Circadian Clock Regulated Gene Expression in the Hypothalamus

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Circadian Clock Regulated Gene Expression in the Hypothalamus

Abstract
The master circadian clock in mammals is the suprachiasmatic nucleus of the hypothalamus (SCN). The SCN generates sustained 24 hour rhythms of molecular oscillation and electrical activity, entrains to light, and synchronizes peripheral tissues. The molecular mechanisms involved SCN cell type specific function, rhythm generation, and signaling to and from the periphery remain incompletely understood. To address basic SCN function, we profiled its RNA expression at 2-hour resolution over 48 hours using Affymetrix 1.0ST arrays. We found ~500 cycling transcripts. These data offer crucial insight into circadian regulation of neuronal connectivity, synaptic transmission, ion homeostasis, splicing factors and protein-folding chaperones. We studied the circadian clock’s response to metabolic perturbations using control, low protein (LP), or high fat (HF) in utero diet during gestation and weaning. We also studied interaction of in utero and adult diet in mice given a control or LP in utero diet followed by a control or HF adult diet. Adult mice from all diet conditions were analyzed for circadian locomotor activity (tau) using running wheels. Adult HF diet had a greater effect than HF in utero diet of lengthening tau in both male and female mice. LP in utero diet lengthened tau in male mice, but did not affect female mice. Circadian CNS response to these in utero and adult diet conditions was measured using PER2::LUCIFERASE expression in the SCN and a neighboring hypothalamic nucleus regulating feeding and satiety, the arcuate nucleus. Period length of PER2::LUC was not affected by diet in the SCN, but both male and female mice exhibited trends of lowered baseline and amplitude of PER2::LUC in all dietary perturbation conditions compared with controls. In the arcuate, dietary perturbations showed trends of increasing baseline and amplitude of PER2::LUC in male mice, while the opposite effect was seen in females. Overall, our data show extensive circadian regulation of gene expression as well as response to metabolic conditions.

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CIRCADIAN CLOCK REGULATED GENE EXPRESSION IN THE HYPOTHALAMUS

Heather I. Ballance

A DISSERTATION

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Presented to the Faculties of the University of Pennsylvania

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2015

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Dedication

This work is dedicated to John, Carol, Rachel, and Kelsey Ballance, with love always.
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Chapter 2:

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ABSTRACT

CIRCADIAN CLOCK REGULATED GENE EXPRESSION IN THE HYPOTHALAMUS

Heather I. Ballance

John. B. Hogenesch

The master circadian clock in mammals is the suprachiasmatic nucleus of the hypothalamus (SCN). The SCN generates sustained 24 hour rhythms of molecular oscillation and electrical activity, entrains to light, and synchronizes peripheral tissues. The molecular mechanisms involved SCN cell type specific function, rhythm generation, and signaling to and from the periphery remain incompletely understood. To address basic SCN function, we profiled its RNA expression at 2-hour resolution over 48 hours using Affymetrix 1.0ST arrays. We found ~500 cycling transcripts. These data offer crucial insight into circadian regulation of neuronal connectivity, synaptic transmission, ion homeostasis, splicing factors and protein-folding chaperones. We studied the circadian clock’s response to metabolic perturbations using control, low protein (LP), or high fat (HF) in utero diet during gestation and weaning. We also studied interaction of in utero and adult diet in mice given a control or LP in utero diet followed by a control or HF adult diet. Adult mice from all diet conditions were analyzed for circadian locomotor activity (tau) using running wheels. Adult HF diet had a greater effect than HF in utero diet of lengthening tau in both male and female mice. LP in utero diet lengthened tau in male mice, but did not affect female mice. Circadian CNS response to these in utero and adult diet conditions was measured using PER2::LUCIFERASE expression in the SCN and a neighboring hypothalamic nucleus regulating feeding and satiety, the arcuate nucleus. Period length of PER2::LUC was not affected by diet in the SCN, but both male and female mice exhibited trends of lowered baseline and amplitude of PER2::LUC in all dietary perturbation conditions compared with controls. In the arcuate, dietary perturbations showed trends of increasing baseline and amplitude of PER2::LUC in male mice, while the opposite effect was seen in females. Overall, our data show extensive circadian regulation of gene expression as well as response to metabolic conditions.
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CHAPTER 1: Introduction

1.1 The Ubiquity and Significance of Circadian Clocks

Circadian Rhythms Are Adaptations to Light and Dark Cycles

Twenty-four hour cycles of light and darkness are an essential component of the environment on Earth. Almost all organisms have evolved to anticipate circadian changes in the environment (Latin circa: “about”, diem: “day”). Single-cell bacteria, plants, and animals all have adaptations to the light-dark cycle and to the accompanying rhythms of heat and cold, as well as seasonal variations in the length of daylight. Multicellular organisms exhibit molecular, cellular, tissue-level, and in some cases behavioral adaptations to the light-dark cycle. These overt behaviors include periods of wake and sleep, feeding and fasting, as well as subtler physiological variations in metabolism of lipids, glucose, and other nutrients [2,3].

Circadian Clocks Offer an Adaptive Advantage

The ability of an organism to anticipate and adapt to circadian changes in the environment offers several evolutionary advantages. At the behavioral level, it allows animals to avoid predators, and in some climates, avoid temperature extremes such as
cold at night or extreme heat during the day in some climates [4–6]. Furthermore, the circadian clock allows the organism to synchronize overt behaviors such as sleep and wake cycles to tissue and cellular level physiological functions. An example includes timing digestion to periods of waking when feeding occurs[7–10].

Circadian Clocks at All Levels of Health and Disease

The ubiquity of circadian function means that disruptions of the circadian clock leads to pathology at all levels. The circadian clock and metabolism interact at the molecular, tissue, and organismal levels. In the best of times, these systems reinforce each other, and circadian rhythms condition animals (including humans) to eat and sleep at the healthiest time points. At the worst of times, disruption of the clock leads to obesity, metabolic syndrome, and diabetes, and dietary problems cause clock malfunction [11–15]. Understanding this system is crucial for therapies for both clock and metabolic disorders. Disruptions of the circadian clock are also associated with cancer, sleep, and psychiatric disorders [16–18]. Furthermore, shift work, jet lag, sleep disorders, and other forms of desynchrony between the environment and the endogenous circadian clock all potentially disrupt the clock and lead to health problems [19,20]. Vertebrate, specifically mammalian, circadian rhythms are of particular interest due to the possibility of modeling human physiology.
1.2 How Clocks Work

In order to regulate timing of diverse behavioral, tissue, and cellular level processes, and tune this timing to the environment, biological clocks must have several characteristics. The clock must have a self-sustained oscillation to ‘keep time’, must entrain to the environment, and must be temperature compensated, so that cycles of heat and cold in the environment do not perturb the clock [2]. In mammals, these functions are carried out through the combination of a molecular clock that is present in all studied tissues, and a master pacemaker in the brain that responds to the environment and synchronizes rhythms in other areas of the brain and peripheral tissues [2,3]. Furthermore, the SCN contains all three elements of a circadian clock, namely, a self-sustaining circadian clock, entrainment to light from the eyes, and temperature compensation through heat shock proteins [3,21,22].

In all tissues studied in multicellular organisms, cellular clocks are thought to be synchronized throughout the body by a central pacemaker located in the brain. In mammals, the chief regulator of circadian rhythms is the suprachiasmatic nucleus of the hypothalamus (SCN). The SCN consist of ~20,000 cells located in the anterior hypothalamus, above the optic chiasm and below the third ventricle. The SCN exhibits twenty four hour rhythms of firing, which are robust enough persist in vitro [23]. SCN rhythmicity is thought to underlie the persistence of circadian locomotor activity in constant darkness, as SCN lesioned animals lose circadian rhythmicity without light cues.
The SCN receives innervation from the retina through the retino-hypothalamic tract (RHT). The RHT stimulates the SCN in response to light, primarily through glutamate and PACAP. This stimulation can affect the firing pattern of the SCN, as well as expression of the molecular clock in SCN cells, and is thought to be the mechanism by which light entrains the SCN, and creates phase shifts circadian locomotor activity [3,24]. The SCN generates twenty-four hours of firing, responds to light stimulation from the environment, and transmits timing information to clocks in other tissues in the brain and periphery through neuronal connectivity, humoral factors, and by regulating behavior [2,3,25].

The most basic unit of the mammalian clock is the molecular transcription/translation feedback loop that sets time in each cell of the body, including in the SCN in the hypothalamus [26]. This multitude of functions in diverse tissues is regulated by a ubiquitous molecular level clock that is conserved from *Drosophila* to mammals [2]. The mammalian molecular clock consists of a transcription-translation feedback loop (TTFL), that is present in all studied tissues (Fig. 1.1) [1,15,27,28]. Activators of transcription, Clock and Bmal1, bind to E-boxes in the promoters of clock controlled genes (CCGs), and facilitate transcription of these genes[2,29]. In some tissues, Npas2 can compensate for loss of Clock [30]. CLOCK and BMAL1 regulate the transcription of other core circadian clock genes, including Per1-3, and Cry1-2. Pers and Crys are translated in the cytoplasm [31]. After dimerization and post translational modification, PERs and CRYs translocate to the nucleus to bind to CLOCK and BMAL1.
This represses CLOCK and BMAL1, lowering expression of their target genes, including Pers and Crys [31]. Over time, PERs and CRYs are degraded, leading to de-repression of CLOCK and BMAL1. CLOCK and BMAL1 resume the cycle by binding to the promoters of specifically E-box regulated CCGs, and the transcription of Pers, Crys, and other CCGs begins again [31]. One complete cycle of the molecular clock takes ~24 hours, thus setting the period of the clock. The period of this process can be lengthened or shortened by alterations in transcription, translation, RNA stability, post-translational modification, protein-stability or degradation of proteins that are involved in the core clock [31].

