The Effects of Fatty Acids on the Molecular Circadian Clock in Immortalized, Clonal Hypothalamic Neurons

by

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

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University of Toronto

2013

Abstract

Diets high in saturated fatty acids are associated with the development of circadian dysregulation, obesity, and type 2 diabetes mellitus. Conversely, unsaturated fatty acids are now known to improve insulin sensitivity, reduce weight gain, and alleviate obesity-induced inflammation. The aforementioned effects of saturated and unsaturated fatty acids have also been identified in the hypothalamus; however, there is a paucity of studies regarding the role of unsaturated fatty acids in circadian rhythms. Therefore, a novel cell model was established to examine the effects of omega-3 fatty acids on circadian rhythms in hypothalamic neurons. The mHypoE-37 cell line expresses Bmal1, Per2, and Rev-erbα in a circadian manner. The saturated fatty acid, palmitate, was found to induce circadian dysregulation of the mHypoE-37 neurons, whereas the unsaturated fatty acid, docosahexaenoic acid, protected against palmitate-induced circadian changes. These studies are the first to identify the potential for unsaturated fatty acids to protect the circadian system.
Acknowledgments

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<td>Agouti-related peptide</td>
<td>AgRP</td>
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<tr>
<td>Alpha-melanocyte-stimulating hormone</td>
<td>αMSH</td>
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<tr>
<td>Arcuate nuclei</td>
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<td>Arginine vasopressin</td>
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<tr>
<td>Basic helix-loop-helix-Period-Arnt-Single-minded</td>
<td>bHLH-PAS</td>
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<td>Beta-transducin repeat-containing protein 1</td>
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<td>Blood brain barrier</td>
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<td>Brain and muscle aryl-hydrocarbon receptor nuclear translocator-like1</td>
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<td>c-jun N-terminal kinase</td>
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<td>Casein kinase I</td>
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<tr>
<td>circadian locomotor output cycles kaput</td>
<td>CLOCK</td>
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<td>Clock</td>
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<td>Cocaine- and amphetamine-related transcript</td>
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<td>Cycle</td>
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<td>Diacyl glycerol</td>
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<td>Term</td>
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<tr>
<td>G-protein coupled receptor</td>
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<td>High fat diet</td>
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<tr>
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<td>Intracerebroventricular</td>
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<td>ipRGC</td>
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<tr>
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<tr>
<td>Liver X receptor</td>
<td>LXR</td>
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<td>Low fat diet</td>
<td>LFD</td>
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<tr>
<td>Medial preoptic area</td>
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<tr>
<td>Messenger RNA</td>
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<tr>
<td>MicroRNA</td>
<td>miRNA</td>
</tr>
<tr>
<td>Mitogen activated protein kinase</td>
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<tr>
<td>Mouse hypothalamic embryonic-(clone number)</td>
<td>mHypoE-XX</td>
</tr>
<tr>
<td>Nicotinamide phosphoribosyltransferase</td>
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</tr>
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<td>Nicotinamide adenine dinucleotide</td>
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<tr>
<td>Nuclear factor-κB</td>
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<tr>
<td>Paraventricular nucleus</td>
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<tr>
<td>Period</td>
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</tr>
<tr>
<td>Peroxisome proliferator-activated receptor</td>
<td>PPAR</td>
</tr>
<tr>
<td>Pituitary adenylate cyclase-activating polypeptide</td>
<td>PACAP</td>
</tr>
<tr>
<td>Term</td>
<td>Abbreviation</td>
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<tr>
<td>Pro-opiomelanocortin</td>
<td>POMC</td>
</tr>
<tr>
<td>Protein kinase C</td>
<td>PKC</td>
</tr>
<tr>
<td>Reactive oxygen species</td>
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</tr>
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<td>Retinohypothalamic tract</td>
<td>RHT</td>
</tr>
<tr>
<td>Retinoic acid-related orphan receptor alpha</td>
<td>RORα</td>
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<tr>
<td>Ribonucleic acid</td>
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<td>Saturated fatty acids</td>
<td>SFA</td>
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<tr>
<td>SKP1-cullin-F-box</td>
<td>SCF</td>
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<td>Simian virus 40</td>
<td>SV40</td>
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<tr>
<td>Sirtuin 1</td>
<td>SIRT1</td>
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<td>Small Ubiquitin-like MOdifers</td>
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<td>Sterol regulatory binding protein</td>
<td>SREBP</td>
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<td>Subparaventricular zone</td>
<td>vSPVZ</td>
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<td>Suprachiasmatic nucleus</td>
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<tr>
<td>Timeless</td>
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<td>Triglycerides</td>
<td>TG</td>
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<td>Type 2 diabetes mellitus</td>
<td>T2DM</td>
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<tr>
<td>Toll-like receptor 4</td>
<td>TLR4</td>
</tr>
<tr>
<td>Tumour necrosis factor α</td>
<td>TNF-α</td>
</tr>
<tr>
<td>Unsaturated fatty acids</td>
<td>UFA</td>
</tr>
<tr>
<td>Vasoactive intestinal polypeptide</td>
<td>VIP</td>
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<tr>
<td>Ventrolateral preoptic nucleus</td>
<td>VLPO</td>
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<tr>
<td>Ventromedial nucleus</td>
<td>VMN</td>
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Chapter 1
Introduction
1.1 Introduction

Obesity has emerged as a major global health concern, affecting at least 10% of the entire world’s population and approximately 25% of the Canadian adult population [1, 2]. Obesity increases the susceptibility of developing cardiovascular disease, diabetes, stroke, and liver disease [3-7]. Together, obesity and its associated health risks have created a serious financial burden on the health care system [1].

Many factors contribute to the development of obesity, such as genetics and dietary composition; however, the underlying cause leading to the obese state is excess energy intake. While feeding and energy expenditure are key factors regulating body weight, evidence now suggests that obesity can also affect physiological processes other than energy homeostasis [8]. It was recently demonstrated that mice fed a high fat diet (HFD) developed atypical behavioural and physiological daily rhythms, commonly referred to as circadian rhythms [8].

Circadian rhythms are endogenous cycles thought to exist in nearly all organisms. From an evolutionary standpoint, these cycles developed to optimize cellular efficiency through the coordination of cellular processes with specific periods of the day [9]. In more complex organisms, circadian rhythms coordinate multiple physiological processes, both independently and in concert with one another. Due to the complex intricacies of the circadian system, it can be easily disturbed by homeostatic insults like obesity. Interestingly, disturbances in the circadian system can independently give rise to obesity and lead to the onset of numerous pathologies [10].

The impact of circadian dysregulation is perhaps most evident in our society among those engaged in shift work. Defined by “anything other than a regular daytime schedule”, shift work is associated with as much as 45% of employees being overweight or obese [11, 12]. Shift work has become increasingly more prevalent due to the changing socio-economic demands of the 21st century. Working in this environment imposes reversed daily cycles and forces the body to undergo certain physiological tasks, such as feeding and physical activity, when their innate circadian cycle is not primed to do so. This effect has also been reproduced in mice, whereby mice fed a HFD during the atypical feed period have an increased incidence of obesity [13].
The HFD used in models of diet-induced obesity (DIO) are designed to mimic Western diets, and often contain high concentrations of saturated fatty acids (SFA). In addition to the association of SFA with obesity and increased energy intake, SFAs are now known to induce central and systemic inflammation. In turn, inflammation exerts positive feedback on energy homeostatic mechanisms that further perpetuates obesity [14]. Conversely, unsaturated fatty acids (UFA) have received much attention due to their potential health benefits, including improving insulin sensitivity and reducing inflammation [15-17].

With recent discoveries identifying the detrimental effects of obesity and SFA on the brain, specifically the hypothalamus, it has become apparent that obesity has a greater impact on holistic health than originally thought. Moreover, this is compounded by the reciprocal relationship between obesity and circadian rhythms. Due to the limited success of developing effective “diet drugs” and the steady growth in the obesity epidemic, it is crucial to gain a better understanding of the delicate relationship between fatty acids and cellular circadian rhythms. However, the heterogeneity of the hypothalamus and previous lack of appropriate hypothalamic models have interfered with efforts to gain insight into this relationship. Therefore, with the use of immortalized, clonal hypothalamic neurons generated in our laboratory, this thesis will explore the effects of both saturated and unsaturated fatty acids on the circadian rhythms in hypothalamic neurons.

1.2 The hypothalamus

Despite its small size, the hypothalamus is an essential region of the brain. In general terms, the hypothalamus is a homeostatic organ, functioning in thermoregulation, reproduction, stress response, fluid and electrolyte balance, circadian rhythms, and energy balance [18]. As suggested by its name, the hypothalamus is situated below the thalamus and above the pituitary [19]. Between the hypothalamus and pituitary, exists the hypophyseal portal system, allowing for endocrine communication between these two structures [19]. The hypothalamus receives and generates outputs in the form of both neuronal and hormonal connections. Interestingly, while the hypothalamus is considered to be within the confines of the blood brain barrier (BBB),
hypothalamic axons have been found to project to the median eminence, void of the BBB, thereby allowing the hypothalamus to sense non-BBB permeable molecules [18]. Furthermore, the hypothalamus receives information through neuronal projections from multiple brain regions including the hippocampus, amygdala, brainstem, and ventrolateral medulla [19]. The hypothalamus sends output signals directly to the dorsal raphe nucleus, ventral medulla, and the nucleus of the solitary tract.

Within the hypothalamus itself, there are three distinct regions, the anterior, tuberal, and posterior hypothalamus [18]. Within these regions exists characteristic subsets of neurons, commonly referred to as hypothalamic nuclei, each of which is associated with a specific set of physiological functions.

1.3 Energy balance

Early lesion studies identified the hypothalamus as a potential regulator of feeding. By specifically targeting the ventromedial nucleus (VMN) and paraventricular nucleus (PVN), rodents become hyperphagic and obese [20-22]. Similarly, stimulation of the rat lateral hypothalamus (LH) area induces hyperphagia and ultimately leads to obesity [23, 24]. However, destruction of the LH leads to aphagia, and eventually starvation [20-22]. Taken together, these initial studies suggested that the hypothalamus may act as the feeding centre of the brain.

With respect to hypothalamic nuclei, the PVN, dorsomedial nucleus (DMN), VMN, LH, and arcuate nucleus (ARC) are classically viewed as the hypothalamic areas responsible for regulating energy balance [25-27]. Energy balance is controlled by two subtypes of feeding related neurons: 1. orexigenic, those that stimulating energy intake or 2. anorexigenic, those that inhibit it. Together, these neurons modulate feeding by expression and secretion of orexigenic and anorexigenic neuropeptides. Agouti-related peptide (AgRP) and neuropeptide Y (NPY) are the classic orexigenic neuropeptides, whereas cocaine- and amphetamine-related transcript
(CART) and α-melanocyte-stimulating hormone (α-MSH), a product of the pro-opiomelanocortin (POMC) pre-propeptide, are the classic anorexigenic neuropeptides [28, 29].

In addition to generating orexigenic and anorexigenic signals, the hypothalamus also acts as an integration centre, receiving inputs from other regions of the brain, as well as the periphery, to modulate satiety. Numerous peripheral satiety signals have been identified. However, with respect to the hypothalamus, much focus has been placed on the adiposity signals: insulin and leptin [30-32]. The potent pancreatic endocrine hormone, insulin, was the first satiety hormone to be identified to act at the level of the hypothalamus to regulate body weight [33-35]. Leptin, which is produced and secreted by adipose tissue in proportion to the total amount body fat, was subsequently discovered as a satiety signal given that its absences results in severely obese mice [36].

With respect to energy homeostasis, both insulin and leptin have actions in the hypothalamus to negatively regulate the activity of orexigenic neurons and stimulate anorexigenic neurons, resulting in an overall decrease in energy intake and increase in energy expenditure [23, 34, 36, 37]. It has also been suggested that during development, leptin acts to promote neurogenesis of projections from the ARC to the PVN, which is required in determining a body-weight set point [38, 39]. However, high levels of circulating adiposity signals, as seen with obesity and type 2 diabetes mellitus (T2DM), can lead to insulin and leptin resistance [25, 40, 41]. Resistance to either adiposity signal impedes the ability of the hypothalamus to properly negatively regulate feeding and often results in weight gain [42, 43].

1.4 Circadian rhythms

Although circadian rhythms are synonymously associated with the sleep-wake cycle, they are involved in a wide array of physiological processes. These biological rhythms follow an approximately 24 hour clock, as suggested by the term - *circa* meaning “approximately” and *diem* meaning “day” [44]. Circadian rhythms are found in nearly all organisms, including fungi
and cyanobacteria [45-47]. However, during seasons of constant light, as found within the arctic, some animals lack circadian rhythms [48].

Circadian rhythms are believed to have their origins in early unicellular organisms, which undergo deoxyribonucleic acid (DNA) replication at night to protect the resultant product from harmful ultraviolet radiation prevalent during daytime [49, 50]. Similarly, possessing an internal clock has been evolutionarily beneficial to organisms by allowing them to efficiently coordinate and anticipate daily activities to regular environmental fluctuations [51].

Biological rhythms must possess four main requirements to be considered a true circadian rhythm [52-56]. Firstly, the rhythm must exhibit an approximately 24 hour cycle. Secondly, the rhythm must persist in the absence of external cues. Rhythms examined in the absence of external cues, typically light, are said to be in a free-running state [57, 58]. For example, humans have a free-running period of 24.18 hours, whereas that of mice is 23.9 hours [52, 53]. A circadian rhythm must also be entrainable to external cues – often referred to as entrainment factors, or “Zeitgebers” (German for “time giver”) [59]. Entrainment allows organisms to adapt to new time zones, but is also the cause of jet-lag. Although light is the strongest Zeitgeber, feeding, temperature, social interaction, and exercise can all act as non-photic Zeitgebers [60-65]. Temperature compensation is the final circadian rhythm criterion, meaning that a rhythm must continue to cycle over a range of physiological temperatures [66, 67].

