demonstrate that activation of the MAPKs JNK and ERK may be required for palmitate to increase Pomc mRNA expression, as this effect is blocked by specific inhibition of each kinase by SP 600125 or PD 0325901 respectively. Palmitate has previously been shown to act via these signalling pathways in the hypothalamus and other metabolic tissues to induce inflammation, ER stress, and insulin resistance [151, 152, 174]. Furthermore, activation of ERK1/2 can regulate Pomc mRNA expression in pituitary corticophrophs [175] and alter glucose-stimulated Pomc mRNA expression in primary hypothalamic neuronal culture [176]. However, this is the first time these pathways have been implicated in the regulation of Pomc by FFAs. Activation of
both JNK and ERK increase the activity of the Sp1 transcription factor [reviewed in [177]]. This represents a potential mechanism for palmitate-mediated activation of MAP kinase to increase Pome, as Sp1 is required for leptin-induced Pome expression [178] and may also be required for FFA induction of Pome. Furthermore, hypermethylation of the Sp1 binding site induced by sustained high-fat diet [179] provides a mechanism of Pome dysregulation in high-fat diet-induced obesity. JNK and ERK are demonstrated to be intracellular targets of ceramide [92, 153, 180-182], and therefore may also act as targets for other lipid metabolites in the regulation of Pome mRNA expression, in addition to palmitate activation via TLR4 [50] and G protein-coupled receptors (GPCR) [47].

4.2.3 Palmitate increases cellular markers of neuroinflammation and ER stress, independent of Pome

Palmitate increased Pome mRNA expression in mHypoA-POMC/GFP-1 and -2 cells and was unchanged in mHypoA-POMC/GFP-3 and -4 cells, while all four cell lines had a significant increase in Il6 and Chop mRNA expression, as cellular markers of neuroinflammation and ER stress respectively.

Palmitate activation of TLR4 and downstream pro-inflammatory signalling through IKKβ/NF-κB is well documented in the hypothalamus [52-54] and confirmed in mHypoA-POMC/GFP-2 cells. A recent report demonstrated that inhibition of IKKβ/NF-κB attenuates changes in Npy mRNA expression by palmitate in an immortalized, hypothalamic NPY-expressing cell line [148]. Additionally, pre-treatment with the commonly prescribed anti-inflammatory sodium salicylate blocks the effects of palmitate on Npy mRNA expression [147]. However, we show that inhibition of TLR4 or IKKβ, by TAK-242 or PS-1145 respectively, attenuates palmitate-mediated Il6, but not the induction of Pome mRNA expression in mHypoA-POMC/GFP-2 cells.
These data provide evidence for a different mechanism for palmitate-mediated changes in Npy compared to Pome mRNA expression, where changes in Pome mRNA expression appear to be independent of IKKβ/NF-κB-induced neuroinflammation. Further support for palmitate-induced increase in Pome mRNA expression independent of neuroinflammation is that all four mHypoA-POMC/GFP cell lines have a robust increase in Il6 mRNA expression, while Pome mRNA expression is only increased in mHypoA-POMC/GFP-1 and -2. This data is in line with a recent report showing that changes in Pome occur before hypothalamic inflammation in animals on a high-fat diet [183].

The increase in Pome mRNA expression in the mHypoA-POMC/GFP-1 and -2 cell, but not the mHypoA-POMC/GFP-3 and -4 cells represents the heterogeneity of POMC neurons. It is known that Pome mRNA expression is significantly elevated in some rodent models resistant to high-fat diet-induced obesity, while Pome mRNA expression is not significantly altered in the diet-induced obese counterparts [184, 185]. Moreover, each cell line may express different levels of intracellular machinery.

While activation of JNK can lead to inflammation [186], it is likely not the downstream pro-inflammatory effect of JNK activation that leads to changes in Pome mRNA expression, since inhibiting JNK in the mHypoA-POMC/GFP-2 cells blocks the palmitate-mediated increase of Pome, while enhancing palmitate-mediated Il6 expression. Additional support for the inflammation-independent role of the MAP kinases in Pome expression is that while inhibition of JNK and p38 MAPK enhance palmitate-mediated Il6 mRNA expression, only JNK inhibition alters Pome mRNA expression, and inhibiting ERK attenuates both Pome and Il6 mRNA expression. Together, this suggests that palmitate acts through the MAPKs to affect Pome, and in part to regulate palmitate-mediated neuroinflammation, but that these downstream effects
occur independently. Palmitate-mediated *Il6* mRNA expression was enhanced by inhibiting JNK or p38 MAPK. These two MAPKs, along with the IKKβ/NF-κB pathway, are downstream of TAK1; thus, an enhancement of *Il6* may be a result of a compensatory increase in IKKβ/NF-κB activation as a JNK/IKKβ/NF-κB activity balance is documented in other cell types [187]. In addition to mediating cellular neuroinflammation, inhibition of each of the MAPKs: JNK, p38 MAPK, and ERK, each attenuate palmitate-induced *Chop* mRNA expression in POMC neurons. The increase in *Pomc* by palmitate remains unchanged with inhibition of p38 MAPK indicating independent mechanisms downstream of MAPK signalling to affect *Pomc* and ER stress.