The core molecular clock has been found in all studied cells, including cells in the SCN. The clock is also thought to regulate at least 40% of the genome at the level of RNA expression [1]. Circadian expression of core clock components and cycling clock regulated molecules have been shown at the RNA and protein levels in the SCN and other tissues [32,33]. However, new components of the core molecular clock are still being discovered and characterized [34].

**Phenotypes of Core Molecular Clock Mutants**

Understanding of the core molecular clock and how it relates to clock output are also compounded by the variety of phenotypes, or lack thereof, which result from mutation or loss of core clock components. Loss of Bmal1 renders animals
Loss of Cry1 results in an hour reduction in period length, while loss of Cry2 increases period length by an hour [29]. The Clock\textsuperscript{Δ19} mutation leads to marked circadian disruption. Complete ablation of Clock, however, is compensated by Npas2 and only results in a small period lengthening effect [30].

1.3 The Mammalian Circadian Clock: The SCN

Introduction

Understanding mammalian clocks in health and disease has involved characterizing the molecular clock shared between the SCN and other tissues, and how the SCN entrains to stimuli from the environment. Early studies provided evidence that the SCN is the master pacemaker, due to its necessity and sufficiency for circadian rhythmic output, and its response to light. The necessity of the SCN for circadian rhythms was demonstrated through ablation of the SCN, which causes arrhythmicity [35–37]. The SCN is also sufficient for generation of circadian rhythms; once the SCN has been ablated, transplantation of the SCN from another animal is sufficient to restore circadian rhythms [35,38,39]. Indeed, the animal receiving the transplant takes on the circadian period of the transplanted SCN [40]. This is particularly striking in the case of transplant of the SCN from WT hamsters into Tau mutant hamsters. Tau mutant hamsters have a short free running period in constant darkness, but transplant of the
WT SCN restores WT circadian free running period [41]. Furthermore, rescue of Clock expression in the forebrain of ClockΔ19 mutant animals is sufficient to restore some degree of rhythmicity in peripheral tissues [42]. Supporting the idea that the SCN is the master pacemaker also includes data that the SCN entrains to timing information from the external environment, particularly light. Evidence that the SCN responds to light include the induction of immediate early genes such as c-fos in the SCN in response to light, as well as upregulation of Per1 expression [24,43,44].

While the SCN is necessary and sufficient for organization of circadian rhythms in mammals, how the SCN actually does this is still an area of ongoing study. Individual cells in the SCN generate rhythmicity through the same molecular clock that is present in all studied tissues as discussed previously [45]. SCN rhythmicity includes rhythmic firing, which is generated by ion channels, some of which are circadianly regulated by the TTFL [24]. The SCN entrains to light stimulus from the environment via PACAP and glutamatergic stimulation from the retinohypothalamic tract (RHT). Glutamate alters calcium, cAMP, and CREB signaling, which in turn can affect transcription of Per genes, and thus shifts the core molecular clock [3,24]. Light stimulation also induces rhythmic VIP secretion, which synchronizes and couples SCN cells [35].

Coupling of SCN cells leads to robust oscillations of the SCN at the tissue level, which are thought to be the basis for circadian output [46,47]. The SCN is thought to synchronize the molecular clock in other brain tissues, including other hypothalamic nuclei as well as larger brain structures such as the brainstem and cerebellum as well as
non-CNS peripheral organs [1,23]. Part of this process occurs through secreted signals such as (e.g., Prokineticin 2, TGF-α, and CIPC) and innervation, although these and other mechanisms are not entirely understood [21,48–50].

**Generation of Rhythmicity in the SCN**

The generation of rhythms in transcription and translation are governed by the TTFL as discussed previously. However, the SCN has a circadian firing pattern that is not shared by all cells harboring a similar TTFL. The SCN is characterized by increased firing during the day, and decreased firing during the night. This electrical activity is conserved between diurnal and nocturnal species, while the consequence on locomotor activity is not [51]. Daily oscillations in SCN firing are thought to be orchestrated through core molecular clock regulations of rhythms of intracellular ions, particularly calcium and potassium, and a variety of constituitively and circadianly regulated ion channels. All of these components are thought to interact, although these interactions are not completely understood [24]. Furthermore, levels of glutamate and GABA signaling in the SCN may show circadian variation, although this phenomenon is not well characterized. These circadian cycles of firing rate and their respective molecular components are thought to shift in response to light stimulation, although how this occurs is not entirely understood [24,52]. This firing is key to SCN function; changes in SCN firing through TTX inhibition lead to locomotor changes, as well as changes in gene
expression. This includes loss of mRNA expression for Prokineticin 2, a peptide secreted from the SCN that is necessary for circadian rhythmicity in locomotor activity [21,53]. The firing of the SCN is regulated by a variety of ion channels, some constitutively active, some circadianly regulated, as well as by oscillation of intracellular messengers such as calcium and cAMP [24,32,35]. These second messengers are thought to connect ion channel activity and gene expression through alterations of CREB phosphorylation, which affects transcription of Per genes [24,35].

**Sodium**

Sodium channels are a key part of SCN firing as in neurons from other tissues. Blockade of TTX-sensitive sodium channels reduces SCN firing. Blockade of SCN firing is known to correspond with locomotor activity output [54]. Intriguingly, TTX-blockade of SCN firing also leads to loss of rhythmicity of Prok2 mRNA transcript [53]. The degree to which TTX blockade of sodium channels affects other ions, particularly levels of intracellular calcium, is still under debate in the literature [55,56]. Voltage gated potassium channels are also a component of SCN firing, and may play a role in locomotor activity output [57].

**Calcium**
Both intra- and extracellular calcium levels play a key role in SCN function, and are thought to be influenced by the output of the TTFL, calcium channels at the plasma membrane, and intracellular calcium cycles. Generation of circadian intracellular calcium rhythms is still incompletely understood, although they have been proposed to be regulated by Bmal1, RyR (ryanodine receptors), and IP3 [48,58]. Intriguingly, Ikeda et al. (2003) have reported that intracellular Ca$^{2+}$ peaks prior to multi-unit activity recorded firing of the SCN [56]. Intracellular calcium levels in SCN neurons are also affected by light, as part of the response to glutamate and PACAP stimulation from the RHT [59]. Increased calcium levels lead to the activation of CAMKIIA, which regulates both calcium channels (L-type) at the plasma membrane, and CREB signaling which in turn regulates the TTFL through Per1 expression [24,56].

An Example of the Role of Ions in SCN Network Function: Temperature Compensation

As mentioned previously, a requirement of circadian clocks is that they are temperature compensated [2,22]. In mammals, temperature compensation of the circadian clock is mediated by the network properties of the SCN. Changes in temperature readily reset peripheral tissues, while the SCN is resistant to shifts in response to temperature, as measured by PER2::LUC expression in SCN and peripheral tissue explants in response to changes in temperature [22]. SCN resistance to temperature shifts is complex, involving ion channels, network connectivity, and the
heat shock response system. Ion channels are necessary for SCN temperature compensation, as treatment with the sodium channel blocker TTX, or the L-type calcium channel blocker nimodipine, rendered the SCN’s phase of PER2::LUC expression sensitive to changes in temperature [22]. Furthermore, network-level connectivity of the SCN is necessary for temperature compensation, as division of the core and shell in culture renders each region sensitive to shifts by temperature [22]. Individual SCN cells are also sensitive to temperature changes. Inhibition of the heat shock response pathway increases temperature sensitivity in SCN PER2::LUC expression in terms of period length [22]. These data revealed that the SCN’s resistance to phase shift by temperature is mediated by heat shock proteins. On the molecular level, HSF1, Heat Shock Factor 1, may bind to the promoter region of the Per2 gene, and provide molecular feedback to the clock [22].

**Coupling of SCN Neurons and Glia**

The molecular clock and ion channels are two key components of SCN rhythm, and generate basic firing patterns of SCN neurons. Coupling between neurons gives rise to the overall tissue level properties of the SCN. Coupling is an essential modulator of the properties of the SCN, especially considering that individual SCN cells are cell autonomous oscillators that remain rhythmic when cultured individually[3,60]. The period length of individually cultured SCN cells varies more than the circadian period
length of animals as measured by locomotor activity output [61]. Coupling between individually oscillating SCN cells is thought to produce the overall period of the SCN. Indeed, the mean of the period lengths of cultured SCN cells approximates the mean period of locomotor activity for WT animals or animals with the Tau mutation, with the mean period of SCN cells from Tau heterozygotes falling between that of WT and homozygous Tau animals [61]. Coupling of SCN cells is also part of the clock’s robustness, as it can partially compensate for loss of some core clock components [47]. Tissue level properties of the SCN also add further complexity to analysis of SCN gene expression. Studies of gene expression in the SCN at the tissue level may yield results that would differ from analysis of single cells. For example, some genes that do not show circadian expression at the tissue level may be rhythmic in some cell types. If these genes are circadianly expressed, but out of phase in different cell types, tissue level analysis might show gene expression to be non-circadian, due to noise from peaks at different phases in different cell types. However, single-cell analysis would show rhythmicity. Furthermore, single cell gene expression analysis may reveal cell-to-cell variation in period length and amplitude of gene expression. Period length varies in SCN cells cultured individually as measured by PER2::LUC expression [3]. Whether cell-to-cell variation in period of RNA transcript expression also occurs in the SCN is unknown, but it is certainly possible given the difference in period lengths observed in PER2::LUC expression.
Despite their importance, mechanisms of coupling between SCN cells are only partially understood. This is partly due to the SCN’s nature as an extremely heterogeneous population of cells. Its most basic division is between the core and the shell. The core is the retino-recipient component of the SCN, and contains VIP (vasoactive intestinal peptide)-expressing neurons[51,62,63]. The shell is the rhythmicity generating part of the SCN, and contains AVP (arginine vasopressin) expressing neurons [64]. The VIP containing core innervates the AVP containing shell. VIP is an essential part of the SCN response to light, and also synchronizes oscillations between individual SCN neurons [51,62]. AVP synchronizes circadian regulation of hormones, including corticosterone and LH (Lutineizing Hormone) [48]. In addition to the basic VIP and AVP expressing neurons, SCN neurons express CALBINDIN, GRP, and almost all cells in the SCN express GABA. The functions of GABA in the literature are often contradictory, although it may serve as a desynchronizing signal to facilitate phase shifts and entrainment [65].