1.4.1 Early studies

Circadian research dates back to the 1700s when Jean-Jacques d'Ortous de Mairan noted that plant leaves have a rhythm of daily opening and closing [68]. More importantly, de Mairan found that this daily pattern persisted in the absence of light. Real strides in chronobiology did not take place until the 1970s, when researchers began to use fruit flies as a research model [9]. By inducing mutagenesis, researchers found that Drosophila melanogaster could possess abnormalities in both pupal eclosion and daily locomotor activity, which could be categorized into three distinct variations: elongated period, shortened period, or completely arrhythmic.
Subsequent studies found that all the mutations associated with these phenotypes were found on the same locus, suggesting that a single gene could be responsible for these irregularities. Similar findings were eventually discovered in the fungus *Neurospora crassa* and hamsters [69, 70].

### 1.4.2 Circadian molecular clock

First discovered in the fruit fly, the molecular circadian clock was found to act through a self-sustaining negative feedback loop [9, 71-75]. In flies, the cycle initiates with the transcription of the regulatory proteins CLOCK (CLK) and CYCLE (CYC). Following transcription and translation, CLK and CYC form a heterodimer and are subsequently phosphorylated, thereby allowing re-entry into the nucleus. Inside the nucleus, the CLK-CYC complex acts as a transcription factor, binding to the E-box cis-regulatory enhance region (CACGTG) and induces the transcription of *Period (Per)* and *Timeless (Tim)*. Like CLK-CYC, PER and TIM proteins heterodimerize and enter the nucleus, inhibiting the interaction of CLK-CYC with DNA, thus blocking the transcription of PER and TIM. Overall, this completes the circadian molecular cycle.

Identifying the *Drosophila* clock model allowed for the mammalian clock orthologs to be discovered shortly after. Like that of the fly, the mammalian clock also functions as a negative feedback loop, although it does contain additional autoregulatory loops. In general, the cycle is commonly viewed in two segments: the positive and negative arm. The positive arm of the mammalian circadian cycle is composed of transcription factors containing binding motifs basic helix-loop-helix-Period-Amt-Single-minded (bHLH-PAS), circadian locomotor output cycles kaput (*Clock*) and brain and muscle aryl-hydrocarbon receptor nuclear translocator-like 1 (*Bmal1*, also called *MOP3*) [76-79]. Mammalian CLOCK and BMAL1 are homologous to *Drosophila* CLK and CYC, respectively. Similar to that of the fly, CLOCK and BMAL1 form a heterodimer and then enter the nucleus to initiate transcription of target genes, including *Period (Per1, Per2, and Per3)* and *Cryptochrome (Cry1 and Cry2)*, all of which contain E-box elements [80-84]. The role of CLOCK in the heterodimer can be interchanged for a paralog, neuronal PAS domain protein 2 (NPAS2); however, this effect appears to be tissue specific [85].
Following transcription and translation of the *Period* genes, the stability of PER is altered through phosphorylation by multiple casein kinase I (CKI) isoforms [86-88], which delays its dimerization with CRY and ensures proper timing of the negative arm. Translocation of the PER-CRY complex into the nucleus signals the end of the molecular circadian cycle, whereby CRY inhibits the activity of CLOCK-BMAL1, thus suppressing its own transcription, as well as other genes induced by CLOCK-BMAL1 [84, 89, 90]. Moreover, PER2 can initiate the positive arm by activating the transcription of *Bmal1*, but the mechanism is not well understood [91]. Overall, the cycling levels of CLOCK and BMAL1 peak during the day, whereas PER and CRY proteins levels are at their highest during the night [92].

In addition to PER-CRY and CLOCK-BMAL1 feedback loops, there exists a second prominent feedback loop consisting of two genes encoding orphan nuclear receptors, retinoic acid-related orphan receptor alpha (*ROR*α) and *Rev-erb*α [91, 93-95]. Transcription of both *ROR*α and *Rev-erb*α are under the control of CLOCK-BMAL1. Like that of other circadian proteins, *ROR*α and REV-ERBα independently translocate to the nucleus and compete for the orphan nuclear receptor response elements (RORE) sequence (AAAGTAGGTCA), found within the *Bmal1* promoter [95, 96]. The activity of *ROR*α and REV-ERBα are antagonistic, whereby *ROR*α stimulates the transcription of *Bmal1* and REV-ERBα inhibits it [97, 98]. Other potential circadian genes, such as *Timeless*, *Dec1*, *Dec2*, and *E4bp4* have been identified through homology and knockout studies, however, their exact role remains unclear [99].

Although the core molecular circadian clock only comprises a small number of genes, their proteins play a significant physiological role acting as transcription factors for an approximated 10% of the entire transcriptome [100, 101].

### 1.4.3 Post-transcriptional circadian regulation

Post-transcriptional modification of the circadian molecular clock acts to ensure proper cycling of circadian rhythms. Studies examining the half-life of *Period* genes were the first to recognize a possible post-transcriptional regulatory role in circadian rhythms [102, 103]. Here,
the *Drosophila Per* transcript displayed a time-dependent stability trend, with the transcript being most stable during the rising phase of *Per* [104]. Subsequent studies found the same property with respect to mammalian *Per2* and *Cry1* [105-108].

More recently, focus has been placed on the 3’ untranslated region (3’-UTR) of circadian transcripts. This region of messenger ribonucleic acid (mRNA) interacts with many RNA binding proteins, which function to modify stability or translation [109]. For example, the heterogenous nuclear ribonucleoprotein (hnRNP) I binds to the 3’-UTR of *Per2*, promoting its degradation [103]. hnRNP I also associates with *Rev-erba* within its internal ribosome entry site (IRES) to promote its translation [110]. Interestingly, while hnRNP I expression is not circadian itself, it does shuttle between the nucleus and cytoplasm in a circadian manner [110]. Similar to hnRNPs, microRNAs (miRNAs) are also known to interact with the 3’-UTR.

miRNAs are small non-coding RNAs that function to degrade mRNA transcripts and inhibit protein synthesis [111, 112]. Although little is known about miRNAs and circadian rhythms, studies suggest that a reciprocal relationship exists. Early research has identified some miRNAs to be rhythmically expressed within the mouse and *Drosophila* [113-116]. Additionally, the expression of the miRNA, miR-219, has been shown to be under the control of CLOCK-BMAL1 and *in vivo* knockdown of miR-219 increases circadian period length [113]. Conversely, interaction of miR-192/194 and miR141 with PER and CLOCK, respectively, decreases their translation [117-119].

1.4.4 Post-translational circadian modification

1.4.4.1 Phosphorylation

The required delay between the positive and negative arm of the circadian molecular clockwork is established not only by transcriptional and post-transcriptional regulation, but also by post-translational modifications, such as phosphorylation and ubiquitination [120-122].
A) Positive Arm

The circadian molecular feedback loop is composed of a positive and negative arm. A) The positive arm begins with the transcription and translation of Clock and Bmal1. In the cytoplasm, CLOCK and BMAL1 form a heterodimer and translocate back into the nucleus, where they initiate transcription of Per, Cry, Rev-erba genes, as well as other genes controlled by the circadian clock.

B) Negative arm

In the negative arm, PER and CRY proteins are phosphorylated by CKI, which inhibits the transcriptional activity of BMAL1. REV-ERBα also acts to inhibit the activity of BMAL1.

Figure 1.1 Mammalian circadian molecular clock schematic

The circadian molecular feedback loop is composed of a positive and negative arm. A) The positive arm begins with the transcription and translation of Clock and Bmal1. In the cytoplasm, CLOCK and BMAL1 form a heterodimer and translocate back into the nucleus, where they initiate transcription of Per, Cry, Rev-erba genes, as well as other genes controlled by the circadian clock. B) In the negative arm, PER and CRY proteins are phosphorylated by CKI, which inhibits the transcriptional activity of CLOCK-BMAL1. REV-ERBα also acts to inhibit the activity of BMAL1.
Phosphorylation has been associated with regulating clock protein entry into the nucleus, protein-protein affinity, and protein degradation. In mammals, the key kinases CKIε and δ, mitogen activated protein kinase (MAPK), and glycogen synthase 3 (GSK3) are known to be involved in phosphorylating clock proteins [123-129].

CKI has opposing actions on clock proteins in the positive and negative arms, whereby CKI acts to promote the transcriptional activity of BMAL1, whereas phosphorylation of the PER and CRY reduces their stability [124, 130, 131]. Conversely, MAPK downregulates the activity of BMAL1 [126]. Altering the stability of PER and CRY prevents early entry into the nucleus, thus creating the necessary time-lag and approximate 24 hour cycle. In humans, the inability for CKI to interact with PER2 leads to the onset of familial advanced sleep phase syndrome (FASPS), a genetic disorder characterized by an advanced sleep-wake cycle [132-134]. Also, mutations in CKIε result in a shorter circadian period, as seen in the tau hamster [88].

The nuclear translocation of PER2 is also accomplished through phosphorylation by GSK3 [125, 129]. GSK3 is also responsible for phosphorylating CRY2, leading to its degradation [125, 129, 135]. Experimentally overexpressing the activity of GSK3 in vitro leads to advanced phase shifts [125, 135].

The molecular time-delay is also accomplished through ubiquitination. PER proteins are marked for ubiquitination by a SKP1-cullin-F-box (SCF) containing E3 ubiquitin ligase, β-transducin repeat-containing protein 1 (β-TrCP1) [123, 136], whereas CRY protein ubiquitination is mediated by another SCF, F-box and leucine-rich repeat protein 3 (FBXL3) [137, 138]. Mutations in the Fbxl3 gene lead to an elongated circadian period [138, 139]. Ultimately, the PER and CRY proteins are degraded by the 26S proteasome.

1.4.4.2 Circadian epigenetic modification

Histone acetylation and DNA methylation are potent modifiers of the circadian system. CLOCK itself possess histone acetylation (HAT) activity, allowing for chromatin remodelling,
and thus altering the expression of target genes [140-142]. Additionally, CLOCK acetylates BMAL1 at lysine (LYS) 537, increasing its ability to interact with CRY proteins [143].

The HAT activity of CLOCK is opposed by sirtuin 1 (SIRT1), a nicotinamide adenine dinucleotide (NAD+)-dependent histone deacetylase (HDAC) [144-146]. SIRT1 itself is expressed in a circadian manner [144]. SIRT1-mediated circadian regulation first requires the recruitment by the CLOCK-BMAL1 complex, following which it can modify the expression of Bmal1, Per2, and Cry1 [144, 146-148]. As well, SIRT1 appears to play a role in the degradation of PER2; however, the mechanism through which it occurs is not clear [144].

Protein stability can also be modified through sumoylation. SUMOylation is the process of attaching Small Ubiquitin-like MOdifers (SUMO) to modulate cellular processes, such as transcription, protein stability, and nuclear translocation [149, 150]. With respect to the clock proteins, BMAL1 is SUMOylated on LYS259, which functions to aid in its entry into the nucleus and regulate its degradation [151, 152].

1.4.5 Circadian hierarchy

While every cell is thought to possess circadian machinery, there exists a ‘master’ clock which controls the rhythm of every ‘slave’ clock. In mammals, the suprachiasmatic nucleus (SCN) is the master clock and is located within the hypothalamus, just above the optic chiasm [153]. Insights into the role of SCN as the master clock came from lesion studies, whereby abolishing the SCN generated arrhythmic animals [154-156]. The SCN was confirmed as the master clock when wild-type SCN grafts transplanted to the tau mutant hamster restored their native period length [157, 158].

The SCN is a bi-lobed structure composed of approximately 20,000 neurons, divided into a ventrolateral, non-rhythmic ‘core’ and a dorsomedial, intrinsically rhythmic ‘shell’ [18, 19, 159-163]. The core is characterized by the expression of vasoactive intestinal polypeptide (VIP) and gastrin-releasing peptide (GRP), whereas the shell contains arginine vasopressin (AVP)-positive neurons. Both the core and shell are γ-aminobutyric acid (GABA)-ergic neurons [159].
In general, the SCN receives various temporal cues, which synchronize the SCN to the external environment and subsequently entrains the rest of the slave clocks. As previously mentioned, light acts as the strongest entrainment factor. The SCN also receives non-photic cues, such as serotonin from the raphe nuclei and NPY from the geniculate-hypothalamic tract [164-166]. These additional connections are believed to entrain the SCN to non-photic cues, such as feeding and social interaction.

The SCN receives photic cues from the retina via a non-visual photoreceptor cell type, the intrinsically photoreceptive retinal ganglion cell (ipRGC) [62, 167, 168]. Unlike rods and cones, ipRGCs express a unique photopigment, melanopsin. Moreover, ipRGCs have a relatively high light-intensity threshold, thereby allowing transient and low light exposure to have minimal impact on the entrainment process [169]. Information received by the ipRGCs is relayed to the SCN via the retinohypothalamic tract (RHT) [170]. The RHT primarily signals to the SCN with the neurotransmitter glutamate; however, asparate and pituitary adenylate cyclase-activating polypeptide (PACAP) have all been identified as secondary signalling molecules [171-175].

More specifically, the RHT directly signals to the SCN core, which induces the expression of clock genes. The exact mechanism through which RHT signalling molecules elicit clock gene expression is not fully understood; however, it appears to employ multiple pathways, including MAPK cascade, nitric oxide, and calcium influx [176-178]. Following the induction of clock expression, the signal is transported to the SCN shell, inducing the rhythmic expression of clock genes [179-181]. From here, the SCN communicates with the hypothalamus, brain, and periphery [182-184].