**4.2.4 Palmitate on insulin signaling**

Palmitate robustly induced markers of cellular neuroinflammation and ER stress in mHypoA-POMC/GFP-2 cells, and palmitate-mediated inflammation and ER stress are reported to be key mechanisms that promote cellular insulin resistance [52, 155, 188], as determined by insulin-stimulated Akt phosphorylation. Therefore, it was unexpected that insulin signalling in mHypoA-POMC/GFP-2 cells was unaltered by palmitate pre-treatment, as it has been reported that 24 hr palmitate exposure in hypothalamic NPY neurons in culture is sufficient to induce cellular insulin resistance [89, 151]. Sustained cellular stress may be necessary to cause dysregulation of insulin signalling in POMC neurons; however, while the mRNA expression of *Il6* and *Chop* is attenuated after 24 hr, phospho-JNK protein levels are elevated. It may be that activated JNK and a subsequent block of insulin signalling by serine phosphorylation of IRS-1 may not be an outcome of FFA exposure in POMC neurons, as it is in the whole hypothalamus [154, 189]. This finding further exemplifies the heterogeneity in the response of specific, defined hypothalamic neurons to nutrients and hormones, as demonstrated in the whole hypothalamus versus clonal NPY and POMC neurons.
4.3 FFA interactions on *Pomc* mRNA expression

When treated alone, palmitate increased *Pomc* mRNA expression, while oleate had no effect on *Pomc* mRNA expression. However, co-treatment of oleate with palmitate completely abolished the increase in *Pomc* mRNA expression by palmitate, as well as the increases in *Il6* and *Chop* mRNA expression. The shuttling of palmitoyl-coA for β-oxidation proposes an intersecting site for oleate action in palmitate-affected POMC neurons. As another lipid and nutrient signal, the presence of oleate may alter palmitate metabolism, contributing to the effect of oleate in blocking palmitate-mediated changes in *Pomc* mRNA expression. Oleate was shown to alter the ratio of palmitate metabolites by increasing the production of TAG while decreasing the production of DAG in kidney [190] and neuronal cells [89], so altering palmitate metabolism may be a mechanism of oleate inhibitory action to palmitate in POMC neurons. Additionally, since we illustrate that oleate is able to block the effects of palmitate on *Pomc, Il6, and Chop* mRNA expression, it may act upstream of the MAPKs. Oleate may have an inhibitory action on TAK1, analogous to the mechanism for the anti-inflammatory effects of DHA in hypothalamic neurons [191]. Additionally, it is suggested that oleate is protective against the deleterious effects of palmitate through activation of protein kinase A (PKA), as inhibiting PKA prevented oleate from blocking the effects of palmitate treatment in neurons [89] and skeletal muscle cells [88]. As PKA activation is shown to inhibit the JNK, ERK, and IκB signaling pathways [192-194] it may be that oleate is blocking the effects of palmitate on *Pomc, Il6, and Chop* in POMC neurons through activation of PKA. However, the mechanisms behind the combinatorial effects of FFAs have not been fully investigated in POMC neurons and provide an avenue for future investigation.
4.4 Limitations

This project specifically examined the effects of the saturated FFA palmitate on Pomp mRNA expression in immortalized hypothalamic POMC-expressing neurons in vitro. This approach allowed us to elucidate the precise mechanisms of palmitate action in POMC neurons. However, in vivo POMC neurons are simultaneously stimulated by many factors including insulin [128, 134], leptin [128, 131], other circulating hormones and nutrients [137], as well as afferent inhibitory innervation from AgRP neurons [128] and ultimately it is the physiological balance of these inputs that regulates food intake and energy expenditure through POMC neurons. Additionally, palmitate is bound to albumin in the in vivo circulatory system, however, as in vitro cell culture is an aqueous system albumin was not used. As well, albumin alone has effects on cultured cells, including antioxidant and anti-apoptotic effects [195] and alters the effects of fatty acids in cell culture by decreasing the unbound fatty acid pool [195, 196]. While this project demonstrates the importance of identifying the specific mechanism of palmitate action in POMC neurons as a therapeutic target, it is unknown how combination with other stimuli, as is the case in vivo, may affect these mechanisms in the control of energy homeostasis.