VIP and intracellular calcium play essential roles in coupling the rhythms of SCN cells at the tissue and cellular levels, respectively. Coupling of SCN cells is to some degree mediated by Gq modulation of intracellular calcium signaling in a VIP dependent manner [62]. Increase in intracellular calcium levels in the SCN precedes CRE promoter activation, followed by increased transcript expression of Per1 and Per2 [62]. In addition to secreted VIP signaling and calcium mobilization, coupling is modulated by synaptic connections. VIP expressing neurons show both circadian variation in axonal
contact between day and night. Glial coverage of the soma of VIP expressing neurons also exhibits day night variation, and may be an important part of circadian connectivity patterns in the SCN [66,67]. While altered neuronal and glial connectivity in the SCN are an intriguing and promising avenue for future study, these phenomena are largely uncharacterized, particularly at the molecular level.

**Entrainment of the SCN**

Overall, the molecular clock, ion channels and homeostasis, glia, neuronal connectivity, and possibly unknown factors generate rhythmic patterns of SCN activity [24,68]. In order to entrain to the environment, these patterns can be reset by external stimuli, particularly by light [51]. Signaling of light from the retina through the retinohypothalamic tract (RHT) to the SCN is largely understood. Furthermore, light stimulation in the early night causes phase delays, while light stimulation in the late night causes phase advances. Phase delays and phase advances are thought to be due to changes in SCN firing, second messengers, and expression of Per1 genes in the SCN during the early night and late night. Also, differential shifts of activation of cAMP, PKA, and ryanodine receptors in response to light may be involved in generating phase delays and phase advances [51]. Post-translational modifications of core clock components may be affected by light and other stimuli as part of entrainment. Many of these post-translational modifications and how they are involved in the molecular clock as well as
its response to stimuli have been partially characterized. However, much work remains to be done [69].

Entrainment involves modulation of behavior and physiology according to shifts in light caused by seasonal day length, as well as by travel. Entrainment can sometimes take several days, e.g., in the case of jet lag. This is thought to be due to desynchronization between the core and the shell of the SCN. The core entrains immediately to light stimuli, while the shell lags behind. Eventually, the shell also adopts the phase of the core. This process is thought to be mediated by transmission of timing information from the core to the shell [70]. Seasonal encoding of day length is thought to be due to phase relationships between the shell and the core. Rhythmicity in the shell of the SCN as measured by PER2::LUCIFERASE, is phase advanced on the order of several hours when compared to the core. On days with short amounts of day light, these phases are thought to be closer together than on long days, when the phases of the shell and core are more divergent [70].

**SCN Signaling to the Periphery**

Part of the SCN’s function as the master pacemaker is synchronizing peripheral tissues. The SCN is thought to transmit timing information to other tissues through secreted signals, and regulate behavior through innervation [48]. The SCN must transmit a timing signal through secretion. SCN contained by a semipermeable
membrane transplanted into SCN-lesioned hamsters are able to restore some degree of circadian rhythmicity without innervation [40]. However, diffusible signals are not the SCN’s only means of transmitting a time signal, as SCN transplants without innervation are not sufficient to restore some forms of output, including glucocorticoids [48,71]. Secreted signals and innervation are also not mutually exclusive mechanisms, as SCN innervation of other brain tissues is thought to be part of transmission of secreted signals, as well as secretion into the third ventricle, which adjoins the SCN [72].

Several factors secreted from the SCN that affect circadian locomotor activity have since been identified. These include, Prokineticin2 (Prok2), TGF-α (Transforming Growth Factor – α), and CIPC (Clock Interacting Protein, Circadian) [21,48–50]. TGF-α and CIPC were discovered through a screen of peptide signals secreted from the SCN, while Prok2 was discovered as a modulator of intestinal function [21,50,72,73]. Prok2, TGF-α, and CIPC are all able to inhibit circadian locomotor activity. These signals are thought to work at the molecular level through modulation of the ubiquitous molecular clock. For example, Prok2 signaling is thought to involve calcium signaling, which affects CREB phosphorylation and expression of Per genes [24,74]. Less is known regarding the molecular mechanisms of other signals such as TGF-alpha [75].

**Remaining Questions in SCN Function**
In addition to what is currently known, there are likely to be a variety of additional mechanisms by which the SCN synchronizes the periphery. In addition to the two peptides characterized by the Weitz group, e.g. TGF-alpha and CIPC, a variety of peptides are known to be expressed in the SCN, although the functions of many have not been characterized [72,76]. Furthermore, understanding of coupling mechanisms at the molecular level between the SCN are not well understood, with questions remaining as to how day length is encoded, and how different period lengths are generated and maintained by individual cells [77]. How the SCN communicates timing information to other regions in the brain is unknown aside from the secreted factors discussed earlier, although many brain regions other than the SCN have circadian rhythms [78]. Furthermore, how the SCN responds to changes in the periphery is largely unknown.

1.4 Clocks Outside the SCN

Introduction

Do peripheral clocks signal back to the SCN? If so, how? It is known that the SCN receives innervation from other brain areas in addition to the RHT. Furthermore, extra-SCN regions are known to have clocks [78]. The clock field has made progress in characterizing clocks in peripheral tissues, although extra-SCN clocks in the brain are only beginning to be recognized and understood [78]. Understanding of peripheral clocks and how they communicate with the SCN is of crucial importance, with growing
recognition that these regions are crucial for regulating clock functions affecting metabolic health [78,79].

**Evidence that peripheral tissues have clocks and may affect the SCN:**

Core clock genes are under intense study in other brain regions and the periphery [78,80–82]. Recent studies suggest that these core clock components may also oscillate in hypothalamic nuclei that share innervation with the SCN [78,82,83]. These nuclei regulate metabolic processes that show circadian variation: increases and decreases in feeding (arcuate nucleus – arc), integration of signals from the gastrointestinal tract (paraventricular nucleus - PVN), satiety (ventromedial nucleus - VMN), and feeding behaviors (dorsomedial nucleus - DMN) [84,85]. From the hypothalamus regions that have evidence for circadian rhythms, we selected the arcuate nucleus for in depth study. The arcuate nucleus has the strongest clock of any of these regions, is anatomically close to the SCN, and shares innervation with the SCN. We therefore began studying these regions, with the understanding that experimental designs and techniques used to study the clock in the arc, if successful, could also be used for later study of the clock in other regions of the hypothalamus, as well as other brain regions [26,78,86].

**The Arcuate Nucleus**
The arcuate nucleus (arc) is a logical place to look for circadian rhythms due to its proximity to the SCN [87]. The arc is also a key part of the hypothalamus, because it acts as an integrator of metabolism by monitoring signals from the other nuclei and the periphery including glucose, fatty acids, leptin, and insulin levels[20,88–90]. In response to these signals, it synthesizes and releases metabolic peptides that either stimulate or repress feeding: the appetite stimulating peptides neuropeptide Y (NPY) and agouti-related peptide (AgRP), and the appetite suppressing peptides proopiomelanocortin (POMC), and cocaine amphetamine regulatory transcript (CART)[84,91–93]. These peptides are a crucial part of the arc’s activities in integrating metabolism: lesions of POMC neurons or NPY receptor expressing neurons alter energy homeostasis[94,95]. Projections from the SCN innervate NPY and POMC containing arc neurons [96]. The arc may also affect the activity of the SCN (Fig. 1.2) [86]. The RNAs for NPY and POMC exhibit circadian rhythms in the rat hypothalamus as a whole, although their circadian rhythms specifically in the arc have not been shown[97]. Inhibition of NPY signaling has effectively treated obesity in a mouse model, and understanding the circadian regulation of the arc may offer insight into timing of administration and dosage parameters of such treatments[97,98].

Furthermore, NPY has the ability to phase-shift circadian rhythms [99]. NPY also signals with GABA, which is a key component of SCN signaling. NPY is thought to be part of non-photic signaling to the SCN from the intergeniculate leaflet (IGL), although NPY
from the arcuate nucleus has not been shown to phase-shift the SCN [25]. Furthermore, alterations in NPY signaling to the SCN from the arcuate nucleus would be most interesting if changes in NPY signaling were due to the arcuate nucleus’s response to the metabolic state of an animal. This would provide key information on how the metabolic state of the periphery might affect circadian rhythms [25,99].

In Utero and Adult Diet Effects on Circadian Rhythms

There is some evidence that an animal’s metabolic state affects circadian rhythms. Adult mice fed a HF diet developed a long period in circadian locomotor activity [100]. Sprague-Dawley rats fed either a control or LP diet in utero and in lactation exhibit altered circadian feeding behavior at postnatal day 35 [101]. C57BL/6J mice fed a control or LP in utero diet exhibit altered light/dark wheel running behavior at 8 weeks of age [102]. Adult mice fed a HF diet developed a long period in circadian locomotor activity [100].

Preliminary studies show that diet affects circadian behavior and transcription in the brain [102]. However, knowledge in this area is in its infancy. The strongest data showing metabolism affecting circadian transcription in the brain come from a handful of intriguing studies that show that in utero diet affects rodent circadian rhythms even in adulthood. Rats exposed to low protein and control diet in utero and during lactation exhibit significant differences in circadian hypothalamic rhythms of the clock transcript
Per1 and of NPY and AgRP as adults [101]. Mice exposed to control or low protein in utero diet exhibited significant differences in cortex levels of core clock transcripts Bmal1, Per1, and Per2 at 8 weeks of age [102].