The SCN regulates the circadian fluctuation in body temperature through an indirect connection to the medial preoptic area (MPOA), via the subparaventricular zone (sPVz) [185-188]. Similarly, through the sPVz, the SCN controls hormonal secretions from the DMH and arousal through the ventrolateral preoptic nucleus (VLPO) and LH [187]. Secondary projections make connections throughout the brain, including the hippocampus, neocortex, and anterior pituitary modifying memory and learning, mental performance, and endocrine secretions,
respectively [188]. Exactly how the SCN synchronizes slave clocks has yet to be fully elucidated; however, it appears to be through a combination of endocrine, autonomic and behavioral signals [189, 190]

1.4.6 Circadian rhythms and Energy homeostasis

The relation between circadian rhythms and metabolism reaches beyond the connection of the SCN to the DMH. Both whole body and cellular energy homeostasis has a close association with circadian rhythms. At the physiological level, most animals display circadian rhythms of insulin, leptin, and glucose levels [191, 192]. Genes related to these metabolic pathways also exhibit rhythmic expression, including glucokinase, glucose-6-phosphate, and glucose transporter 2 [193, 194]. Studies have shown that caloric restriction increases levels of the nutrient sensor NAD⁺, thereby increasing the activity of SIRT1 and in turn regulates the expression of clock genes [195-197]. Conversely, NADH, the reduced form of NAD+, increases the activity of CLOCK-BMAL1 [198].

AMP-activated protein kinase (AMPK) also acts as an intermediary between cellular energy status and circadian rhythms. Activated by exercise and fasting, AMPK can phosphorylate CRY1, as well as activate nicotinamide phosphoribosyltransferase (NAMPT), the rate-limiting enzyme in NAD⁺ salvage pathway [199, 200]. Moreover, transcription of Nampt is under the control of CLOCK, resulting in the expression of NAMPT and NAD⁺ in a circadian manner [147, 148].

Nuclear receptors (NR) also have a reciprocal role with metabolism and circadian rhythms [201]. NRs sense fat-soluble molecules and can in turn modify various aspects of physiology, including lipid and carbohydrate metabolism [202-204]. In mice, 28 of the 49 NRs exhibit circadian rhythms in various tissues [200]. Of the core circadian proteins, the NR REV-ERBα plays a role in regulating hepatic gluconeogenesis, adipocyte differentiation, and lipid
Figure 1.2 The SCN and rhythm generation

A) The mammalian ‘master’ clock is the SCN. Photic signals are transmitted to the SCN via the RHT from the non-visual ipRGCs. Signals from the RHT induces the expression of clock genes in the SCN ‘core’, which is then relayed to the ‘shell’, generating the rhythmic expression of clock genes. B) In addition to light, food, exercise, and temperature can also act as synchronization factors. The SCN uses a multitude of signalling mechanisms to relay temporal information to ‘slave’ clocks. The SCN regulates the rhythms within and outside of the hypothalamus, including thermoregulation, feeding, and arousal. Adapted from Maury et al. 2010 [205]
metabolism [206, 207]. Contrary to REV-ERBα, which inhibits the transcription of Bmal1, peroxisome proliferator-activated receptor (PPAR) α induces the expression of Bmal1 [208, 209]. Additionally, BMAL1 creates a positive feedback loop by activating PPARα. Activation of PPARα by either BMAL1 or FAs can lead to FA oxidation and alter expression of genes involved in lipid homeostasis [210].

Similar to PPARα, PPARγ positively regulates the expression of Bmal1 and is rhythmically expressed [211, 212]. PPARγ knockout models have reduced rhythmic expression of clock genes [211]. The activity of PPARγ can be suppressed by SIRT1, resulting in increased fat mobilization during the fasted state [213]. Conversely, SIRT1 activates PPARγ’s transcriptional activator PGC-1α [197]. Like PPARγ, PGC-1α regulates the expression of Bmal1 and oscillates in a circadian manner [214]. Moreover, Pgc1α knockouts have abnormal temperature, metabolism, locomotor activity rhythms, in addition to altered clock and metabolic gene expression [214].

1.4.6.1 Circadian and metabolic dysregulation

The relationship between circadian rhythms and metabolism becomes evident once the system falls into dysregulation, as seen in shift work and obesity. As a result of the industrial revolution and modern society operating 24 hours a day, shift work has become increasingly prevalent – shift workers account for nearly 20% of employees in certain industries [215, 216]. Shift work desynchronizes most activities with native circadian rhythms. This misalignment has been linked to increased incidence of diabetes, obesity, cancer, and cardiovascular disease [215-217].

Symptoms associated with shift work have been experimentally replicated with humans placed on a forced desynchrony protocol, whereby participants were found to have increased blood pressure, inverted cortisol rhythms, and reduced insulin sensitivity [11, 12]. Similarly, diabetics are known to have dampened rhythms in insulin secretion and glucose tolerance [12,
While the mechanism linking circadian dysregulation and metabolism is not well understood, evidence has connected sleep quality to energy homeostasis.

Like shift work, poor sleep quality or short sleep duration is associated with metabolic conditions, such as diabetes, obesity, and increased appetite [192, 219-221]. This connection is manifested in narcoleptics who have random sleep patterns, which is believed to cause an increase body mass index (BMI) and obesity [222-225]. Similarly, individuals with night eating syndrome (NES) consume the majority of their caloric intake during the night and also present with phase shifted levels of metabolic hormones, including ghrelin, insulin, and cortisol, as well as an inverted 24-hour blood glucose rhythm [226-229]. The atypical eating pattern of NES patients is reminiscent of the Clock mutant mice, which consume significantly more calories during the normal inactive period [10].

The Clock mutant mice, which possess a deletion in exon 19 of the Clock gene, displays altered feeding rhythms, with overall increases in total food consumption, in addition to increased feeding during the light cycle [10]. Most importantly, these mice demonstrate key features of the metabolic syndrome, including hyperglycemia, hyperlipidemia, hyperphagia, and hyperleptinemia [10]. The obese phenotype of the Clock mutant is amplified with HFD studies, whereby mice show increases in both fat and lean body mass, compared to paired-fed control mice. The obese phenotype and associated hyperphagia appears to be a result of altered neuropeptide expression, given that the mice have relatively low expression levels of CART, POMC, and ghrelin [10].

Mutations in other core clock genes also lead to changes in metabolism. Similar to the Clock mutant, Per2 knockout mice fed a HFD gain more weight and have elevated adiposity, in addition to possessing a shortened circadian period [230]. Interestingly, while the Bmal1 knockout mice have impaired gluconeogenesis, hyperleptinemia, glucose intolerance, dyslipidemia circadian arrhythmia, they are not obese [231-233].

Metabolic dysregulation appears to have a reciprocal role with circadian dysregulation. Wild-type mice given a HFD have increased free running period and altered clock gene
expression [8]. Additionally, wild-type mice fed a HFD exclusively during the inactive phase gained more weight than mice fed an isocaloric diet during the active phase [13]. Moreover, mature-onset obesity model, histamine H(1)-R knockout mice, restricted to active phase feeding were resistant to weight gain [234]. While an obvious directional relationship exists between metabolism and circadian rhythms, the mechanism behind this phenomenon is not fully understood.

1.5 Fatty acids

Fatty acids (FAs) are essential molecules required for proper cellular function [235]. Structurally, FAs are composed of a carboxylic acid group and hydrocarbon tail consisting of 2 to 30 or more carbons. FA can be grouped as either saturated fatty acids (SFA), those with a straight hydrocarbon chain lacking double bonds, or unsaturated fatty acids (UFA), those with at least one double bound within its chain [236]. Additionally, UFAs containing more than one double bond, such as the omega-3 fatty acids, are referred to as polyunsaturated fatty acids (PUFA) (as opposed to monounsaturated fatty acids (MUFA)).

FA can exist in either free fatty acid (FFA) form or bound to proteins, such as plasma albumin [237-239]. When bound, albumin acts as a delivery system, transferring FA where needed. At the cellular level, FFA were initially thought to gain entry into cells through a simple ‘flip-flop’ diffusion method, however, more recent evidence points to carrier proteins playing a much larger role than diffusion. Four major groups of FA protein transporters exist: FA translocases (FAT), FA transport proteins (FATP), caveolins, and FA binding proteins (FABP) [240]. FAT, FATP, and caveolins are all plasma membrane proteins aiding in transporting FAs into the cell. Once inside the cytoplasm, FAs are bound to FABP and directed to various organelles to modulate membrane composition, generate ATP through β-oxidation, or modified to signalling molecules [241-243]. Interestingly, FABPs exhibit a circadian expression pattern, suggesting the existence of optimal times for FA utilization [241].
Table 1.1 Physiological effects associated with circadian gene mutations

<table>
<thead>
<tr>
<th>Gene Mutation</th>
<th>Circadian Effect</th>
<th>Metabolic Effect</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Clock</strong></td>
<td>4 hour period elongation and arrhythmic in constant condition [77]</td>
<td>Develop metabolic syndrome [10]</td>
</tr>
<tr>
<td><strong>Bmal1</strong></td>
<td>Arrhythmic [76]</td>
<td>Dampened plasma glucose and triglyceride rhythm [232]</td>
</tr>
<tr>
<td><strong>Cry1 and Cry2</strong></td>
<td><em>Cry1&lt;sup&gt;−/−&lt;/sup&gt;</em> have shorten period [244]. <em>Cry2&lt;sup&gt;−/−&lt;/sup&gt;</em> mice longer period [83]. <em>Cry1</em>- <em>Cry2</em> double knockout mice are arrhythmic [245].</td>
<td>N/A</td>
</tr>
<tr>
<td><strong>Per1</strong></td>
<td>Shortened period [84, 246]</td>
<td>N/A</td>
</tr>
<tr>
<td><strong>Per2</strong></td>
<td>Short period and arrhythmic in constant conditions [190, 247]</td>
<td>N/A</td>
</tr>
<tr>
<td><strong>CKIε</strong></td>
<td>Increased period in <em>CKIε&lt;sup&gt;−/−&lt;/sup&gt;</em> mice [248] and shortened period Tau hamster [88]</td>
<td>N/A</td>
</tr>
<tr>
<td><strong>CKIδ</strong></td>
<td>Elongated period in fibroblasts and liver explants of <em>CKIδ&lt;sup&gt;−/−&lt;/sup&gt;</em> [86]</td>
<td>N/A</td>
</tr>
<tr>
<td><strong>Rev-erba (Nr1d1)</strong></td>
<td>Decrease in periodicity [95]</td>
<td>Elevated plasma triglycerides [249]</td>
</tr>
<tr>
<td><strong>Rora</strong></td>
<td>Decrease in periodicity [98]</td>
<td>Lower serum triglycerides and HDL [250]</td>
</tr>
<tr>
<td><strong>Ppargc1a</strong></td>
<td>Elongated period [214]</td>
<td>Increased insulin sensitivity [251]</td>
</tr>
</tbody>
</table>
Various tissues, including adipose tissue and the liver, can also synthesize *de novo* FFAs [252]. Of all synthesized FFAs, the 16-carbon SFA, palmitate, is the primary product of fatty acid synthesis [253]. Additionally, palmitate accounts for approximately 30% of all the FFAs found in humans. For these reasons, palmitate is commonly used for experiments examining the effects of FFAs.

### 1.5.1 Fatty acid gene regulation

While numerous studies have examined the physiological effects of FAs, the mechanisms have only now started to be elucidated, including their capacity to modulate gene expression [254-258]. In particular, FAs are able to regulate the activity of transcription factors, including PPARs, liver X receptors (LXRs), and sterol regulatory binding proteins (SREBPs) [259-264].

As previously mentioned, PPARs are NRs that detect nutrient levels, including FAs, FA derivatives, and molecules structurally similar to FAs [265, 266]. Of the PPARs, PPARα has received the considerable attention with respect to FA-associated gene regulation. In mice, PPARα is robustly activated by dietary and *de novo* FAs [267]. Moreover, changes in hepatic gene expression induced by UFA are almost entirely associated with the activity of PPARα activity [268]. Overall, altering the activity of PPARα mediates changes in the expression of genes involved in ketogenesis, gluconeogenesis, and lipoprotein metabolism [265, 266].

Closely related to the PPARs, LXRs are activated by derivatives of cholesterol and act to regulate FA synthesis, genes involved in lipogenesis, and cholesterol metabolism [262, 269, 270]. Experimental inhibition of LXRs contributes to the development of hyperlipidemia and other metabolic disorders [271]. The action of LXRs are mediated by its binding to promoter regulatory regions, in addition to its interaction with SREBPs [272]. Similar to LXRs, SREBPs regulate fatty acid and cholesterol metabolism by acting as transcription factors [269, 273]. In particular, the control of SREBP1 over genes involved in lipogenesis and MUFA synthesis is potently suppressed by the omega-3 FA, docosahexaenoic acid (DHA) [274, 275].
1.5.2 Saturated fatty acids and obesity

The prevalence of obesity has a direct correlation with the rise in the consumption of processed foods. Fats account for approximately 30-40% of a typical Western diet. While SFA exist naturally in many animal- and plant-derived foods, excessive consumption of SFA can lead to physiological disturbances.

In normal physiology, the majority of fat is stored in adipose tissue in the form of triglycerides (TG) with only a small amount circulating in the blood stream [276]. Plasma levels of FFA peak during the night and act as the primary energy source during the inactive phase [277]. Plasma FFA levels are primarily regulated by hydrolysis of TG from adipocytes, but circulating levels also increase following fat consumption [278, 279]. Post-prandially absorbed FFAs are directed to adipose tissue in the form of chylomicrons, where they are hydrolysed by lipoprotein lipase (LPL) and incorporated into adipocytes [280, 281]. Depending on the amount of fat and activity of LPL, a percentage of hydrolysed fat will escape and join the circulating levels of FFAs [281-283].