This project uses many small molecule inhibitors to antagonize receptors and inhibit specific signaling proteins to identify the mechanism of palmitate action in POMC neurons. However, while inhibition of specific proteins altered palmitate-induced mRNA levels, the effectiveness of inhibitors and percentage of inhibition was not confirmed by quantifying protein activation. To address this limitation, phosphorylation levels of the inhibited proteins can be measured by western blot to confirm decreased activation.
It is reported in the literature that rodents can become obese on a high-fat diet or be resistance to the diet, and that different levels of *Pomc* are a factor contributing to the different phenotypes [184, 185, 197]. In the case of resistance to high-fat diet-induced obesity the animals have higher levels of *Pomc* compared to animals that become obese [184, 185]. While these studies highlight the importance of *Pomc* in overall energy homeostasis and contribution to metabolic dysregulation in the development of obesity, they also show that POMC neurons respond differently between individuals. Even within a study cohort of genetically similar animals on a high-fat diet some animals become obese, while others remain resistant to the diet and are excluded from study [198]. Each POMC neuronal cell line used in this study was generated from a POMC/GFP mouse, but how these specific animals respond to a high-fat diet or palmitate exposure has not been investigated. Identifying the underlying mechanisms for how POMC neurons from diet-induced versus diet-resistant animals respond differently to high-fat exposure would be instrumental to further characterize the direct effects of FAs in POMC neurons and the role in metabolic dysregulation and obesity.

The cell lines used in this project were immortalized with SV40 large T-antigen, which targets and inactivates p53 causing the cell to enter S phase and promoting DNA replication [199]. Inactivation of this transcription factor may also alter the expression of genes independent of the cell cycle. Therefore, the expression profiles of the mHypoA-POMC/GFP cell lines may be altered as a result of immortalization. Generation of the mHypoA-POMC/GFP cell lines ideally immortalized all of the hypothalamic POMC neurons creating a non-clonal population [144]. However, passaging the cells may favour some cell characteristics, specifically rate of proliferation, over others, ultimately changing the overall phenotype with each passage. Cell cultures were therefore maintained under thirty total passages for all experiments to reduce the inherent population selection that may occur.
4.5 Future directions

4.5.1 Determine the effect of prolonged palmitate exposure in POMC neurons

mHypoA-POMC/GFP-2 neurons exposed to a single, 50 µM palmitate treatment show an acute response, as the mRNA expression of cellular markers of neuroinflammation and ER stress return to near basal levels 24 hr after exposure. This parallels an in vivo report showing that rats fed a high-fat diet have increased mRNA levels of inflammatory markers after one day in the hypothalamus, and that the inflammatory response is resolved after one week of a high-fat diet [200]. However, prolonged palmitate exposure, representative of sustained high-fat diet, may differentially regulate Pomc mRNA expression than with acute exposure, as this group found that markers of hypothalamic inflammation returned with prolonged high-fat diet [200], suggesting that the neuroprotective responses to high-fat diet were exhausted. To determine the effect of chronic palmitate exposure on Pomc mRNA expression, mHypoA-POMC/GFP cells can be repeatedly exposed to media containing fresh palmitate every 24 hr and Pomc mRNA levels measured at 72 hr or 96 hr. This is important to determine how the palmitate signalling mechanisms may be altered with prolonged exposure compared to the mechanisms that control Pomc under acute exposure.

4.5.2 Determine whether the increase in Pomc mRNA expression with palmitate is due to increased transcription or increased stability of the transcript

While palmitate increases Pomc mRNA expression, it is unknown whether this increase is due to an increase in transcription of the gene, or whether it is due to an increase in the stability of the transcript. To determine whether the increase is due to stability or transcription, transcriptional
inhibitors such as actinomycin D (ActD) and 5, 6-Dichloro-1-β-D-ribofuranosylbenzimidazole (DRB) can be used. Use of these two different inhibitors may be necessary as they have different mechanisms for inhibiting transcription. ActD inhibits RNA elongation in transcription [201] while DRB targets cyclin dependent kinase [202]. While pre-treatment with transcriptional inhibitors will not determine if palmitate is directly affecting transcription of Pomc, it can determine if palmitate is causing the transcription of some factor that is then increasing Pomc transcription and can rule out mRNA stability as a cause for increase Pomc mRNA expression.

4.5.3 Secretion assay

Pro-opiomelanocortin is the prohormone produced from the Pomc gene. However, enzymatic cleavage of this product to the mature α-MSH peptide is required for the metabolic consequences of decreased food intake and increased energy expenditure [127]. While, Pomc mRNA expression and expression of the enzyme Pc1/3 are increased with palmitate treatment, it is not known if α-MSH secretion is increased. To determine if palmitate increases the secretion of mature α-MSH peptide, an enzyme immunoassay can be performed.