Summary

The effect of in utero diet on adult circadian rhythms is an intriguing, but highly uncharacterized biological phenomenon. The in utero diet has the potential to be a powerful model for the effect of metabolic state on circadian rhythms because the effect persists even after a return to normal diet in adulthood. Furthermore, such long lasting effects may involve epigenetic mechanisms, a less characterized area of the molecular circadian clock [103,104].

1.5 Knowledge Gaps in Understanding Signaling Within the SCN, and Between the SCN and Peripheral Clocks

Over the past several decades, circadian research has made extraordinary progress in understanding how the molecular clock functions, and characterizing the molecular clock in the SCN and peripheral tissues. Many questions remain at the tissue and systems level. How the SCN communicates timing information to other clocks in the brain and periphery is largely unknown, aside from a few secreted factors. Furthermore, little is known regarding how information about the state of the periphery
is transmitted to the SCN. How the SCN is modulated by the metabolic state of peripheral tissues is a particularly important question, given the severe metabolic phenotypes exhibited by some clock mutant animals [13,14]. Overall, though much progress has been made regarding how the core clock functions in the SCN, many questions remain regarding how the SCN transmits information to other regions of the brain and periphery, and how the brain and periphery in turn signal to the SCN.

My Work in Context:

To address knowledge gaps in how the SCN encodes and transmits circadian information, we designed the most complete RNA expression profile of the mouse SCN ever performed, consisting of a microarray profile at 2 hour resolution over 48 hours, and an RNA-Seq profile at 6 hour resolution over 48 hours. Previous studies have shown that SCN function relies on the molecular clock, diverse cell types, and circadian changes in coupling of neurons and glia [2,66]. The SCN clock also functions through intricate signaling to respond to light, darkness, and temperature changes [22]. All of these processes are only partially understood. We therefore hypothesized that we would see cycling transcripts representing the core clock, neuronal connectivity factors, and heat shock chaperones in our time course. In Chapter 2 we discuss the results of this study.

Current research shows that in utero diet impacts adult metabolism [103]. The effect of LP in utero diet on adult circadian rhythms in metabolism is a recent and
intriguing discovery [101,102]. But if or how this affects period length in adult animals is an open and important question. If so, it’s possible that early diet could impact adult behavior, e.g. chronotype. Kohsaka et al. (2007) have shown that adult mice fed a HF diet show long period circadian rhythms of locomotor activity[100]. However, the effects of in utero high fat diet on adult circadian period have not been studied. To address these questions, we developed a model of in utero dietary perturbation where female mice received a control, LP, or HF diet beginning with mating and continuing through pregnancy, parturition, and suckling. Offspring were then weaned to a control adult diet, and circadian phenotype was characterized in adult male and female mice of 2-4 months of age. We further tested the interaction between in utero and adult diet by weaning male and female mice from control and LP in utero diet to an adult control or HF diet before characterization of circadian phenotype. We hypothesized that in utero diet would affect adult circadian locomotor activity, and tested this hypothesis by measuring circadian period length of wheel running activity in constant darkness of adult mice from our in utero diet model. We furthermore hypothesized that circadian gene expression in the SCN and the arcuate nucleus would be affected by in utero diet. We tested this hypothesis using the PER2::LUCIFERASE heterozygous mice exposed to the same in utero and adult dietary conditions as mice from the circadian locomotor activity model[23]. SCN and arcuate nuclei were cultured from male and female mice in each dietary condition. PER2::LUCIFERASE expression in these SCN and arcuate nuclei was then analyzed for period length, amplitude, and baseline PER2::LUC expression in
each condition. In Chapter 3 we discuss the effects of in utero and adult diet on adult circadian locomotor and PER2::LUC expression phenotype of male and female mice.

Our experiments address circadian gene regulation in the hypothalamus at the basic and translational research levels. The gene expression profile of the suprachiasmatic nucleus addresses knowledge gaps of the molecular function of the SCN in generating firing rhythms, entraining to the environment, and synchronizing peripheral tissues. How the SCN responds to changes in the periphery, such as metabolic changes are addressed by our experiments investigating the effect of LP and HF in utero diet on circadian period length in adult animals. Furthermore, these data may lead to translational studies, as a HF in utero diet addresses high fat diet concerns common in the Western world. LP in utero diet addresses problems of inadequate diets that are high fat and calories, yet still do not contain enough protein.

In sum, these studies highlight the important role of the SCN in coordinating rhythms in the CNS and periphery, as well as underscore the reciprocal nature of these interactions.
**Fig. 1.1: The Mammalian Molecular Circadian Clock**

The Mammalian Molecular Circadian Clock

**A**
- Dimer of Transcription Factors CLOCK and BMAL1
- Translocation to Cytoplasm

**B**
- Transcription
- Binding to CLOCK and BMAL1

**C**
- Repression of Transcription
- Translocation to Nucleus

**D**
- Repressors PER and CRY re-enter the nucleus and repress CLOCK and BMAL1
- Old transcripts for PERs and CRYs are degraded and not replaced

**E**
- Clock Controlled Genes (~10% of repressed genes)
- Translocation to Cytoplasm

Adapted from: [https://www.hhmi.org/biointeractive/mammalian-molecular-clock-mode](https://www.hhmi.org/biointeractive/mammalian-molecular-clock-mode), Liu et al. [2007]

**Fig. 1.1:** The Mammalian Molecular Circadian Clock. The mammalian clock is a transcription-translational feed back loop (TTFL). **A**) Transcription factors CLOCK and BMAL1 activate transcription of repressors. Some transcripts translocate to the cytoplasm. **B**) Transcripts are translated in the cytoplasm. **C**) Repressors PER and CRY re-enter the nucleus and repress CLOCK and BMAL1. **D**) Transcription of Per and Cry genes by CLOCK and BMAL1 is repressed. PER and CRY are degraded and levels of these proteins drop. **E**) Drop in PER and CRY proteins de-represses CLOCK and BMAL1. CLOCK and BMAL1 resume transcription [19,47,105].
Fig. 1.2: Hypothalamic Circuitry of Circadian Rhythms and Metabolism.

The suprachiasmatic nucleus (SCN) is the master circadian clock in mammals. An adjoining nucleus of the hypothalamus, the arcuate nucleus, regulates feeding and satiety. Key peptides expressed in the arcuate include Neuropeptide Y (NPY), which stimulates feeding, and Pro-opiomelanocortin (POMC), which regulates satiety [48,86,106].
CHAPTER 2: Circadian Regulation of Gene Expression in the SCN

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Data deposition: the microarray and RNA-Seq data from this paper have been deposited in the NIH-GEO (National Institutes of Health – Gene Expression Omnibus) database, accession number: GSE70392.
2.1 Abstract

We performed a circadian RNA expression profile of the mammalian master pacemaker, the suprachiasmatic nucleus (SCN) in mice, at 2-hour resolution using microarrays, and at 6-hour resolution using RNA-seq. We found hundreds of transcripts that cycle in the SCN, two dozen of which cycle across all other studied brain regions. We identified a rush hour of cycling transcripts regulating axonal synaptic connectivity and neurotransmission, extending to intracellular signaling through a panoply of cycling kinases and phosphatases. Further, we found cycling regulators of ER calcium stores, protein folding, and RNA metabolism. Tdp-43, Prnp, and transcripts for other aggregation prone proteins cycle in phase with heat shock chaperones, underscoring the function of the circadian clock in maintaining cellular homeostasis. Our profile of SCN RNA expression highlights the rapidly expanding panorama of circadian regulation of physiology in health and disease.
2.2 Introduction

The suprachiasmatic nucleus (SCN) of the hypothalamus is the master circadian synchronizer in mammals. There are ~10,000 cells in each bilateral SCN located above the optic chiasm [107]. This small network of cells entrains to light and dark cycles, heat and cold levels, and other environmental stimuli [2]. The master pacemaker harbors a cellular-level molecular clock and entrains clocks throughout the body through neural and humoral processes [26].

The SCN responds to light through glutamaterigic signaling from the retinohypothalamic tract (RHT) [59]. RHT stimulated glutamate release alters calcium levels and homeostasis in the SCN [59]. SCN firing in turn signals to other CNS regions through reciprocal innervation, and through secretion of the signaling proteins PROK2, TGF-alpha, and VIP ([96], [48]). Previous work has investigated clock regulation of RNA transcripts and proteins in the SCN [32,108–110]. Progress has also been made in showing how coupling between individual neurons in the SCN contributes to SCN network level activity [3].

Although progress has been made in understanding how the SCN generates rhythmicity, the mechanism through which the SCN regulates circadian rhythms in other oscillating tissues, particularly other CNS tissues, is only partially understood. Evidence for circadian regulation can also be found in recent studies of Per1-luciferase and PER2::LUC mice, showing circadian oscillation in extra-SCN brain regions [78,79]. The importance of clock function in the brain is underscored by a recent study showing that
brain specific rescue of the clock function in ClockΔ19 mice restores rhythmic expression of some peripheral clock function [42]. Furthermore, our lab recently characterized circadian RNA expression in mouse hypothalamus, brainstem and cerebellum using DNA-arrays and RNA-Seq [15]. We showed hundreds of transcripts cycling in each region.