In the case of obesity, reduced LPL activity results in higher circulating levels of FFA [282]. Increased plasma FFAs can lead to fat accumulating in non-adipose tissue [284]. Continual accumulation can lead to cytoplasmic lipases breaking down FFAs into harmful metabolites, such as ceramide and diacyl glycerol (DAG) [285]. In particular, this can lead β-cell death and insulin resistance, furthering the possibility of developing T2DM [286]. High levels of SFAs have also been shown to attenuate insulin signalling in hypothalamic neurons [247].

Additionally, SFAs can modulate the expression of hypothalamic neuropeptides [247]. Many in vivo studies have reported that animals fed a diet rich in SFA have significantly lower expression of the AgRP and NPY [287, 288]. Interestingly, these reports are seemingly contradictory to the concomitant weight gain and obesity often associated with animals fed a HFD.
1.5.3 Saturated fatty acids and inflammation

Obesity-induced inflammation was first observed when mice fed a HFD had increased adipose macrophage infiltration [289, 290]. New evidence suggests that the obesity induced low grade chronic inflammation may have a causal relationship with additional obesity-associated pathophysiological changes.

The inflammatory response seen in adipose tissue is regulated in part by the cellular membrane receptor, toll-like receptor 4 (TLR4) [291]. Indeed, TLR4 knockout mice fed a HFD are resistant to obesity-associated inflammation and insulin resistance [292]. Primarily activated by SFAs, TLR4 modulates the expression of inflammatory cytokines through the transcription factor, nuclear factor-κB (NF-κB) [291, 293, 294]. Typically held in the cytoplasm by its inhibitor, IκB, NF-κB activation results in the transcription of inflammatory cytokines, such as tumour necrosis factor α (TNF-α) and interleukin-6 (IL-6) [288-290]. Indeed, with respect to obesity and inflammation, both TNF-α and IL-6 are known inflammatory cytokines involved in the pathophysiological response [295-297].

During the onset of obesity, circulating levels of the pro-inflammatory cytokine TNF-α are characteristically elevated [298, 299]. Obesity-associated increases in TNF-α have been demonstrated to induce β-cell apoptosis, thereby furthering progression of the obesity-induced pathophysiological conditions, including insulin resistance and T2DM [300, 301]. As with TNF-α, the pro-inflammatory cytokine IL-6 increases following weight gain and obesity [302, 303]. Studies have shown that palmitate increases both the expression and secretion of IL-6 in adipocytes [304]. In addition to inflammation, IL-6 is involved in lipid metabolism, whereby IL-6 decreases adipose LPL activity, thus reducing TG accumulation [305, 306]. In turn, this effect can contribute to circulating levels of FFA and thus further increase inflammation.

While systemic inflammation is principally studied in obesity-related inflammation, recent research has emerged demonstrating that the hypothalamus is also subject to inflammation [14, 307]. As seen in the periphery, hypothalamic obesity-induced inflammation results in increased levels of TNF-α and IL-6 [307]. Hypothalamic inflammation can impair normal
hypothalamic function, such as feeding and energy expenditure. Inhibition of inflammatory pathways by intracerebroventricular (ICV) infusion in obese rats results in reduced feeding and weight gain [308, 309]. Similarly, neuron-specific disruption of the TLR4 or NF-κB pathway prevents DIO [294, 310, 311]. Exactly how inflammation mediates changes in feeding and energy expenditure remains unclear; however, evidence suggests that inflammation modulates signalling molecules in the insulin and leptin signalling pathways, especially SOCS3 [311, 312].

1.5.4 Unsaturated fatty acid and obesity

The relationship between FAs and obesity is not solely restricted to SFAs. Of late, UFAs have received much attention due to their potential anti-obesity effects, especially with respect to fat accumulation, insulin resistance, and inflammation [313-318].

Multiple groups have reported that supplementing rodent diets with PUFA dramatically reduces fat mass gain [319, 320]. Further insight into these findings suggests that UFAs, especially DHA, reduce adipocyte size through reductions in fat accumulation [318, 320, 321]. Several studies have reported a myriad of potential mechanisms resulting in reduced fat accumulation, including reduced energy intake, increased energy expenditure, increased thermogenesis, and lipid metabolism [316, 317, 322-325]. Within the broad term of lipid metabolism, PUFAs, have been demonstrated to reduce adipocyte fat deposition by concomitantly reducing the activity of LPL and increasing the activity of enzymes involved in β-oxidation and peroxidation [325-328]. In addition to improving fat accumulation, PUFAs have been proven beneficial in certain models of metabolic disorders.

Obese and diabetic mice fed a HFD complemented with PUFAs are resistant to weight gain seen in HFD-alone control animals [17, 318]. Similarly, dietary intervention with PUFAs following the onset of obesity was found to reduce weight gain [329]. Here, mice were fed a HFD for 13 weeks and then subsequently placed on to one of three diets: the original HFD, HFD supplemented with PUFAs, or low fat diet (LFD). The body weight of both the PUFAs supplemented and LFD groups were significantly reduced to the levels of the control animals.
Likewise, rats administered with PUFAs through ICV injections following 8 weeks on a HFD exhibited reduced food consumption, decreased NPY and increased POMC and CART mRNA expression, and improved insulin sensitivity [330].

1.5.5 Unsaturated fatty acids and inflammation

The anti-obesity properties of PUFAs also extend to inflammation. While the ability for PUFAs to reduce inflammation in adipose tissue has been well documented, it was only recently determined that these effects are mediated through the previous orphan binding receptor, G-protein coupled receptor (GPR) 120 [331, 332]. Activation of GPR120 by both native PUFAs and synthetic agonists significantly suppresses the inflammatory response through a reduction in TNF-α and IL-6, as well as other key markers [332]. Indeed, this effect has also been demonstrated in the hypothalamus, whereby ICV PUFAs injections reverted DIO-associated inflammation. Overall, the ability of PUFAs to suppress the inflammatory response appears to be accomplished through NF-κB and PPARγ [333, 334]. PPARγ can directly suppress inflammation through its activity as a transcription factor or indirectly by interfering with the NF-κB pathway. Induction of PPARγ by DHA in vitro significantly reduces inflammatory cytokines following TLR4 stimulation [335].

1.6 Cell lines

1.6.1 Circadian rhythms and cell lines

Early cellular circadian studies investigated the avian pineal gland, which functions similar to the SCN in mammals. Here, cultured pinealocytes still demonstrated their native oscillatory properties [336-338]. Once the mammalian master circadian regulator was identified, cultured SCN grafts were found to have the same rhythmic properties as pinealocytes [158]. Although SCN explants were helpful in identifying its role and properties, it was not until the advent of circadian cell lines that the underlying molecular mechanisms of the circadian clock were uncovered.
Of the existing SCN cell lines, the SCN 2.2 have been instrumental in furthering the understanding of the SCN [339-341]. Like the in vivo SCN, these cells rhythmically express clock genes, can be phase shifted, and can induce rhythmicity in SCN lesioned rats and rat-1 fibroblasts [342]. Furthermore, studies using this cell line have discovered the dependency of the SCN on voltage-gated calcium channels, rhythmicity of PKC activity, and rhythmic cycling of NAD+/NADH [343, 344].

More recent studies examining the SCN have utilized brain slices from the Per2::luciferase knock-in mouse, providing a novel method for real time analysis of Per2 expression [345]. Additionally, non-SCN cell lines have been generated from the Per2::luciferase mouse; however, these cultures require a supplemental serum shock protocol to induce rhythmicity [346, 347]. The serum shock protocol allows for non-SCN cells to be synchronized to neighbouring cells and rhythmically express clock genes [348]. This discovery has provided the necessary tools to investigate the properties and mechanism underlying peripheral slave clocks. Specifically, studies utilizing peripheral cell lines have aided in the understanding of clock protein shuttling, importance of CKI, and the interaction between CLOCK-BMAL and associated NRs [349-351].

The serum shock method has also been used to further hypothalamic research. The widely used hypothalamic, gonadotropin-releasing hormone (GnRH) expressing GT1-7 cells were shown to have altered GnRH secretion following circadian dysregulation [352]. More recently, the Belsham laboratory has generated a novel set of hypothalamic cell lines, of which a subset has been identified to robustly express circadian clock gene [353]. Additionally, a study from the Belsham laboratory demonstrated that a neuronal cell model, mHypoE-44, exposed to SFAs alters the expression of the clock genes Bmal1, Clock, Per2, Rev-erba, as well as NPY [354].
1.6.2 Hypothalamic Cell lines

Animal models, especially knock-outs rodents, have been instrumental in advancing the understanding of various aspects of physiology. However, due to the myriad of complexities found in vivo, gaining insight into the cellular and molecular mechanisms of physiological processes often proves difficult. Indeed, this is especially true of the hypothalamus given its intricate anatomy, both within the hypothalamus itself and the connections made with the remainder of the body. For these reasons, the reductionist approach of cell lines proves advantageous given that most native factors can be experimentally controlled.

Despite the need for hypothalamic cell lines, it was only recently that such cell lines were established. As previously mentioned, the Belsham laboratory generated an array of clonal, immortalized hypothalamic neuronal cell lines [353]. Conversely to the previously generated hypothalamic cell lines, including the GT1-7s, these novel cell lines do not originate from tumours. In brief, the cell lines were generated by isolating the hypothalamii of embryonic mice and retrovirally transfected with the SV40 T-antigen, containing a neomycin resistance gene. Successfully transfected cells were selected using geneticin and then serially diluted, thus creating clonal populations. The cell lines are designated as mouse hypothalamic embryonic-(clone number) (mHypoE-XX). Experiments carried out in this thesis employed the mHypoE-37 neurons.

This method has generated a unique set of cell lines, each expressing a different profile of neuropeptides and neuronal morphologies, enabling various aspects of hypothalamic physiology to be studied. With respect to energy homeostasis, the mHypoE-46 neurons have been utilized to demonstrate that hypothalamic exposure to insulin downregulates the transcription of NPY and AgRP mRNA [190]. Furthermore, native insulin signalling has been shown to be attenuated following palmitate treatment in the mHypoE-44 cell line [247]. Additionally, characterization of the mHypoE-44 neurons has found this cell line to exhibit robust circadian rhythms, as well as, the ability for palmitate to disrupt the expression profiles of circadian genes [189, 354].
1.7 Hypothesis and Aims

Countless studies have demonstrated the detrimental effects SFAs have on energy homeostasis, inflammation, and the progression of obesity. Conversely, PUFAs have recently been identified as putative agents to ameliorate the negative effects associated with obesity. The effects of both SFAs and PUFAs exerted on obesity, in part, are accomplished through modulating hypothalamic physiology. Moreover, while the bi-direction relationship between obesity and circadian rhythms has been recognized, few studies have examined the effects of SFA on the circadian molecular clock. Furthermore, the role of PUFAs on circadian rhythms remains to be elucidated. Thus, the mHypoE-37, which endogenously express key circadian molecular genes, will be used to test the general hypothesis that **PUFAs can protect hypothalamic neurons against SFA-induced changes in circadian rhythms.**

This hypothesis will be tested in three aims. In Aim 1, the clock gene expression profile of the mHypoE-37 cell line will be characterized. In Aim 2, the effects of the SFA, palmitate, and the omega-3, DHA, on circadian clock expression will be assessed. In Aim 3, it will be determined if DHA can protect against palmitate-associated changes in circadian gene expression.
Figure 1.3 Immortalization protocol for clonal, hypothalamic cell lines.

A) 1. Murine embryonic hypothalamic were harvested at embryonic days 15, 17, and 18 and were triturated into primary culture.  2. Cultures were retrovirally transfected with the SV40 T-antigen, with a neomycin resistance cassette.  3. Successfully transfected cells were selected using geneticin.  4. Resistant colonies were serially diluted to generate clonal cell lines. B) Image of the mHypoE-37 neurons obtain by Jennifer Chalmers (unpublished data, Belsham laboratory).
Chapter 2
Materials and Methods
2 Materials and Methods

2.1 Cell culture techniques

mHypoE-37 neurons were grown in Delbucco’s modified eagle media (DMEM, Sigma-Aldrich, Oakville, Ontario, Canada) containing 4.5 mM glucose, supplemented with 5% fetal bovine serum (FBS, GIBCO, Burlington, Ontario, Canada) and 1% penicillin-streptomycin (pen-strep, GIBCO, Burlington, Ontario, Canada). Cultures were kept in standard cell culture conditions (37°C, 5% CO₂, in humidified incubators). Cells were grown in 150 mm culture dishes to 95% confluency prior to being transferred to 60 mm culture dishes for experimental trials. Cells were dissociated using a 1x-trypsin-EDTA solution (GIBCO, Burlington, Ontario, Canada), resuspended, and diluted using the previously mentioned growing media.

2.2 Experimental protocols

The mHypoE-37 neurons were used in four distinct sets of experiments: (1) untreated to examine basal expression patterns, (2) treated with the saturated fatty acid palmitate, (3) treated with the unsaturated fatty acid DHA, and (4) first pre-treated with DHA followed by a combination of fatty acid treatments. All sets of experiments were repeated with a minimum of three replicates, with each independent trial completed on a separate day and using a different cell passages.