4.5.4 Chromatin immunoprecipitation

If it is determined that increased transcription underlies the palmitate-induced increase in Pomc mRNA expression, then it is important to determine the transcription factor(s) that are responsible for the increase. Chromatin immunoprecipitation (ChIP) can be used to investigate binding of potential transcription factors to areas involved in Pomc transcription and how they are altered with palmitate treatment.
4.6 Conclusions

Overall, these results demonstrate that palmitate can act directly in POMC neurons to increase Pomc mRNA expression as a normal physiological response and expands our understanding of the role of FFAs in the central regulation of energy homeostasis. These findings provide a differential mechanism of action of FFAs in POMC neurons compared to NPY neurons, underscoring the complexity of the hypothalamic neuronal circuitry and the necessity to study individual neurons in order to define potential neuron-specific therapeutic targets.
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Appendix I

mHypoA-POMC/GFP-1
Supplemental Figure S1. Palmitate increases cellular markers of neuroinflammation and ER stress in mHypoA-POMC/GFP-1 cells. mHypoA-POMC/GFP-1 cells were treated with 50 μM palmitate or water control for 8 hr and the mRNA expression of markers of cellular neuroinflammation and markers of ER stress were assessed by qRT-PCR; n = 4. Data are expressed as mean ± SEM; **P < 0.01, ***P < 0.001, ****P < 0.0001, palmitate to water control. Statistical analysis was performed by unpaired t-test.
Supplemental Figure S4.2. In mHypoA-POMC/GFP-1 cells the effects of a single palmitate treatment are acute. mHypoA-POMC/GFP-1 cells were treated with 50 µM palmitate or water control for 4, 8, or 24 hr and Pomc (A), Il6 (B), and Chop (C) mRNA expression was assessed by qRT-PCR; n = 3 - 4. Data are expressed as mean ± SEM; **P < 0.01, ***P < 0.001, ****P < 0.0001. Statistical analysis was performed by two-way ANOVA with Bonferroni post hoc test.
Supplemental Figure S4.3. Palmitate does not alter insulin signaling in mHypoA-POMC/GFP-1 cells. mHypoA-POMC/GFP-1 cells were pre-treated with 50 µM palmitate or water control for 24 hr, serum starved for 1 hr, then challenged with 10 nM insulin or water control for 15 min. Relative Akt (A) and JNK (B) protein phosphorylation was assessed by western blot; n = 4. Data are expressed as mean ± SEM; **P < 0.01, ****P < 0.0001. Statistical analysis was performed by two-way ANOVA with Bonferroni post hoc test.
Neither TLR4 does not mediate the palmitate-mediated increase in *Pomc* mRNA expression. mHypoA-POMC/GFP-1 cells were pre-treated with 10 µM TAK-242 (TLR4 inhibitor) or DMSO control for 1 hr, then treated with 50 µM palmitate for 8 hr. The mRNA expression of *Pomc* (A), *Il6* (B), and *Chop* (C) were assessed by qRT-PCR; n = 4. Data are expressed as mean ± SEM; **P < 0.01, ***P < 0.001, ****P < 0.0001. Statistical analysis was performed by two-way ANOVA with Bonferroni post hoc test.
Supplemental Figure S5. The palmitate-mediated increase in Pomc mRNA expression is dependent on palmitoyl-coA synthesis. mHypoA-POMC/GFP-1 cells were treated with 50 µM palmitate, 50 µM methylpalmitate, or water control) for 8 hr. Pomc mRNA expression was assessed by qRT-PCR; n = 4. Data are expressed as mean ± SEM; **P < 0.01. Statistical analysis was performed by one-way ANOVA with Bonferroni post hoc test.
Supplemental Figure S6. C16-ceramide does not alter Pomc mRNA expression in mHypoAPOMC/GFP-1 cells. mHypoA-POMC/GFP-1 cells were treated with 1 µM or 10 µM C16-ceramide or ethanol/dodecane control for 8 hr. *Pomc* mRNA expression was assessed by qRT-PCR; n = . Data are expressed as mean ± SEM. Statistical analysis was performed by one-way ANOVA with Bonferroni post hoc test.
Supplemental Figure S7. Oleate blocks the effects of palmitate on the mRNA expression of Pomc and markers of cellular neuroinflammation and ER stress. mHypoA-POMC/GFP-1 cells were treated with 50 µM palmitate, 50 µM oleate, 50 µM palmitate + 50 µM oleate, or water control for 8 hr. The mRNA expression of Pomc (A), Il6 (B), and Chop (C) were assessed by qRT-PCR; n = 4. Data are expressed as mean ± SEM; ****P < 0.0001. Statistical analysis was performed by one-way ANOVA with Bonferroni post hoc test.