Given the wealth of circadian data generated by these experiments, we sought to create a similar profile of RNA expression in the SCN. Previous work on the SCN has shown circadian control of transcript expression of many key pathways and genes [32,110]. Although revealing, this work was limited by 4 hour time resolution, which limits the detection of cycling genes, is prone to false positives, and is poor at accurate estimation of phase similarities and differences of transcript expression between the SCN and other tissues. Furthermore, prior work was limited to arrays that lacked a full complement of protein encoding genes. Here we performed a time-course RNA expression profile of the SCN at 2 hour resolution using Affymetrix MoGene 1.0 ST arrays and 6 hour resolution using RNA-seq.
2.3 Materials and Methods

Animals:

120 male C57BL/6J mice of 6-8 weeks of age were ordered from Jackson Laboratories. Mice were entrained to a 12:12 (LD) cycle for three weeks and then released into constant darkness (DD). Tissue collection began at Circadian Time (CT) 18 and 3 mice were sacrificed every 2 hours for 48 hours. All animal experiments were performed with the approval of the Institutional Animal Care and Use Committee of the University of Pennsylvania.

Tissue Collection:

Mice were euthanized by CO2 inhalation followed by cervical dislocation under red light illumination. Eyes were covered with black electrical tape prior to dissection. Whole brains were dissected, frozen in ice-cold isopentane, and stored at -80 °C. Brains were thawed to -20°C, mounted in a cryostat, and SCN samples were taken using a 1 mm punch from between bregma -0.22 and bregma -0.92 [111]. Each SCN punch was put in in 500 uL of Trizol Reagent (Ambion/Life Technologies, 15596018) and then stored at -80 °C.
**RNA Extraction:**

RNA was extracted using QIAGEN RNeasy (Cat. No. 74106) with the following modification: Following centrifugation and transfer of aqueous phases to new tubes, 1.5 volumes of 100% ethanol were added to each sample. At the end of the protocol, samples were resuspended in 25 uL of Rnase free H2O. RNA was quantified using High Sensitivity RNA Qubit quantification (Invitrogen/Life Technologies, Ref. Q32866). Equal quantities of RNA from each of three SCN were combined at each time point.

**Microarray:**

30 ng of each pool of SCN samples from each time point were amplified, converted to cDNA, and biotin labeled using the Pico WTA V2 (NuGen, 3302) and Encore Biotin Module (Nugen, 4200). Samples were then hybridized to Affymetrix MoGene 1.0 ST Arrays (901171) at the Array Core at the University of Pennsylvania.

**Data Analysis:**

Cel files were processed by the RMA algorithm [112]. Data were processed as in (Zhang and Lahens et al., 2014)[1], to select for probesets corresponding to protein-coding genes. These 19,788 probesets were used for downstream analysis. All probesets
were analyzed by JTK-CYCLE [113], using a period length of exactly 24 hours. Probesets were designated as cycling if they had a JTK-CYCLE q-value <0.05. For JTK-CYCLE analysis of the Time of Day data set, (Chiang et al. (2014))[109], period lengths analyzed by JTK-CYCLE were between 8 and 24 hours, since proteins in this data set were found to have periods ranging from 8 to 24 hours [109,113].

For ease of visualization, microarray data were transformed (2x=y) as in (http://circadb.hogeneschlab.org/query)[114]. Data comparing cycling transcripts within the SCN are expressed as median normalized array intensity. Due to greater differences in amplitude across tissues than within tissues, data comparing cycling transcripts across tissues are expressed as max normalized array intensity, to allow more complete visualization of cycling genes in each tissue. A loess value of 0.5 was used to plot each transcript in ggplot2 () in R [115].

For generation of the heatmap of microarray expression in cycling RNA transcripts, median normalized cycling transcripts (q<0.05), were phase-sorted according to lag (phase) in Excel. Data were imported into Mev [116], and max, median, and minimum color values were adjusted to allow visualization of the pattern of cycling transcript expression. To generate the heat map of the SCN RNA-Seq data, median normalized cycling transcripts (Adj. p <0.05) were phase-sorted. Data were imported into Mev, and max, median, and minimum color values were adjusted to allow visualization of the pattern of cycling junction expression.
For analysis of overlap with the circadian transcriptome, cycling transcripts from the SCN were compared with those from the 12 organs measured in (Zhang and Lahens et al., 2014) using Vennerable() in R [117]. The Chow-Ruskey diagram was also generated using Vennerable().

For PSEA analysis (Zhang et al., In Review), cycling transcripts and their respective phases from array data meeting the $q < 0.05$ criterion were analyzed with default PSEA parameters, with the exception that “Max gene names to label” was changed to 60. The gene set used was C5: GO Gene sets, BP: GO biological process, from the GSEA MSigDB Collection from the Broad Institute [118].

**RNA-Seq:**

**Library Construction:**

33.3 ng from each of three SCN RNA samples were combined at each of eight time points, CT 22, 28, 34, 40, 46, 52, 58, and 64. Libraries were prepared using the standard Low-Throughput protocol of the TruSeq Stranded kit, using poly-A selection and two minutes of fragmentation. The resulting libraries were quantified using Qubit DNA high sensitivity quantification (ng/uL). Equal quantities of each library were pooled to yield 10 uL of a 10 nM pool.
Libraries were sequenced in a two-stage process. To estimate yield, pooled SCN samples were sequenced on an Illumina MiSeq. Data were de-multiplexed and mapped using STAR [119]. Mapped read counts for each library were calculated. To equalize reads across libraries, we calculated a normalization factor for each independent library so that each would have a roughly equivalent number of reads. Samples were repooled and sequenced on an Illumina HiSeq using a a 100 x 100 PE RapidSeq protocol (www.illumina.com).

**RNA-Seq Mapping:**

The raw reads were mapped to the mm9 genome build provided on UCSC using the RNAseq aligner STAR (version STAR-2.3.0e) [119–121]. The algorithm was given known gene models (provided by RefSeq) to achieve higher mapping accuracy [122].

**PORT normalization:**

To normalize the read counts from each time point, PORT, was used. PORT is an in house developed algorithm that is not yet published but can be found at https://github.com/itmat/Normalization. The release used was v0.6.3-beta 2/2/2015 and the normalization was conducted on the gene, exon, intron and junction level.
Gene models provided from UCSC were used [121], as well as the ones available from ENSEMBL [123].

**Alternative-splicing analysis:**

During alignment, STAR identifies intronic regions from those reads with spliced alignments to the reference genome. First, all intronic regions that were present in less than four time points were filtered out, to ensure that all introns and junctions analyzed were present in at least half of profiled time points. Alternative splicing events were identified by searching for cases where introns shared the same start coordinates, but had different stop coordinates (or vice versa). These sets of introns representing alternative-splicing events are referred to as alternatively-spliced loci. Next, any alternative-spliced loci were merged with overlapping genomic coordinates, if they were present on the same strand. Lastly, annotation information was assigned to each junction and alternatively-spliced locus using the Ensembl gene annotation [123].

Having assembled this list of alternatively-spliced junctions, read counts were assigned to all of them from the PORT-normalized SCN RNA-seq data. Next, the major and minor spliceforms were identified at each of these alternatively-spliced loci using the Ensembl annotation information. Only those junctions that could be assigned to a minimal set of spliceforms were considered.
These junction counts were then analyzed for oscillating patterns using JTK_CYCLE (parameters set to search for rhythms with periods of exactly 24 hours; oscillators defined as $p < 0.05$). For ease of visualization, read counts of each splice junction were median normalized across all CTs, and plotted with a loess value of 0.75 in ggplot2() and Cowplot() in R [115,124].

2.4 Results and Discussion

The master mammalian circadian clock is in the SCN of the hypothalamus [125]. Over the last two decades, many studies have shown that the core molecular clock drives transcriptional rhythms in downstream, output genes [2]. To identify the circadian output genes in the SCN, we created an RNA expression profile of the SCN at 2 hr sampling resolution over 48 hours using microarrays. In parallel, we studied this same expression profile using poly-A RNA-seq at 6 hr resolution. These data were analyzed as described in (Zhang and Lahens et al., 2014) (See Materials and Methods for full details). We found 523 transcripts cycling with JTK-CYCLE q-values < 0.05 (Fig. 2.1A), which is similar to the number of oscillating transcripts found in other brain regions [113]. Like elsewhere in the brain and body, the pattern of circadian transcript expression in the SCN exhibits dawn and dusk “rush hours” (Fig. 2.1B and 2.1C). These results show that circadian gene expression in the SCN is strikingly similar to gene expression in the cerebellum, whole hypothalamus, and brainstem. The expression profiling show cycling
genes offering fresh insight into how the SCN regulates (i) the core molecular clock, (ii) neuronal connectivity (iii) synaptic activity and signaling, (iv) ER homoestasis, and (v) RNA and protein stability, compared with numbers of cycling transcripts in the periphery identified by Zhang and Lahens et al. (2014) [1,2,26]. We therefore analyzed these transcripts for circadian function both within the SCN and between tissues. Furthermore, we identified 654 splice junctions cycling with an adjusted p < 0.05 as determined by JTK-CYCLE (see Materials and Methods. Supplementary Digital File 2.S210), and examined them for biological meaning in context of other circadian data (Fig. 2.1D-F).

We compared our microarray data with the SCN time course carried out in DD from Hatori et al. (2014) [126]. Data from the 430 MOE Affymetrix high density expression arrays from this data set were analyzed the same way as our data, including JTK-CYCLE (Supplemental Digital File 2.S11) [113,127]. Many genes that cycled in our microarray data also had highly significant BHQ values in the Hatori et al. (2014) data set (Supplementary Figure 2.S1). Genes also showed high agreement of phase in both data sets with a median phase difference of 3 hours (Supplementary Figure 2.S2A-B).

We then compared transcripts that cycle in the SCN with those that cycle in the hypothalamus, brainstem, and cerebellum as found in Zhang and Lahens et al. (2014)[1]. We found that only twenty-three transcripts cycled in all brain regions, confirming earlier findings that circadian regulation is largely tissue specific (Fig. 2.2A)[32]. Many transcripts that cycle across tissues are components of the core molecular clock,
including *Per1-3, Nr1d1-2, Dbp*, and *Chrono* [34,128]. Other transcripts that cycle across the brain are part of the heat shock response, the immune system, calcium signaling, and neuronal connectivity, all of which have been previously shown to be key areas of clock regulation (Fig. 2.2B)[22,59,66,129].