In all cases, the cells for each replicate were transferred from a single mother 150 mm culture dish to 60 mm culture dishes 2 days prior to start of the experiment, allowing cultures to reach an approximate confluency of 60%. The media was then aspirated and replaced with fresh growing media lacking FBS for 12 hours. Following the serum starvation period, cultures were shocked with 30% FBS (by volume) for 30 minutes. The serum shock allows for individual cells to become synchronized with one another.
2.2.1 Basal expression

In the first experiments examining basal circadian mRNA rhythms, cells were placed into the standard growing media following serum shock, this was considered time zero. RNA was then isolated every 3 hours over the entire 36 hours period (Figure 2.1A).

2.2.2 Effects of fatty acids

The second experiment tested the effects of saturated fatty acids. Following serum shock, at time zero, cells were placed in growing media containing either 25 µM palmitate or water vehicle. Similarly, in the third experiment, cells were exposed to 25 µM docosahexaenoic acid (DHA) or dimethylsulfoxide (DMSO, Sigma-Aldrich, Oakville, Ontario, Canada) vehicle immediately following serum shock. RNA was isolated every 3 hours over 36 hours for both experiments 2 and 3 (Figure 2.1 B).

2.2.3 Effects of pre-treating with DHA

In the final experiment, cells were first exposed to either 25 µM DHA or DMSO vehicle for 1 hour following the starvation period. The culture were next serum shocked and then placed in growing media containing either DMSO vehicle, 25 µM DHA, or a co-treatment of 25 µM DHA and 25 µM palmitate. Again, the cells were isolated for RNA every 3 hours over a 36 hour period (Figure 2.1 C).

2.3 Fatty acid preparation

An initial 100 mM stock concentration of palmitate was made by heating 27.8 mg of sodium palmitate (Sigma-Aldrich, Oakville, Ontario, Canada) in 1 mL of molecular grade water (Thermo Scientific, Nepean, Ontario) at 60°C. Similarly, a 100 mM stock concentration of DHA was prepared in DMSO. A subsequent 25 mM working stock was made for both palmitate and
DHA using water and DMSO, respectively. The final experimental concentration of 25 µM palmitate and DHA was made by diluting the working concentration in growing media with or without FBS.

2.4 RNA isolation, cDNA synthesis, and real-time RT-PCR

2.4.1 RNA isolation

RNA was extracted using a modified guanidinium thiocynate method. Prior to isolation, a denaturation solution was made (per sample) composed of 500 µL of water saturated phenol, 500 µL of guanidium thiocynate solution, 50 µL of 2 M sodium acetate, and 3.6 µL of β-mercaptoethanol (Sigma-Aldrich, Oakville, Ontario, Canada).

1 ml of the denaturation solution was added per 60 mm culture dishes. The solution was spread using a plastic scrape tool and collected into a 1.5ml Eppendorf tube. A micropipette was then used to triturate the sample, ensuring all cells were lysed. Once all the samples had been collected, 100 µL of chloroform-isooamyl alcohol (49:1) (Sigma-Aldrich, Oakville, Ontario, Canada) was added to each sample, vortex for 10 seconds, and placed on ice for 15 minutes. Next, the samples were centrifuged for 20 minutes at 4°C at 14,000 rpm, allowing for the sample to separate. The top aqueous layer containing RNA was removed and placed into a new, sterile Eppendorf tube, containing a 20:1 solution of ethanol and 1 M acetic acid. The tubes were inverted and stored at -20°C overnight, allowing for the RNA to precipitate.

The following day, the RNA was pelleted by spinning the samples at 4°C at 14,000 RPM for 30 minutes. The supernatant was discarded and replaced with 1 mL of 75% ethanol. The samples were spun once more at 4 °C at 14,000 RPM for 10 minutes to wash the pellet of remaining organic contamination. The supernatant was carefully aspirated and the pellets were air dried on ice in a laminar flow hood to prevent contamination.
Figure 2.1 Experimental protocols for circadian time courses

All time courses were plated 2 days prior to the serum starve, allowing cells to grow to approximately 60% confluency.  

A) For the analysis of basal circadian rhythms, neurons were serum starved for 12 hours and then received a serum shock. Following the serum shock, the media was replaced with growing media.  

B) Effects of individual treatment of either saturated or unsaturated fatty acids. Following the serum shock, cells were exposed to either 25 μM DHA or 25 μM palmitate.  

C) DHA pre-treatment. Prior to the serum shock cells were exposed to 25 μM DHA or DMSO control for 1 hour. Cells were then shocked and subsequently exposed to DMSO, 25 μM DHA alone, or both 25 μM DHA and 25 μM palmitate.
Finally, the RNA pellets were diluted in molecular grade water and subsequently quantified using the NanoDrop 2000c spectrophotometer (Thermo Scientific, Nepean, Ontario) to determine concentration and purity.

2.4.2 Complementary DNA (cDNA) synthesis

Due to the sensitive nature of real-time RT-PCR, DNA found within each sample must be removed to ensure that only the desired mRNA is amplified. Genomic DNA was eliminated with the Turbo DNase kit (Ambion, Streetsville, Ontario, Canada) as per manufacturer’s protocol. The DNAase was inactivated with EDTA in order to protect the subsequently synthesized cDNA. Single stranded cDNA required for real-time reverse transcriptase polymerase chain reaction (real-time RT-PCR) was synthesized using the High-Capacity cDNA Reverse Transcription Kit (Applied Biosystems, Streetsville, Ontario, Canada) as per manufacturer’s instructions.

2.4.3 Real-time RT-PCR (qRT-PCR)

cDNA was amplified using a qRT-PCR master mix, gene-specific primers (15 μM), and platinum Taq (pTaq) (5U/μL). The qRT-PCR master mix was prepared with 0.3X SYBR® Green, 1X ROX reference dye, 2 mM dNTP, 50 mM MgCl₂, and 1X PCR buffer (Invitrogen, Burlington, Ontario, Canada). Samples and reagents were loaded in triplicate into 384-well plates and placed in an Applied Biosystems (ABI, Streetsville, Ontario, Canada) Prism 7000 Sequence Detection System machine for amplification and detection. Results were analyzed using the ABI Sequence Detection System (SDS) version 2.4 software.

The PCR thermocycling protocol had 5 distinct phases: 2 minutes at 50°C, 10 minutes at 95°C, 40 amplification cycles of 95°C for 15 seconds and 60°C for 1 minutes, followed by the final dissociation step of 95°C for 15 seconds, 60°C for 15 seconds, then 95°C for another 15 seconds.
2.4.4 Primers

Primers were designed using the Integrated DNA Technologies (IDT) website’s program, Primer Quest (www.idtdna.com). Primers were also synthesized by IDT. Theoretical primer products were cross-referenced using the National Centre for Biotechnology Information (NCBI) basic local alignment search tool (Primer BLAST) program (www.ncbi.nlm.nih.gov/tools/primer-blast/).

All newly designed qPCR primer products were run on an agarose gel to confirm amplicon size using a 50 base-pair ladder (Fermentas, Burlington, Ontario, Canada). The newly designed qPCR primer products were also isolated and purified using the QIAquick PCR product purification kit (Qiagen, Mississauga, Ontario, Canada) and sent for sequencing at The Centre for Applied Genomics (TCAF) (The Hospital for Sick Children, MaRS Centre, Toronto, Ontario, Canada). Sequencing results were again cross-referenced against the BLAST database.

2.5 Statistical analysis

2.5.1 qRT-PCR analysis

The relative mRNA expression was calculated by first normalizing the gene of interest to the expression of histone 3a. Normalized values were then standardized by averaging all of the normalized values in each replicate and then dividing each individual normalized value by the average. The standardized values from each treatment-specific time point within each replicate were averaged and then the corresponding standard error was calculated.

2.5.2 Circadian analysis

The period, amplitude, and acrophase was calculated for each Bmal1, Per2, and Rev-erba for each replicate using the statistical software OriginPro 8.5 (OriginLab Corp, Northampton,
MA). These values were calculated using cosinor analysis, which uses the least squares method to fit a cosine wave to the input values. The average and standard error was then calculated for the period, amplitude, and acrophase of each gene. One-way analyses of variance (ANOVA), followed by Bonferroni’s multiple comparison test, and student’s t-test were conducted using GraphPad Prism 6 (GraphPad Software Inc., San Diego, CA) software to determine statistical differences between treatment groups. Differences were considered to be statistically significant if p < 0.05.

Figure 2.2 Representative circadian cosinor graph

Following mRNA analysis, the expression profile of clock genes were examined using a cosinor analysis. This thesis examined three of the key components of the cosinor output: period, amplitude, and acrophase. Period length refers to the time it takes for one complete cycle. Amplitude is a measurement of the difference between the highest point and the mid-point of the cycle, or mesor. Acrophase is the time at which the cycle peaks.
Table 2.1 qRT-PCR primer sequences

<table>
<thead>
<tr>
<th>Gene</th>
<th>Primer Sequence (5’ – 3’)</th>
<th>Amplicon Size</th>
<th>Annealing Temp</th>
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</thead>
<tbody>
<tr>
<td>AgRP</td>
<td>F: CGGAGGTGCTAGATCCACAGA</td>
<td>69</td>
<td>60°C</td>
</tr>
<tr>
<td></td>
<td>R: AGGACTCGTGAGCAGCCTTACAC</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Bmal1</td>
<td>F: GGGAGGCCCACAGTCAGATT</td>
<td>78</td>
<td>60°C</td>
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<tr>
<td></td>
<td>R: GTACCAAAAGAAGCCAAATTTCATCAA</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Clock</td>
<td>F: CACCGACAAGATCCCTACTGAT</td>
<td>151</td>
<td>60°C</td>
</tr>
<tr>
<td></td>
<td>R: TGAGACATCGTGCTGCTGT</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Histone 3a</td>
<td>F: CGCTTCCAGAGTGAGCAGCTATT</td>
<td>72</td>
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<tr>
<td></td>
<td>R: ATCTTCAAAAAAGGCCAACCAGAT</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Per2</td>
<td>F: TCATCATTTGGAGGCACAAA</td>
<td>135</td>
<td>60°C</td>
</tr>
<tr>
<td></td>
<td>R: GCATCGTAGCCGTTGGGTT</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Rev-erbα</td>
<td>F: TGGAGACACAGCAGCGAGCTG</td>
<td>114</td>
<td>65°C</td>
</tr>
<tr>
<td></td>
<td>R: CATAGTGGAAACCTGAGGCA</td>
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<td></td>
</tr>
<tr>
<td>IL-6</td>
<td>F: GGGCTAAGGACCAAGCAACAA</td>
<td>85</td>
<td>60°C</td>
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<td></td>
<td>R: GGTGGGCGGAGTACAAGAGAAGACTCA</td>
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<td>TLR4</td>
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<tr>
<td></td>
<td>R: GCTCAGATCTAAAGTCTCTTGG</td>
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<td></td>
</tr>
<tr>
<td>IκB</td>
<td>F: TGCTGGCCAGTGTAGCAGTCTT</td>
<td>150</td>
<td>60°C</td>
</tr>
<tr>
<td></td>
<td>R: CAAAGTCACCAAGTGCTCCACGAT</td>
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Chapter 3
Results
3 Results

3.1 The mHypoE-37 neurons exhibit robust circadian rhythms

Studies suggest that every cell possesses the ability to exhibit a circadian rhythm. Additionally, it is well known that transcript levels of core circadian genes oscillate in an approximately 24-hour rhythm [247]. If the connection between a cell and the master regulator is lost, a cell will lose it rhythmicity over time. However, cells in culture can be synchronized to one another using a serum shock [348].

In order to test this theory and establish a functional cellular circadian model, the mHypoE-37 neuronal cell line were screened using RT-PCR to determine whether they express the core circadian genes, as well as a multitude of hypothalamic neuropeptides, receptors, and signalling molecules. Screening results indicated that the mHypoE-37 cells strongly express the core circadian genes, Bmal1, Per2, Rev-erba, and Clock. These neurons were also found to express genes involved in both energy homeostasis and inflammation, such as AgRP, IL-6, and TLR4. Furthermore, under confocal microscopy, mHypoE-37 cells exhibited classical neuronal morphology in culture (Figure 1.3 B).

Next, to evaluate whether the mHypoE-37 neurons express the core circadian genes, Bmal1, Per2, Rev-erba, and Clock, in a circadian manner, RNA was harvested from culture every 3 hours following serum shock for 36 hours. All three genes showed consistent, robust rhythms in each replicate (n = 3). Bmal1, Per2, and Rev-erba were all found to have period lengths of approximately 24 hours (26.52 h ± 1.39, 22.52 h ± 0.48, and 26.28 h ± 0.62, respectively) (Figure 3.1 A). The transcriptional profile of the circadian gene Clock was also examined; however, this gene was not found to oscillate in a circadian manner (Figure 3.1 A).

Finally, it is well-established that the mRNA transcripts of the negative and positive arms of the circadian cycle oscillate in opposing phases [84, 89, 90]. Indeed, a superimposition of the transcriptional profiles of Bmal1-Per2 and Bmal1-Rev-erba revealed that these genes cycle in opposing phases (Figure 3.2 B). Given that the mHypoE-37 neurons possess the necessary
cellular circadian machinery and cycle in a rhythmic manner reflective of the physiological arms of the circadian clock, this cell line functions as a viable model for circadian molecular studies.

3.2 Saturated fatty acids significantly alter circadian rhythms

To explore the effect that saturated fatty acids have on the molecular circadian system, the mHypoE-37 neurons were treated with the sixteen-carbon saturated fatty acid, palmitate. Palmitate is the most common product of fatty acid synthesis and accounts for approximately 30% of all total free fatty acids [253]. Transcriptional analysis included an examination of the expression profile of each gene for changes in period length, amplitude, and acrophase, to identify circadian changes induced by fatty acid treatment.