The clock is thought to function similarly throughout the body. Whether the phase and amplitude are similar between the SCN and other tissues remains an open question, however. Therefore, we compared expression of core clock transcripts in the SCN and the twelve brain and peripheral tissues. Notably, dawn phase activators of the core clock, *Bmal1 (Arntl)* (BH q<0.100) and *Npas2*, are in phase across tissues, but show markedly lower amplitude in the SCN and other brain regions when compared with peripheral tissues (Fig. 2.2C & D). Low amplitude oscillations may be advantageous for phase shifting clocks in the brain like the SCN clock [24]. An evening phase cycling transcript, Dbp, exhibits similar amplitude in the brain and other tissues, and peaks in phase in the SCN and other areas (Fig. 2.2E). The evening phase components of the core clock, *Per1-3*, peak several hours earlier in the SCN than in other brain regions and in the periphery (Fig. 2.2F-H). These data are consistent with PER2::LUC expression peaking in the SCN before peaking in peripheral tissues [23]. Although all functions of *Per1-3* are not understood, *Per1-3* expression in the SCN preceding expression in other tissues may play a role in tissue level organization of the circadian clock [1].

**Neuronal Connectivity:**
The core molecular clock enables both periodicity and synchronization of all tissues. In the SCN, the molecular clock regulates oscillation of individual SCN cells, which couple through complex patterns of neuronal connectivity [3]. This SCN network function is important for both rhythm generation and resetting. Therefore, we examined known regulators of neuronal connectivity. Intriguingly, tight junction transcripts cycle with peak expression in the dark phase, while factors regulating rearrangement of synaptic connectivity peak at the start of the light phase. We note that Ahnak, a tight junction protein expressed in the blood brain barrier, cycles in phase across the brain, with RNA transcript expression peaking in the early half of the dark phase [130]. In the SCN, Jam2, a junction adhesion molecule, peaks in phase with Ahnak, adding additional regulation of tight junctions in the SCN [131](Figs. 2.3A-2.3B).

Intriguingly, tight junctions cycle ~8 hours out of phase with modulators of synaptic plasticity. This may be to allow increased space between neurons for modulation of neuronal connectivity [132]. We noted that Mmp14 cycles in all four brain regions (Fig. 2.2A, 2.3C). The fly homologue of Mmp14, Drosophila Mmp1, is required for circadian locomotor activity and modulation of circadian axonal connectivity through interactions with Fasc2 and EcoR [133]. The transcript for the mammalian homologue of Fasc2, Ncam1, is in phase with Mmp14, as is L1cam [134,135]. L1cam and Ncam1 (Ncam1 q< 0.0549) (Fig. 2.3D) modulate neuronal connectivity [136]. MMP14 interaction with L1CAM also modulates downstream
signaling and neuronal connectivity in the rat brain [135]. During rat brain development, L1CAM and NCAM localize to the SCN, and in the case of L1CAM, this expression is light-dependent [137].

The evening rush hour of neuronal connectivity cycling transcripts is accompanied by a regulator of actin polymerization, Kank1, and a regulator of neuronal migration, Gpr56, both of which cycle across brain tissues (Fig. 2.3E, F). Kank1 and Gpr56 may both limit cellular motility, providing a counterbalance to synaptic plasticity [138–140].

Overall, cycling transcripts regulating junctions and neuronal connectivity may regulate tighter connections between neurons during times of activity, and decreased adhesion at others to allow synaptic reorganization. Furthermore, cross brain cyclers Ahnak, Mmp14, Kank1, and Gpr56 may carry out these cycles in many brain regions in addition to the SCN. These transcripts offer insight into how the circadian clock may interact with other synaptic processes, such as those of learning and memory, all of which require periods of synaptic reorganization, and therefore may be facilitated by temporal periods of decreased synaptic adhesion [132].

**Secreted Axon Guidance Factors:**

In contrast to global regulation of neuronal connectivity, intricate patterns of cycling transcripts are also present in the SCN. The SCN is a heterogeneous population
of cells, which may couple with different strengths and using diverse mechanisms[3]. How this occurs is not well understood. We identified anti-phase cycling of secreted axonal guidance signals and their receptors (Figs. 2.3G-2.3J)[141]. The secreted repulsion factor Slit1, shows peak transcript expression in the early part of the light phase (CT 5.0), while expression of the receptor, Robo4, peaks at dusk (CT 11.0)(Fig. 2.3G). Drosophila expressing a hypomorphic form of ROBO exhibit a shortened period length, and exhibit early cytoplasmic localization of PER [142]. Here we show that Robo4 is clock-regulated in mammals, peaking in phase with Pers 1-3 (Fig. 2.2B). The expression of another secreted signal, Sema3a, peaks at dusk (CT 13), while transcripts for components of the SEMA receptor complex, Nrp2 and Plxna4, peak at CT 0.0 and CT 2.0, respectively (Fig.2.3H), [141,143]. Splice junctions of semaphorins and plexins both cycle, specifically PlxnB1, PlxnB3, and Sema6c cycle (Figs. 2.3I, 2.3J). Different cell types in the SCN may express Sema3e, Plexin4A, Nrp2, and Robo4, allowing differential coupling at different times of day. Furthermore, circadian perturbations from the environment or metabolism may have different effects on transcript expression of each of these secreted signals and its receptors. This may cause greater changes in neuronal connectivity in some cell types than in others, changing the overall coupling of SCN cell types, and modulate firing patterns of the SCN.

Interactions between different isoforms of semaphorins, plexins, neuropilins, slits, and robos are not fully characterized [144,145]. We note that many of the secreted factors and receptors are non-canonical pairings. Daily neuronal connectivity cycles may
necessitate weaker and more transient interactions than those that guide permanent neuronal pathways during development. We also cannot discount the role of non-circadian members of these protein families that are expressed but not clock regulated in the SCN (http://circadb.hogeneschlab.org/query), [114]). We furthermore note that in some cases interactions between semaphorins and plexins are repulsive, while in other cases they are adhesive [141].

As mentioned previously, modulation of neuronal connectivity may require increased space between neurons [132]. In the SCN this may be modulated by Ncam1. NCAM1 is modified by addition of poly-sialic acid (PSA), which leads to repulsion between pre-and post-synaptic neurons [132]. NCAM1 expression may therefore increase synaptic space, allowing modulation of synaptic connectivity by MMP14, L1CAM, and other proteins [132,135,146]. Nrp2 is in phase with Ncam1. NCAM1 and NRP2 are both modulated by the addition of poly-alpha2,8-sialic acid (PSA), [147]. ZBTB16, which is encoded by a cross-brain cycling transcript (Fig. 2B), interacts with GNE, the enzyme that modulates cellular levels of sialic acid, the monomer of PSA [148]. PSA expression in the SCN is increased by glutamate, and is required for glutamate induced phase-shifts [149]. This organization may be a mechanism whereby global signals such as PSA levels modulate specific synaptic connections in response to overall levels of excitation or inhibition in the SCN.

**Synaptic Transmission:**

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Neuronal connectivity regulates synaptic activity, which is itself clock regulated. The synaptic release machinery, glutamatergic and GABAergic signaling, as well as sodium and calcium channels, all display circadian regulation in the SCN. The overall excitation or inhibitory state of the SCN is carefully regulated by neurotransmitters, vesicle release, receptor expression, and downstream signaling. Neurotransmitter release is regulated by vesicle release machinery Snap25, Stxbp1, and synapsins, Syn2 and Syn3 (CTs 2.0-5.0), [150,151], (Fig. 2.4A). Neurotransmitter receptors including NMDA and AMPA receptors Grin1 and Gria1, as well as GABA receptor subunits Gabbr2 and Gabra3, peak between CT 3 and 4 (Fig. 2.4B). Receptors and downstream signaling are organized in space by scaffolding molecules, specifically PSD-95 (CT 4.0) [152]. Glutamate and GABA regulate Ca2+ influx into the cell, and cycle in phase similar to calcium channels which peak just prior to or during the light phase [59,153]. These are Cacna1c (L-type), Cacna1b (N-type) (CT 5.0), Cacna2d2 (voltage dependent), Cacna1a (P/Q type), and Cacna1g (T-type)(CT 0-5), [24,154], (Fig. 2.4C). In addition, some calcium channels may be active pre-synaptically as well [151]. Glutamate and GABA induced calcium influx signals to intracellular calcium through kinase activity [59]. Key intracellular kinase CALMODULIN (Calm1 CT 1.0), activates calmodulin-dependent kinases, Camk2a (CT 4.0), [155,156]. CAMKIIA is required for SCN calcium signaling and affects the molecular circadian clock through calcium dependent regulation of Period gene transcription, and circadian locomotor activity [24,44,157,158]. Many synaptic
signaling molecules are present in a canonical form, while a homologous transcript cycles in a similar phase. Examples include Camk2a and Camk2b, Calm1 and Calm3, and PSD-95 and PSD-93 (Fig. 2.4B). These isoforms may mediate cell type specificity of circadian activity. On the other hand, both isoforms may cycle in the same cell, but exhibit differential localization, or one isoforms may be differentially regulated by stimuli. It is particularly intriguing that Camk2a-b, Calm1-3, and PSD-93-95 are all components of long-term potentiation. It may be that these homologues modulate long-term potentiation in response to different stimuli. In the SCN, they may facilitate LTP in response to zeitgebers such as light, thereby providing a temporal memory of the stimulus [159]. Notably, these cyclers peak slightly later than cyclers mediating of neuronal rearrangement, which would facilitate rearrangements of PSD-95 scaffolds in response to stimuli [132].