Following serum shock synchronization and palmitate exposure, only Bmal1 exhibited significant changes in its periodicity. Treatment with palmitate (P) was found to increase the Bmal1 period length by 24.40 hours, compared to control (water control (C) = 31.53 h ± 1.07 vs P = 55.93 h ± 1.674, n=4, p < 0.001) (Figure 3.2 A). The period length of both Per2 and Rev-erba were nominally affected by palmitate (Figure 3.3 A, Figure 3.4 A). Palmitate was also found to modify the circadian expression profile of Bmal1 by significantly increasing its amplitude (C = 0.4508 ± 0.0321, P = 0.9139 ± 0.0396, n=4, p < 0.001) (Figure 3.2 B). Similarly, the Per2 rhythm was also found to have an elevated amplitude (C = 0.5555 ± 0.4591, P = 0.6991 ± 0.0248, n=4, p < 0.05) (Figure 3.3B). No changes were detected with respect to the amplitude of Rev-erba (Figure 3.4 B).

Finally, the circadian profiles were examined for changes in acrophase – a measurement of the time at which a cycle peaks. Palmitate exposure altered the acrophase of both Bmal1 and Rev-erba in the mHypoE-37 neurons. The Bmal1 acrophase was significantly increased from 6.922 h ± 0.5433 to 51.60 h ± 1.519, a difference of 44.67 h (n = 4, p < 0.001) (Figure 3.2 C). Similarly, the Rev-erba acrophase shifted from 17.31 h ± 0.6599 to 19.59 h ± 0.4495 in response to palmitate (n = 4, p < 0.05) (Figure 3.4 C). No significant variations were found in the Per2
Figure 3.1 Basal expression of core circadian genes in the mHypoE-37 cell line

The mHypoE-37 neurons were placed in serum free media for 12 hours prior to receiving a 30% serum shock. Following synchronization, total RNA was harvested every 3 hours for 36 hours. RNA was subsequently converted into cDNA and used for qRT-PCR with Bmal1, Per2, Rev-erbα, and Clock specific primers. All mRNA values are normalized to histone 3a expression. N=3 for all experiments. A) Bmal1, Per2, and Rev-erbα in the mHypoE-37 cell line are expressed in circadian manner with a period length of 26.52 h ± 1.39, 22.52 h ± 0.48, and 26.28 h ± 0.62, respectively. Transcription of Clock is not rhythmic. B) Superimposition of Bmal1-Per2 and Bmal1-Rev-erbα expression profiles reveals that the genes are cycling in opposing phases. Values are plotted as mean values ± SEM.
acrophase. Taken together, these results suggest that palmitate can dramatically alter all aspects of certain clock genes (Figure 3.3C).

3.3 25 µM Palmitate does not induce inflammation

Fatty acids have recently been identified as mediator of hypothalamic inflammation, commonly associated with obesity [307]. Therefore, the effect of palmitate on the transcript levels of key inflammatory genes was investigated. In the mHypoE-37 neurons, the inflammatory genes, TLR4, IκB, and IL-6, were found to be expressed at levels detectable by qRT-PCR. qRT-PCR analysis revealed minor, insignificant increases in IL-6 and TLR4 transcript levels, while non-appreciable changes were observed in IκB (Figure 3.5 A-C).

Furthermore, fatty acids have been found to alter levels of neuropeptides involved in energy homeostasis [287, 288]. Therefore, transcript levels of the orexigenic neuropeptide AgRP were examined following palmitate treatment. Contrary to the literature, AgRP levels were not found to fluctuate upon fatty acid exposure (Figure 3.5 D).

3.4 Omega-3 fatty acids have little effect on circadian rhythms

Conversely to the negative effects attributed to the saturated fatty acids, omega-3 unsaturated fatty acids are thought to illicit beneficial, protective health effects [313-318]. To test whether this hypothesis also applies to the circadian system, the mHypoE-37 cell line was treated with the omega-3 fatty acid, docosahexaonic acid (DHA), the primary structural component of the cerebral cortex that represents nearly 40% of all unsaturated fatty acids in the brain [348]. As with the palmitate studies, cultures were exposed to 25 µM of DHA immediately following serum shock.

Similar to that of the palmitate experiment, the period length of Bmal1 was increased upon exposure to DHA compared to DMSO control (36.29 h ± 0.2781, 43.22 h ± 0.7213, n=4, p < 0.001, Figure 3.2 A). However, it should be noted that the DHA-induced increase in period length was approximately 17 hours less than that observed with palmitate treatment. DHA
treatments were also found to slightly decrease the period length of both *Per2* and *Rev-erba*, but these changes did not reach statistical significance (Figure 3.3 A and Figure 3.4 A). Likewise, cultures exposed to DHA had no significant changes in either amplitude or acrophase for any of the circadian genes (Figure 3.2-3.4).

### 3.5 DHA downregulates IL-6 mRNA expression

As with the saturated fatty acid experiments, the effects of omega-3 fatty acids on the cellular inflammatory response were also studied. Transcript levels of the pro-inflammatory cytokine, *IL-6*, were found to be significantly downregulated between the hours of 21 and 33, inclusively (Figure 3.6 A). DHA did not alter the expression of *IkB* nor *TLR4* (Figure 3.6 B, C). However, *IkB* was slightly elevated during the second half of the time course, although this did not reach significance. Moreover, no changes were found in the transcription of *AgRP* throughout the duration of the time course (Figure 3.6).

### 3.6 DHA protects against palmitate-induced circadian effects

In order to evaluate whether DHA could confer its protective effects and prevent the previously described alterations in circadian rhythms induced by palmitate, cultures were exposed to 25 μM DHA or DMSO control for 1 hour prior to synchronization. Following synchronization, cultures were then treated with either vehicle control (DMSO-DMSO), 25 μM DHA alone (DHA-DHA), or a 25 μM DHA and 25 μM palmitate co-treatment (DHA-DHA+P).

The observed increase in the period length of *Bmal1* following palmitate treatment was nearly eliminated in the presence of 25 μM DHA. In the initial palmitate experiment, exposure to palmitate alone resulted in a 24.40 hour increase in *Bmal1* periodicity; however, this increase was reduced to 4.28 hours in the DHA-DHA+P group, compared to DMSO-DMSO control (DMSO= 37 h ± 3.033, DHA-DHA = 37.40 h ± 1.629, DHA-DHA+P = 41.46 h ± 1.920, n=4, Figure 3.2 A). As expected, there were no statistical differences detected in either *Per2* or *Rev-erba* period length (Figure 3.3 A and Figure 3.4 A).
DHA pre-treatment was also found to abolish the previously described increase in the amplitude of *Bmal1* (Figure 3.2 B). In contrast, pre-treatment did not protect against palmitate-induced increase in the amplitude of *Per2* (Figure 3.3 B). Indeed, it should be noted that pre-treating the mHypoE-37 cell line with DHA followed by an exposure to DHA alone lowered *Per2* amplitude, compared to DMSO-DMSO control. However, with the addition of palmitate in the co-treatment experimental group, *Per2* amplitude was still found to be elevated compared to both DMSO-DMSO and DHA-DHA (DMSO-DMSO = 0.6326 ± 0.0419, DHA-DHA = 0.5980 ± 0.0195, DHA-DHA+P = 0.7934 ± 0.0463, n = 4, p < 0.05, Figure 3.3B).

Finally, exposing the mHypoE-37 neurons to with DHA prior to palmitate also protected against the palmitate-associated changes in acrophase. The large palmitate-induced shift seen in the *Bmal1* acrophase was reversed upon pre-treatment with DHA (Figure 3.2 C). The same restorative effect was also found with respect to *Rev-erba* (Figure 3.4C). However, the acrophase of *Per2* did not change following palmitate treatment alone, and no differences were found between the three treatment groups (Figure 3.3 C).

Overall, these data suggest that DHA possesses the potential to confer a protective effect against saturated fatty acid-induced changes in the molecular circadian system.
Figure 3.2 Effects of saturated and unsaturated fatty acids on the expression profile Bmal1

The effects of fatty acids on the circadian system in the mHypoE-37 neurons were completed in two sets of experiments: (1) exposure to an treatment of either 25 μM DHA or palmitate following serum shock - first and second column - and (2) pre-exposure to 25 μM DHA prior to serum shock and then co-treatment with 25 μM DHA and palmitate, post-serum shock – the last column. Total RNA was harvested every 3 hours for 36 hours and subsequently used for qRT-PCR. All values were normalized to histone 3a. Results were subjected to cosinor analysis to determine the period length, amplitude, and acrophase of each gene. Treatment with 25 μM palmitate resulted in an increase in the period and acrophase of Bmal1 by 24.40 h and 44.67h, respectively (A, C). Treatment with 25 μM DHA resulted in an increase in the period of Bmal1 by 6.92h (A). Palmitate treatment also elevated the amplitude of Bmal1 (B). Pre-treatment with 25 μM DHA prevented all palmitate-induced modifications in the expression profile of Bmal1 (A, B, C). Values are plotted as mean values ± SEM. N=4 for all experiments. *** = P < 0.001.
The effects of fatty acids on the circadian system in the mHypoE-37 neurons were completed in two sets of experiments: (1) exposure to either 25 μM DHA or palmitate following serum shock - first and second column - and (2) pre-exposure to 25 μM DHA prior to serum shock and then co-treatment with 25 μM DHA and palmitate, post shock – the last column. Following synchronization, total RNA was harvested every 3 hours for 36 hours and subsequently used for qRT-PCR. All values were normalized to histone 3a. Results were next subjected to cosinor analysis to determine the period length, amplitude, and acrophase. Treatment with 25 μM palmitate resulted in an increase in the amplitude of Per2 (B). No significant changes were found in the expression profile of Per2 following DHA treatment. Pre-treatment with 25 μM DHA was not successful in reducing the palmitate-induced increase in the acrophase of Per2 (B). Values are plotted as mean values ± SEM. N=4 for all experiments. * = P < 0.05.
Figure 3.3 Effects of saturated and unsaturated fatty acids on the expression profile of Rev-erba

The effects of fatty acids on the circadian system in the mHypoE-37 neurons were completed in two sets of experiments: (1) exposure to an treatment of either 25 μM DHA or palmitate following serum shock - first and second column - and (2) pre-exposure to 25 μM DHA prior to serum shock and then co-treatment with 25 μM DHA post shock – the final column. Total RNA was harvested every 3 hours for 36 hours and subsequently used for qRT-PCR. All values were normalized to histone 3a. Results were next subjected to cosinor analysis to determine the period length, amplitude, and acrophase. Treatment with 25 μM palmitate resulted in an increase in the acrophase of Rev-erba (C). No significant changes in the expression profile of Rev-erba following DHA treatment alone (A, B, C). Pre-treatment with 25 μM DHA was found to revert the increase in the acrophase of Rev-erba (C). Values are plotted as mean values ± SEM. N=4 for all experiments. * = P < 0.05.
Figure 3.4 Effects of saturated fatty acids on inflammatory and metabolic gene transcription

The mHypoE-37 cell line was treated with 25 μM palmitate following serum shock. Total RNA was harvested every 3 hours for 36 hours and subsequently used for qRT-PCR. All values were normalized to histone 3a. Exposure to palmitate did not alter the expression of any examined inflammatory genes, IL-6, TLR-4, or IκB, or the orexigenic neuropeptide, AgRP (A-D). Values are plotted as mean values ± SEM. N=4 for all experiments.
Figure 3.5 Effects of unsaturated fatty acids on inflammatory and metabolic gene transcription

The mHypoE-37 cell line was treated with 25 μM DHA following serum shock. Total RNA was harvested every 3 hours for 36 hours and subsequently used for qRT-PCR. All values were normalized to histone 3a. A) Exposure to 25 μM DHA significantly reduced the expression of *IL-6* between the hours of 21 and 33, inclusively. However, DHA treatment did not alter the expression of *TLR-4*, *IκB*, or *AgRP* (B-D). Values are plotted as mean values ± SEM. N=4 for all experiments. * = P < 0.05.
Chapter 4
Discussion
4 Discussion

4.1 General discussion

Circadian rhythms are vital to the well-being of all organisms. In mammals, the hypothalamic SCN functions to coordinate the proper biological rhythms with the appropriate time of day. Dysregulation of the circadian system leads to detrimental alterations in normal physiology, as clearly demonstrated with obesity and shift workers [10, 215-217]. Recently, studies have identified the potential for dietary SFAs to contribute to circadian dysregulation [8]. Moreover, the association between SFA and the onset of obesity suggests that both FAs and obesity can positively feedback into circadian dysregulation, thus furthering the progression of both conditions.

The adverse effects of SFA on both energy homeostasis and circadian rhythms have also been documented in the hypothalamus [307, 308, 354]. Conversely, recent evidence has demonstrated that PUFAs can potently alleviate the negative physiological effects induced by obesity and T2DM [295, 320, 332]. However, the role of PUFAs on circadian rhythms has yet to be examined. Given that the hypothalamus is a key endocrine regulator of numerous key physiological processes, the purpose of this study was to investigate the individual and combined effects of SFA and PUFAs on hypothalamic regulation of circadian rhythms. Here, the mHypoE-37 cell line served as a model for circadian molecular research. Next, the individual effects of both SFA and PUFAs on circadian rhythms of hypothalamic neurons were analyzed. Finally, it was examined whether PUFAs could prevent SFA-induced changes to hypothalamic circadian rhythms.

4.2 Molecular circadian rhythms in mHypoE-37 neurons

In order to test the validity of the mHypoE-37 neurons as a model for circadian studies, mRNA from the cell line was isolated and screened for key circadian genes. Of importance, the mHypoE-37 cell line strongly expressed Bmal1, Per2, Rev-erba, and Clock mRNA transcripts. Once the presence of core circadian genes had been confirmed, the rhythmicity of these
transcripts was evaluated by collecting the total RNA every 3 hours over a 36 hour time course. However, without a synchronization protocol, the mRNA expression profile of the circadian genes from the mHypoE-37 cells do not exhibit any predictable circadian pattern (data not shown).