We also note several cycling splice junctions from Nelf/Jacob/Nmsf (NMDA receptor synaptonuclear signaling and neuronal migration factor) (Fig. 2.4D). NELF responds to NMDAR stimulation by translocating to the nucleus in an ERK1/2 phosphorylation dependent manner, where it affects gene transcription [160]. Nelf therefore may be a messenger mediating nuclear changes in response to synaptic stimuli, just as rearrangements of synaptic components respond at the cell surface.

We noted that in addition to the canonical calcium/CAMKIIA signaling cascade, recent research has pointed to a variety of additional signaling mechanisms regulating SCN function. SCN firing has been shown to regulate circadian locomotor activity [54].
Particularly important is tetrodotoxin (TTX) sensitive firing of the SCN, as TTX is capable of greatly damping the circadian rhythm of Prokineticin2 (Prok2) transcript expression in the SCN [53]. PROK2 is a key circadian signal secreted by the SCN, known to be important for circadian regulation of locomotor activity [21,161], (Fig. 4E). Intriguingly, three cycling sodium channel subunits Scn3b (CT 1.0), Scn3a (Nav1.3) (CT 1.0), and Scn9a (Nav 1.7) (CT 23.0) (Fig. 4F), peak prior to Prok2 (CT 5.0) transcript expression [162]. These cycling channels peak at dawn, near the peak of the vesicle release machinery, and they may have multiple functions.

Synaptic connectivity is a prerequisite for synaptic activity. We found that synapse components including glutamate and GABA receptor subunits, synaptic scaffolds, and downstream signaling molecules including the canonical CAMKIIA cascade are clock regulated. Synaptic signaling includes key circadian signals, namely, TTX-sensitive potassium channels, Prok2 expression, and CAMKIIA, which regulates transcription of Per genes through CREB signaling [24,162], (Fig. 2.4E-F, 2.4B). The rush hour of transcripts peaking in the light phase codes for regulators of core clock function, organization of synaptic connections within the SCN, as well as SCN output in the form of excitability, firing, and secretion of PROK2.

**Intracellular Signaling:**
We performed phase-set enrichment analysis (PSEA) (Zhang et al. (In Review)), to identify pathways enriched in our data, and pathways that are enriched at particular times (Fig. 2.5A). Many pathways were identified (Supplemental Digital File 2.S12). A particularly intriguing result identified an intracellular signaling cascade that interweaves kinases, phosphatases, and inositol signaling (Fig. 2.5A). The cascade begins with \textit{Slc20a1}, a phosphate transporter that peaks early, at CT 1.5, (Fig. 2.5B), in anticipation of kinases and phosphatases. Examples include the light induce kinase, \textit{Salt-inducible kinase 1 (Sik1 CT 5.0)}, which dampens responses to phase shifts, as well as a light induced phosphatase, \textit{Dusp4} cycles (CT 6.0) [69,163]. Like other synaptic components described previously, \textit{Sik1} has a cycling homologue, \textit{Sik3}, similar to other synaptic components mentioned previously [164]. Additional modulators of synaptic homeostasis cycle, including \textit{Polo-like kinase 2 (Plk2)}, and \textit{Tnik} (CT 5.0), which interacts with PSD-95 [165,166]. Map kinases and interacting proteins also cycle, including \textit{Map4k2}, and mapk-interacting protein transcripts \textit{Mapk8iP2}, and \textit{Mapk8ip3} (CT 4-7) (Fig. 2.5C), [167–169]. These data provide further evidence that Map kinase signaling is involved in SCN function, as has been investigated previously [170,171]. However, the map kinase cascade is extensive, and specific map kinase cascade components upstream of ERK are not all characterized.

MAPK signaling may interweave with inositol signaling, and we noted several inositol cycling transcripts in the SCN [172]. Inositol transporter \textit{Slc5a3} (CT 22.0), peaks prior to inositol dependent cycling transcripts, including phosphatidylinositol-4-
phosphate 5-kinase, type 1 alpha (Pip5k1a, CT 3)), and (3-phosphoinositide-dependent kinase (Pdpk1/Pdk1), (CT 1), [173,174]. PDK1 upregulates protein synthesis as part of its role in the PI3K-AKT MTOR pathway. In synapses, it does this in responses to synaptic signals, such as insulin induced PSD-95 upregulation [152]. Furthermore, PDK1 was previously shown to be involved in MAPK dependent-SCN response to light through modulation of RSK1 [175]. We note that an RSK1 isoform, RSK4 (Rps6ka6) (CT 23.0), cycles, although RSK4 is relatively uncharacterized. (Fig. 2.5D) [174,176,177]. RSK4 may add layers of circadian clock regulation in the SCN, as a cycling RSK isoform.

Phospholipase C Epsilon 1 (Plce1), an important component of inositol signaling, peaks later in the dark phase (CT 18.0) in the SCN [178](Fig. 2.5E). PLCE1 signals through IP3 induced Ca2+ release from the ER [178]. Cytosolic Ca2+ rhythm has previously been shown to cycle in the SCN [179], although the mechanism is not entirely understood. Furthermore, synaptic signaling through calcium has been thought to interact with circadian cycling of intracellular calcium levels [59,180]. Notably, Plce1 cycles in all studied brain regions (Fig. 2.6A). Plce1 domains respond to Rho/GEF, cAMP, and even other forms of PLC, and may therefore integrate a variety of GPCR-coupled secreted signals [181]. PLCE1 also has notably long activation lasting an hour or more, as opposed to seconds to minutes in the case of some other phospholipase C isoforms [182]. Furthermore, the cycling of Plce1 in all studied brain regions means that it could integrate different signals in different brain tissues, through a multitude of pathways, with long lasting kinetics to increase or lower cytosolic Ca2+ levels over a long time
period. Modulation of calcium levels across the brain as well as the SCN may therefore be a key aspect of clock function.

**ER Calcium Homeostasis, and RNA and Protein Stability and Aggregation:**

*Plce1*, a transcript coding for a protein that regulates IP3 mediated calcium release from the ER, cycles in phase across the brain, peaking in the dark phase (Fig. 2.6A) [178]. Intriguingly, *Calr*, the transcript for CALRETICULIN (CALR), a calcium binding protein that maintains ER calcium levels, and lowers cytosolic calcium levels, cycles across the brain and peaks close to the start of the light phase (Fig. 2.6B). CALRETICULIN, (CALR) can also counteract release of calcium into the cytosol from IP3 signaling [183], and contributes to ER Ca2+ storage by binding to calcium through an EF-hand domains [183].

CALR also regulates protein folding and quality control. It does this through complex formation with the constitutively active heat shock protein coded HSP90B1 (Fig. 2.6C) [184]. *Calr* (CT 23.0) and *Hsp90b1* (CT 0.0), transcripts cycle in phase across all studied brain regions. Transcripts for HSP90B1 interacters AHSA1 and STIP1, also cycle in phase, at CT 22.0 (Fig. 2.6D). The transcript for an activator of HSP90B1, *Ahsa1*, cycles in phase with *Hsp90b1* (Fig. 2.6C). AHSA1 facilitates ATP-binding of HSP90B1 allowing HSP90B1 to dimerize, and therefore bind to ‘client’ proteins. STIP1 slows ATP-hydrolysis to facilitate HSP90b1 association with client proteins [185]. STIP1 also interacts with the
endogenous prion protein, PRNP which cycles in phase [186](Fig. 2.6D). PRNP, a
disordered protein prone to aggregation, is therefore in phase with Hsp90b1, which
responds to unfolded proteins [187,188]. Interestingly, CALR can also facilitate
disaggregation of prion protein [189].

PRNP also has non-pathogenic circadian roles. PRNP deficient mice exhibit a
striking behavioral phenotype of lengthened circadian period and altered sleep patterns
[190]. The molecular basis for PRNP interaction with the clock is not well understood,
although it has been shown the E4BP4 (NFIL3) binds to the Prnp promoter [191].
Interestingly, STIP1 signaling through PRNP has been shown to stimulate protein
synthesis via the PI3K-AKT-MTOR pathway [186]. Stip1 and Prnp also cycle in additional
tissues, as does the PI3K pathway (Fig. 2.6E), [1,114].

In addition to regulating prion protein aggregation formation, CALRETICULIN
regulates RNA aggregation through the scaffolding of RNA stress granules [192,193].
Components of RNA transcription, splicing, and transport are tightly co-regulated in
both space and time. In conditions of cell stress, these transcripts are also co-regulated
by formation of stalled translational clusters known as stress granules. Intriguingly, a
number of transcripts for regulators of RNA metabolism with the potential to form
stress granules cycle in phase with Calreticulin in the SCN. A key mediator of miRNA
metabolism, Drosha (CT 2.0), shows peak RNA expression just prior to dawn (Fig. 2.6F).
DROSHA interacts with RNA-splicing factors including TDP-43 (CT 0.5), although DROSHA
itself does not localize to stress granules [194,195]. In addition to TDP-43, other RNA-
binding splicing factors Rbm4b, Srpk1, and Srpk2 also cycle (CT 0-3), [196], (Fig. 2.6F). The essential splicing reaction mediated by U1 and U2, and U4-6 is regulated by these transcripts. SRPK1 is associated with U1 function, while SRPK2 is associated with U4-6 function[197]. Splice junctions of U2 auxiliary factors U2af1 and U2af2 also peak at the beginning of the light phase, as do U6 component Lsm6 and a lesser-characterized LSM gene, Lsm14b [198–201], (Fig. 2.6G, 2.6H). Furthermore, a large number of Hnrnp splice junctions cycle in the same phase. HNRNPs may regulate sequence-specific splicing reactions, sometimes by competing for binding with other splicing regulators, and human homologues of many HNRNPs interact with human TDP-43 [202–204], (Fig. 2.6I).