Although ex-planted SCN tissue and SCN cell lines can transcribe mRNA in a circadian manner in culture, all other cells types in vitro require a synchronization protocol [339-341]. Previous work in the Belsham laboratory has demonstrated that a 20% serum shock is sufficient to induce circadian synchronicity; however, preliminary experiments with the mHypoE-37 neurons found that 20% did not generate robust rhythms (data not shown) [354, 355]. Therefore, cells were next exposed to a 30% FBS shock for 30 minutes, which in turn generated robust, circadian clock gene expression. To date, the exact factor(s) responsible for inducing synchronization in vitro are not known. Elucidating this phenomenon has proved difficult owing to the extensive and complex composition of serum; however, studies suggest that synchronization activates pathways involved in PKC, cAMP, and calcium influx [356].

Unlike in vivo where the SCN consistently synchronizes slave clocks, the serum shock protocol lacks the ability to continually keep cells synchronized to one another and therefore, non-SCN cultured cells will eventually desynchronize from one another [186, 357]. As seen in each of the experiments in this thesis, the expression profiles of all the circadian genes exhibits dampening over duration of the time course. Circadian dampening is thought to be a result of the drifting of mRNA transcription amongst individual cells and the changes in available nutrients and cellular by-products, including secreted factors, in the culture media [357].

Following the serum synchronization protocol and qRT-PCR analysis, statistical analysis is required to determine if the mRNA transcript profile is circadian or not. Many early circadian mRNA studies used ANOVAs comparing peak and troughs to identify significant trends [358-360]. While, this method may prove beneficial for in vivo studies where collecting numerous data points may not be feasible, this statistical analysis overlooks many transcriptional events between data points and does not compare the data to a rhythmic function. More recent work has
developed a cosinor analysis method, which utilizes a least squares method to fit all data points in a data set to a cosine wave curve [361, 362].

Using the cosinor circadian analysis, the mHypoE-37 cells were found to rhythmically express the core circadian genes, \textit{Bmal1}, \textit{Per2}, and \textit{Rev-erba}, as seen in Figure 3.1 A. All three genes exhibited robust circadian rhythms, cycling with an approximately 24 hour period. The transcriptional profile of \textit{Clock} was also analyzed, but was did not display circadian properties (Figure 3.1 A). While the non-rhythmic expression of \textit{Clock} in the mHypoE-37 cell line conflicts with previous reports from the Belsham lab given that the mHypoE-44 neurons expressed \textit{Clock} in a circadian manner with an approximate period of 25 hours [189], many other groups have reported non-rhythmic expression of \textit{Clock} in other cell types, including the immortalized vascular smooth muscle cell line, Movas-1, GT1-7 neurons, primary retinal tissue, and even primary SCN tissue [77, 244, 245, 348, 358]. Here, it is possible that the paralog of CLOCK, NPAS2, was actively substituting for Clock, thus reducing the expression of \textit{Clock}.

The circadian mRNA expression profile of the mHypoE-37 model is comparable to that of other serum shock cell lines [354, 358, 363]. Post serum shock, the acrophase for each \textit{Bmal1}, \textit{Per2}, and \textit{Rev-erba} was approximately 8 hours, 1 hour, and 18 hours, respectively. Given that the expression of \textit{Bmal1} peaks inbetween that of \textit{Per2} and \textit{Rev-erba}, its negative transcriptional regulators, this is indicative that these genes are oscillating in correct phase to one another. This phenomenon was confirmed by superimposing the transcriptional profiles of \textit{Bmal1-Per2} and \textit{Bmal1-Rev-erba}, as seen in Figure 3.1 B, whereby the individual mRNA transcripts cycle in opposing phases. Overall, given that the mHypoE-37 cell line possesses the necessary cellular circadian machinery and that the core circadian genes are expressed in the correct temporal-rhythmic manner, these data validate this cell line as a functional model for circadian molecular studies.
4.3 Transcriptional effects of palmitate on mHypoE-37 neurons

HFDs and SFAs are recognized as major contributors to obesity, as well as obesity-associated pathologies, including circadian dysregulation [8, 10, 308]. In 2007, Kohsaka and colleagues clearly demonstrated that HFDs possesses the ability to disrupt circadian rhythms in mice [8]. Here, animals were placed on a HFD for 6 six and maintained on a standard 12:12 light/dark cycle. Elongation in circadian period length was detected as earlier as 2 weeks, and continued to increase over the 6 week duration. Additionally, mice on the HFD had altered expression of core circadian genes, including Clock, Bmal1, Per2, and Rev-erba, as well as modified transcript levels of the hypothalamic neuropeptides, AgRP, NPY, and POMC. In a similar fashion, the mHyoE-37 neurons were indeed found to have altered circadian rhythms following SFA exposure. Of particular importance, palmitate induced a dramatic increase in the period length of Bmal1, increases in the amplitude of both Bmal1 and Per2, and significant shifts in the acrophase of Bmal1 and Rev-erba.

Variations in circadian periodicity and acrophase can alter the time-appropriate expression of clock genes. Similarly, increases in clock gene amplitude can potentially increase availability of clock proteins, given that more transcripts are available for translation. Additionally, considering that clock proteins act as transcription factors for 10% of the transcriptome, variations in the presence and availability of clock proteins not only alters core circadian rhythms, but also changes the expression of genes involved in nearly every aspect of physiology [100, 101].

SFAs are thought to induce their cellular effects through numerous methods, such as generation of reactive oxygen species (ROS) and endoplasmic reticulum (ER) stress, impaired glucose action, and activation of signalling molecules [247, 364]. More recent evidence has recognized a role for SFAs in modulating miRNA levels, particularly in the regulation of circadian transcript stability. Currently, only miRNA 194 has been implicated in both palmitate and circadian regulation; however, it has only been demonstrated to decrease the translation of the Per genes and has not been shown to regulate mRNA stability [117-119, 307, 365]. Given
that miRNA studies are still in their infancy, it is difficult to rule out the role of palmitate in miRNA-mediated changes in clock gene mRNA stability.

With respect to signalling molecules, GSK3β is activated by SFAs and known to modulate acetylation status [126, 129, 366]. For instance, previous studies have reported the potential for palmitate to induce BMAL1 acetylation causing its cytoplasmic sequestration by CRY1, and thus preventing CLOCK-BMAL1 mediated transcription [354]. In turn, this reduces the transcription and translation of REV-ERBα, subsequently prolonging the transcriptional inhibition of Bmal1 by REV-ERBα. Additionally, activation of GSK3β can alter the stability of REV-ERBα, thereby further reducing the suppression of Bmal1. Conversely, it is important to note that inhibition of GSK3β upregulates the transcription of Per2, which is contradictory to the palmitate-induced increase in the amplitude of Per2 [367].

In a similar method, palmitate-induced circadian changes may be a result of modified HDAC activity, such as that of SIRT1. Activated by NAD+, the activity of SIRT1 is upregulated during fasting and energy expenditure, and reduced upon feeding [144-146, 195-198]. Supplementing the media with palmitate produces a positive energy environment for mHypoE-37 cells and consequently reduces the levels of NAD+ and activation of SIRT1. With reduced SIRT1 HDAC activity, acetylated levels of BMAL1 would remain high, again delaying the entry of CLOCK-BMAL1 into the nucleus, preventing transcription of the negative arm of the feedback loop. While a reduction in SIRT1 accounts for the increase in Bmal1 transcription, it conflicts with the increase in Per2 amplitude. Considering the conflicting evidence with regards to GSK3β and SIRT1, the palmitate-mediated modulation of the Per2 expression profile seen in the mHypoE-37 neurons opposes all the currently reported mechanisms for Per2 regulation, and therefore requires further investigation.

In addition to the association of SIRT1 with NAD+, SIRT1 also interacts with other metabolic sensors, including the PPARs, presenting an additional link between metabolism and circadian rhythm [211, 212]. The NRs PPARs are transcription factors activated by various nutrients, including FAs and their derivatives. Conversely, the activity of PPARγ is suppressed by the induction of SIRT1. Activation of both PPARγ and PPARα induces the transcription of
Bmal1 [208, 209]. Taken together, the suppression of SIRT1 and activation of the PPARs by palmitate provides a potential mechanism for the dramatic changes seen in expression profile of Bmal1.

Although palmitate severely impacted molecular circadian rhythms, palmitate did not alter the expression of either inflammatory nor AgRP mRNA. Numerous studies have indeed reported that HFD and SFAs modulate the expression and secretion of the pro-inflammatory cytokines, including IL-6, and orexigenic neuropeptides, NPY and AgRP [287, 288, 291, 293, 294]. While the IL-6 expression in mHypoE-37 neurons following palmitate treatment was slightly elevated, it did not reach statistical significance. Supplemental experiments attempted to induce inflammation with higher concentrations of palmitate; however, initial trials indicated that the mHypoE-37 neurons could not tolerate the increased concentration as seen through a loss in cell viability. Overall, the limited effects induced by palmitate on inflammatory cytokines suggests that the hypothalamic molecular clock is more susceptible to the detrimental effects of palmitate, compared to that of the hypothalamic inflammatory response.

Taken together, these data establish the negative effects palmitate exerts on the circadian clock in the hypothalamic mHypoE-37 cell line. This information was then used as a set point to determine whether omega-3 FAs can protect against SFA circadian effects.

### 4.4 Transcriptional effects of DHA on mHypoE-37 neurons

PUFAs have recently gained much attention for their potential to combat obesity and T2DM [15-17]. Omega-3 FAs are now known to exert actions in the hypothalamus; however, there is a paucity of studies examining their effects on circadian rhythms [330, 332]. To date, omega-3 FAs have only been implicated in the daily rhythm of melatonin, osteocalcin, and urinary N-telopeptide [368, 369]. These studies have identified that diets deficient in PUFA weaken the circadian rhythms of these hormones, but do not present any supporting data. Given this lack of molecular evidence, the mHypoE-37 cell line was utilized to investigate a potential role of omega-3 FAs in circadian molecular rhythms.
Compared to the SFA experiments, mHypoE-37 neurons exposed to DHA demonstrated minimal effects on the expression profiles of circadian genes. DHA had no significant effects on the acrophase or amplitude of any of the clock genes analyzed. Similarly, DHA was not found to alter the period length of Per2 or Rev-erba. However, DHA did elongate the period of Bmal1. Changes in the expression profile of Bmal1 may be accounted for through activation of DHA’s membrane receptor, GPR120. Given that GPR120 is a Ga_{q/11}, binding of omega-3 FAs activates phospholipase C, cleaving phospholipid phosphatidylinositol 4,5-bisphosphate into DAG and inositol 1,4,5-trisphosphate, resulting an overall influx of cytosolic calcium [332]. This increase in calcium may modulate circadian gene expression, given that non-selectively blocking the voltage-dependent calcium channels in SCN 2.2 cell abolishes the circadian rhythm of Bmal1 [370]. As such, this can be confirmed by synthetically activating GPR120 with GW9508 and examining the resultant changes in Bmal1 expression [332].

The effect of DHA on the expression profile of Bmal1 may also be induced by the same mechanism hypothesized with the palmitate-induced circadian changes. As previously mentioned, activation of both PPARα and PPARγ by omega-3 FAs can stimulate the expression of Bmal1, thus resulting in an atypical expression profile [210-212]. Additionally, PPARγ may also explain the robust downregulation in IL-6 mRNA expression following DHA treatment. This corresponds with many other studies, whereby ICV injections and dietary supplementation with omega-3 FAs significantly reduces inflammation, including the transcription and secretion of IL-6 [295, 320, 332]. Omega-3 FAs are thought to suppress inflammation through PPARγ a two-pronged approached [333-335]. Firstly, PPARγ is a functional transcription factor which can directly reduce inflammation. Secondly, PPARγ is thought to inhibit the inflammatory response induced by NF-κB, however, it is still unclear whether this effect is accomplished by omega-3 FAs directly or indirectly through metabolites [371]. While NF-κB is classically considered as the key mediator of the inflammation response, a recent study has demonstrated that NF-κB activation was suppressed following co-treatment with palmitate and DHA without actually altering the expression of IL-6, suggesting that regulation of NF-κB and IL-6 may occur through independent mechanisms [304].
Although DHA was found to have minimal effects on the circadian molecular clock, this does not provide insight into potential protective abilities of DHA. Therefore, in combination with the results collected from the palmitate experiments, these data were used to address the protective effects of DHA.

4.5 DHA protect mHypoE-37 neurons from SFA-induced effects

The recent increased incidence of obesity is generally associated with an overall concomitant decrease in physical activity and increase in poor diet, including those rich in SFAs. Studies are now beginning to understand the varying effects different FAs impose on adiposity, insulin sensitivity, and energy homeostasis [235, 257, 263, 308, 318]. Specifically, PUFAs, especially the omega-3 FAs, have received considerable attention for their anti-obesity effects. Of importance, Cintra et al. examined the role of omega-3 FAs in the hypothalamus of obese rodents [330]. Here, using ICV injections, PUFAs were demonstrated to reduce diet-induced inflammation and adiposity. While PUFAs have been shown to alleviate obesity, as well as its associated symptoms, research has yet to determine if PUFAs can combat the effects of obesity and SFA on circadian dysregulation.

To investigate the whether or not DHA could protect the circadian clock against the circadian effects induced by palmitate, mHypoE-37 neurons were first pre-treated with DHA to potentially confer protective effects and then were co-treated with DHA and palmitate. Overall, DHA did protect the neurons from the effects palmitate exerted on the core circadian genes. Specifically, pre-exposure and co-treatment with DHA dramatically improved the status of the circadian expression profile of Bmal1. Initial experiments found that palmitate increased the periodicity of Bmal1 by 24.4 hours; however, pre- and co-treatment with DHA almost completely abolished this effect, seen through an increase of only 4.28 hours. Similarly, DHA also conferred protective effects with respect to the acrophase of Bmal1, given that the acrophase differed by only 2 hours compared to that of the control group.
Although DHA was successful in reverting the majority of palmitate-induced effects on circadian rhythms, DHA was not able to restore the amplitude of Per2. Activation of SIRT1 by DHA was initially thought to restore native Per2 expression as a result of increased BMAL1 nuclear translocation following deacetylation [354, 372, 373]. While DHA may have combated the regulation of SIRT1 activity induced by palmitate, the level of activation may not have been sufficient enough to suppress the actions of palmitate. As such, the exact reason for the persistent increase in the amplitude of Per2 requires further examination.

In addition to SIRT1, DHA may impart its protective effects through PPARγ activity. However, it is difficult to solely associate these benefits with PPARγ given that it is activated by both saturated and unsaturated FAs. Overall, the protective effects of DHA are more likely to occur through an increased ability for the neurons to block the action of palmitate or to positively utilize palmitate. At the level of the cellular membrane, omega-3 FAs are capable of competing with SFAs for binding of TLR4, thereby subsequently inhibiting the activation of downstream effectors, including the NF-κB inflammatory pathway [374]. Though membrane receptor inhibition may factor into the protective effects of DHA, this may not be a large contributing factor given that palmitate treatment did not appear to activate TLR, as seen through negligible changes in IL-6 mRNA expression.

The potential for omega-3 FAs to combat the effects of SFAs are not restricted to receptor inhibition. In particular, omega-3 FAs have been demonstrated to upregulate FA utilization by stimulating β-oxidation [325-328]. Increases in β-oxidation can occur through multiple pathways. With respect to PUFAs, consumption of the dietary omega-3 FA, eicosapentaenoic acid, activates AMPK [375, 376]. In general, AMPK acts to increase the mitochondrial FA transporter, carnitine parmitoyltransferase 1, thereby leading to an influx of FAs into the mitochondria, and in turn results in FA oxidation [377, 378]. Overall, DHA-induced activation of AMPK may act to decrease the cellular concentration of palmitate, thereby removing the action of palmitate on the circadian clock. It should also be noted that increased AMPK activity can inhibit the transcriptional activation of both PPARα and PPARγ, thereby reducing the induction of Bmal1 expression [379].
Increases in AMPK activity are also known to improve whole cell viability, by reducing levels of ROS and ER stress [380]. Conversely, SFAs are known to elevate levels of ROS and ER stress [247, 364]. Circadian rhythms have a close connection with cellular stress markers, given that circadian knockout models also present with increases in ROS production and ER stress [381]. Whether direct induction of ROS and ER stress causes circadian dysregulation has yet to be determined; however, based on other reciprocal relationships regarding circadian dysregulation, a bi-directional effect seems likely. If confirmed, activation of AMPK by DHA would lower cellular stress markers, in turn removing the negative effect of ROS and ER stress on the circadian molecular rhythms.

The ability for AMPK to revert circadian effects induced by palmitate may be accomplished through a more direct connection to the molecular circadian system. In addition to increasing FA oxidation, activation of AMPK results in higher cytosolic concentration of NAD+ [382]. DHA-induced activation of AMPK would increase the activity of SIRT1, thereby decreasing the acetylation status of BMAL1 and assisting in its nuclear translocation. In turn, this would allow for transcriptional initiation of the negative arm of the circadian cycle, and completion of the entire cycle. Additionally, AMPK acts to phosphorylate the CRY proteins, in turn reduce their stability and potentially shortening the circadian period [199].

4.6 Limitations

The advent of cell lines has significantly contributed to the understanding of both cellular and molecular mechanisms. These in vitro models provide the opportunity to examine complex cellular mechanisms, such as signal transduction pathways that govern both the transcription and translation of essential circadian genes, which is currently not possible in the in vivo hypothalamus. Additionally, the hypothalamic cell line utilized in this thesis allows for the study of a specific set of stimuli, in a controlled environment. The hypothalamic cell lines generated by the Belsham laboratory display distinct neuronal morphology, exhibit contact inhibition, and strongly express an array of neuropeptides, clock genes, receptors, and signalling molecules.
At cellular membrane, DHA can block the binding of palmitate with TLR4, thus preventing its signal transduction. Additionally, DHA can activate AMPK, which can potentially alleviate the circadian impingement induced by palmitate. AMPK upregulates CPT1, thereby reducing the cellular concentration of palmitate through β-oxidation. Alternatively, AMPK acts to inhibit the transcription factor PPARγ, which is activated by palmitate, and can upregulate the transcription of Bmal1. Conversely, transcription of the negative arm can be induced by increases in NAD+ as a result of AMPK activation. Subsequently, SIRT1 deacetylates BMAL1, thus promoting its nuclear translocation and transcription. SIRT1 has also been identified a suppressor of PPARγ activity. Elevated levels of ROS and ER stress by palmitate are reduced by AMPK; however, their relationship with the circadian molecular clock has yet to be elucidated.
[383]. However, for these same reasons, the results presented in this thesis are not without their limitations.

The mHypoE-37 cell line is derived from a single immortalized embryonic neuron [353]. Due to the fact that the embryonic hypothalamus is undergoing continual neurogenesis and that the SV40 immortalization protocol requires dividing cells, embryonic cells are optimal candidates for the generation of cell lines. Since the mHypoE-37 cell line is of embryonic nature, this model may not be representative of fully developed neurons and thus, may function differently than mature adult neuronal cells. Similarly, the use of SV40 T-antigen in the immortalization process can potentially interfere with cellular processes. In particular, inhibition of T-antigen through short-hairpin RNA knockdown has been shown to increase the basal activity of signalling molecules, including Akt, JAK2, and STAT3 (Belsham, unpublished data). Though, it should be noted that cell lines generated amongst the mHypoE-37 neurons have been demonstrated to function similar to adult neurons, including glucose sensing, insulin and leptin signalling, and AMPK activation [190, 246, 247, 384].

Another inherent limitation with the mHypoE-37 neurons is that they lack the heterogeneity present in the in vivo hypothalamus. The experiments conducted in this thesis were performed on a single neuronal cell type. As such, conclusions based on the results found herein cannot be generalized to all neurons nor to the hypothalamus as a whole. Additionally, given that the mHypoE-37 neurons lack the native synaptic connections present within the hypothalamus in vivo, the synthetic environment of the immortalized neuronal cultures does not provide inhibitory or excitatory modulatory effects, as well as lacks the regulatory mechanisms governing the accumulation of secreted cellular products.

Within this thesis, the serum shock protocol and FAs were used to analyze the differential effects fats have on the transcription of essential circadian genes. Although the serum shock protocol allows for cellular synchronization, as seen in vivo, it does not recreate the continual synchronization exerted by the SCN. For this reason, circadian rhythms of cells in culture are not 24 hours. Additionally, the effects of FAs on the circadian system may be amplified due to the time-dependent increase in circadian dysynchrony. Moreover, the experimental use of
constant FA exposure is not necessarily representative of the native circadian fluctuations in FAs availability. Furthermore, this study focuses on mRNA expression and as such, conclusions made regarding changes in protein levels can only be speculative. Although, it should be noted that previous studies have shown that the expression profile of clock proteins is representative of the transcription profile of core circadian genes following the appropriate time-delay.

Overall, while the results presented in this thesis are novel and promising, certain limitations must be considered when evaluating their importance.

4.7 Future directions

This thesis illustrates the effects of both SFA and omega-3 FAs on the expression of key circadian genes. The experiments presented in this thesis demonstrate the important relationship between fats and circadian rhythms; however, many key mechanistic questions remain. The current study has employed the use of the serum shock protocol to synchronize cultures. Initial studies of this research found that the mHypoE-37 neurons required a greater concentration of FBS to induce synchronization; however, once synchronized, these cells demonstrated robust cycling of circadian genes. The precise serum factor(s) that act to generate circadian synchronicity in cell cultures have yet to be determined. Given the robust cyclic expression pattern of the mHypoE-37 cells, it would be valuable to observe if post-serum shock cultured media could itself act to synchronize other non-shocked cultures. Additionally, this thesis has demonstrated that FAs can modulate the circadian system; however, this result calls into question the potential for FAs themselves to act as synchronization factors.

Further, this thesis identified the differing effects both SFAs and PUFAs imposed on the circadian system. While these experiments are novel, these studies did not identify the mechanism by which FAs exert their effects. Of interest, this thesis has presented multiple potential candidates that can act as intermediaries between FAs and circadian rhythms, including the PPAR family and AMPK. The activation of both PPARα and PPARγ by FAs has been implicated with an increase in the transcription of Bmal1. Evidently, further studies are required to analyze the exact ability of both palmitate and DHA to activate the PPARs and in turn, the
ability of the PPARs to modulate clock gene transcription in the mHypoE-37 cell line. Numerous specific PPARα and PPARγ inhibitors have been generated, including MK886 and T0070907, and are readily available for purchase [385-388]. Co-treatment with either palmitate or DHA and a specific PPAR-isofrom inhibitor could determine if the observed effects of FA-associated increases in Bmal1 transcript levels are a result of PPAR activation, as well as whether a specific isoform of PPAR plays a greater role. In a similar manner, inhibition of PPARγ could provide insight into the mechanism behind the DHA-induced suppression of Il-6 expression.

Similarly, the acetylation status can dramatically impact the expression level and timing of clock gene transcription. Given that palmitate is believed to delay the deacetylation of BMAL1, thereby prolonging the initiation of the negative arm of the circadian cycle, the use of synthetic SIRT1 activators would effectively examine the role of HDACs in palmitate-induced circadian modifications [389, 390]. These experiments would further provide insight into the persistent increase in amplitude of Per2, regardless of DHA treatment.

Final experiments examined the protective role of DHA against palmitate-mediated transcriptional changes in the core circadian genes in hypothalamic neurons. Here, DHA appears to exert its effects by increasing the activity of AMPK, thereby restoring native circadian rhythms. A more complete understanding of the role of AMPK in protecting circadian rhythms can be gained by pre-treating the mHypoE-37 neurons with an AMPK activator, such as AICAR or metformin, prior to palmitate exposure [391, 392]. Additionally, inhibiting AMPK with compound C upon co-treatment with DHA and palmitate would further confirm the involvement of AMPK in the protective ability of DHA [393].

Given that this research has solely focused on transcription of clock genes, complementary studies are required to better understand the effects of FAs on circadian rhythms. It would indeed be valuable to replicate these results in an additional cell line. Duplicating these experiments in the novel Per2::Luciferase cell lines would facilitate a cost-effective and simple method to examine the effects of FAs on the rhythmicity of Per2 [346, 347]. Confirming these results would provide a proper foundation for potential future in vivo experiments. Moving
forward, using the model established by Cintra et al., whereby PUFA ICV injections reverted DIO and inflammation, it would be relevant to determine whether DHA could also prevent the HFD-associated increase in circadian rhythms [330].

4.8 Conclusion

The current thesis has characterized a novel hypothalamic cell model for the study of the transcriptional and molecular events underlying circadian rhythms. Herein, the mHypoE-37 cell line has been shown to possess robust rhythmic expression of the circadian clock genes, Bmal1, Per2, and Rev-erba, and demonstrates rhythms representative of those found in vivo. Additionally, the expression profiles of these genes are influenced by the exposure to both SFA and PUFA. Multiple studies have now identified the negative impact of SFA and HFD on obesity, T2DM, and circadian rhythms. Conversely, omega-3 FAs are considered as putative therapeutics for their ability to alleviate the progression of insulin resistance, weight gain, and inflammation. However, little is currently known with respect to the role of omega-3 FAs and circadian gene expression. Thus, this thesis aimed to examine the potential protective abilities of omega-3 FAs on circadian rhythms. As hypothesized, this study is the first to demonstrate that the omega-3 FA, DHA, can ameliorate the negative circadian effects induced by the SFA, palmitate. Taken together, these results contribute to the growing understanding of the various roles FAs play in the circadian system, as well as the hypothalamus.
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Appendix A: OriginPro cosinor analysis output for basal circadian expression of clock genes in mHypoE-37 neurons

- **Per2 mRNA Expression**
  - Period: 22.52 h
  - Amplitude: 0.531
  - Acrophase: 1.43 h

- **Bmal1 mRNA Expression**
  - Period: 26.51 h
  - Amplitude: 0.410
  - Acrophase: 7.94 h

- **Rev-erb mRNA Expression**
  - Period: 26.28 h
  - Amplitude: 0.62
  - Acrophase: 17.87 h
## Appendix B: OriginPro cosinor analysis output for palmitate treatment on clock genes in mHypoE-37 neurons

### Relative Gene Expression

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<thead>
<tr>
<th>Gene</th>
<th>Treatment</th>
<th>Period</th>
<th>Amplitude</th>
<th>Acrophase</th>
</tr>
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<td>Palmitate</td>
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<td>H₂O</td>
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<td>Palmitate</td>
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Appendix C: OriginPro cosinor analysis output for DHA treatment on clock genes in mHypoE-37 neurons

<table>
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<th>Gene</th>
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<th>Acrophase</th>
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<td>DHA</td>
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<td>DHA</td>
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Appendix D: OriginPro cosinor analysis output for DHA pretreatment on clock genes in mHypoE-37 neurons

<table>
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