In times of cellular stress such as heat shock, splicing and translation of RNAs is stalled and transcripts and regulators of RNA metabolism collect in stress granules. Many cycling transcripts participate in this process. The human homologue of TDP-43 has been shown to interact with CAPRIN1 (CT 0.0) [204], which is a stress granule component and mediator of stress granule assembly, along with G3BP1 and G3BP2 (CT 1.0),[205–208]. SRPK1 phosphorylates RBM4, causing it to aggregate in cytoplasmic stress granules [209], although this has not been shown for RBM4B, specifically. The human homologues of SRPK1 and TDP-43 interact, although to our knowledge the functional significance of this interaction has not been further characterized [204]. Intriguingly, the human homologue of SRPK1 forms complexes with AHSA1 and HSP90 [210]. This interaction may be important, given the role of heat shock proteins in disassembling stress granules [211]. Human TDP-43 forms complex with human HSPH1
(CT 23.0), [204]. Hsph1 is a cross-brain cycler (Fig. 2.2B, 2.5J) that regulates disaggregation of proteins [212].

RNA metabolism is a potent area for circadian regulation of gene expression, as splicing factors could regulate both core clock components, and many clock output genes. Thus, many RNAs that aggregate in stress granules may also have none-pathogenic clock functions. Tdp-43, Srpk1, and Rbm4b modulate RNA splicing under baseline conditions. Rbm4b (Lark2) is a homolog of Drosophila Lark, which modulates period length in flies through PER modulation [213,214]. Rbm4b cycles antiphase to Per1-3 (Fig 2.6F, Figs. 2.2F-H, respectively). Intriguingly, RBM4 is also involved in hypoxia related changes in translation, and stress granule formation, although once again RBM4B has not been investigated individually [209]. Its role in oxidative stress perturbations and clock dysfunction warrant further investigation [215]. Human homologues of TARDBP and CAPRIN1 interact [204], and CAPRIN1 is predicted to play a role in the core clock [34]. Interestingly, TDP-43 stress granule formation is driven by oxidative stress, heat shock, and other stressors [205,216], which may also be involved with effects of circadian disruption on neurodegeneration [215].

Expression of transcripts related to RNA and protein metabolism peak in phase in the SCN. These include RNA binding proteins that control RNA stability, splicing, and contribute to relocalization of these transcripts to stress granules during cellular stress. Aggregation-prone proteins such as PRNP and its ligand STIP1 are also in phase (Fig. 2.6D). We noted coordinated expression of heat shock proteins, which respond to
misfolded proteins and disassemble stress granules. Coordinated expression of stress granule related transcripts and heat shock proteins may be a clock-regulated homeostatic response to stress granules. Loss of temporal coordination of heat shock proteins and stress-granule relating splicing factors may be part of the recently discovered connection between loss of core clock function and increased markers of neurodegeneration [215]. Loss or weakening of the circadian clock through aging, genetic polymorphisms in molecular clock components, oxidative stress or other factors may alter circadian expression of *Tdp-43, Caprin1*, and other transcripts, so that their peak expression no longer coincides with peak expression of heat shock factors. Heat shock factors might then be less efficient at disaggregating stress granule components, contributing to neurodegeneration.

**Comparison of Phase of mRNA and Protein Expression in the SCN:**

Given the importance of protein regulation in the SCN, we then sought to determine the relationship between phase of cycling RNA expression and phase of cycling protein expression in the SCN. We compared our data with data from an SCN proteomics data set from Chiang *et al.* (2014), consisting of 6 time points over 24 hours, with 4 replicates at each time point. We analyzed the proteins that showed variance over the time of day (The Time of Day Proteome) using JTK-CYCLE to determine phase of these proteins (see Materials and Methods). Twenty-eight transcripts were represented
in both data sets [109]. Comparison of phase of peak RNA transcript expression with phase of peak protein expression shows a median difference of 7 hours (Fig. 2.7) (see Materials and Methods, Supplementary Digital File 2.S13). Furthermore, 13 of the 28 genes had RNA – protein phase differences of less than 6 hours, while 15 had phase differences greater than 6 hours. These results reflect phase differences between peak circadian RNA and protein expression in other tissues, such as the liver. Robles et al. (2014) reported ~50% of genes had 2-6 hours of phase difference, and 40% had greater than 6 hours between peak mRNA and protein expression[33]. The wide range of time between peak mRNA expression and peak protein expression may mean that translation of mRNAs is extensively regulated post-translationally, awaiting future investigation [33].

Summary:

Our data show that most clock regulation of transcription in the SCN is largely SCN-specific. 523 transcripts cycle in the SCN, and 23 transcripts cycle across the SCN and other studied brain regions. The largest group of cross-brain cycling transcripts consists of clock components. Most clock components are in phase in the SCN and other tissues. Activators Npas2 and Bmal1 show lowered amplitude in the SCN, possibly to facilitate resetting the clock in the brain. Per1-3 show advanced phase in the SCN relative to other tissues. Additional cycling transcripts provide clues to circadian
organization of brain function. Tight junction transcripts *Ahnak* and *Jam2* peak in the dark, while modulators of synaptic connectivity *Mmp14, L1cam,* and *Ncam1* peak at the beginning of the light phase. *Mmp14* may extend circadian neuronal connectivity throughout the brain as a whole. We also describe anti-phase cycling of secreted axon-guidance factors and their receptors. Neuronal connectivity is extended to the level of synapses, where neurotransmitter release, receptors, ion channels, and downstream signaling are clock regulated at the transcript level. The overall excitation level of the SCN is modulated through glutametergic and GABAergic signaling, as well as an abundance of ion channels. Regulation of ion homeostasis is particularly crucial in the case of calcium, which is regulated by many types of cycling channels, the CAMKIIA kinase cascade. This cascade connects to intracellular calcium signaling, which is regulated across the brain. Intracellularly, cascades of calcium signaling may connect synaptic transmission and intracellular homeostasis. This cascade connects to intracellular calcium signaling, which is regulated across the brain. *Plce1* mobilizes calcium and peaks in the dark phase. *Calr* sequesters calcium in the ER. *Calr* and *Hsp90b1* cycle across the brain, regulating aggregation and disaggregation of aggregation-prone proteins such as PRNP, and stress granule components. Overall, we show circadian expression of transcripts regulating RNA and protein stability, synapse activity, neuronal connectivity, and the core molecular clock.
2.5 Figures

Figure 2.1: Profile of Circadian RNA Transcript Expression in the Suprachiasmatic Nucleus
**Figure 2.1: Profile of Circadian RNA Transcript Expression in the Suprachiasmatic Nucleus (SCN).** A) Heat map of 523 phase sorted transcripts cycling in the SCN with a JTK-CYCLE q-value of q < 0.05. Circadian time (CT) is on the x-axis, with peak expression indicated in yellow, and trough expression in blue. B) Polar plot of peak phase of cycling transcripts. CT is indicated on the circumference of the plot with each circle representing a different number of transcripts, with the smaller to the larger circle indicating 20-80 transcripts. C) A frequency histogram of the phase of peak expression, with circadian time (CT) indicated on the x-axis. D) Heat map of 654 phase sorted splice junctions cycling in the SCN with an adjusted P-value of p < 0.05. Circadian time (CT) is on the x-axis, with peak expression indicated in yellow, and trough expression in blue. E) Polar plot of peak phase of cycling splice junctions. CT is indicated on the circumference of the plot with each circle representing a different number of splice junctions, with the smaller to the larger circle indicating 50-200 transcripts. F) A frequency histogram of the phase of peak splice junction expression, with circadian time (CT) indicated on the x-axis.
Figure 2.2 Overlap of Transcripts that Cycle in the SCN, Brain, and Peripheral Tissues
Figure 2.2: Overlap of Transcripts that Cycle in the SCN, Brain, and Peripheral Tissues.

A) Chow-Ruskey diagram of cycling transcripts in the SCN overlapping with those from hypothalamus, brainstem, and cerebellum. B) List of transcripts cycling in all four brain regions. C-H) Expression plots for core clock genes across tissues with SCN in red, brain tissues in blue, and peripheral organs in black. Data are plotted as fraction of peak transcript expression. All non-SCN data are from Zhang and Lahens et al., (2014)[1].
Figure 2.3 Circadian Regulation of Neuronal Connectivity

A

Ahnak

B

Jam2

C

Mmp14

D

Kank1

E

Mmp14, L1cam, Ncam1

F

Sema3e, Nrp2, PlxnA4

G

L1cam

H

Gpr56

I

Sema6c Junction

J

PlexinB Junctions

K

PlxnB

SCN Brain tissues
**Figure 2.3: Circadian Regulation of Neuronal Connectivity.**

A) Tight junction cycler *Ahnak* is plotted in SCN (red), brain regions (blue).  
B) *Junction adhesion molecule 2, Jam2*, is plotted in SCN (brown).  
C) *Mmp14* is plotted in SCN (red), brain regions (blue).  
D) SCN neuronal connectivity cyclers are plotted as *L1cam* (yellow), *Mmp14* (orange), and *Ncam1* (pink).  
E-F) *Kank1*, and F) *Gpr56* are plotted in SCN (red), and other brain regions (blue).  
G-J) Gene expression profiles for axon guidance factors and receptors.  

The receptor transcript *Robo4* is indicated in green. The secreted signal *Slit1* is indicated in purple.  
H) Transcripts from the receptor complex are plotted as *Nrp2* (green) and *PlxnA4* (blue), and the secreted guidance factor is plotted as *Sema3e* (purple).  
I) The transcripts for receptor splice junctions from *PlxnB1* (light blue) and *PlxnB3* (yellow).  
J) The axon guidance factor transcript *Sema6c* splice junction is plotted in red. All non-SCN data are from Zhang and Lahens et al., (2014)[1].
Figure 2.4 Circadian Regulation of Synaptic Transmission

A. Synaptic Vessicle Release

Legend
- Snap25
- Stxbp1
- Syn2
- Syn3

B. Neurotransmitter Response

Legend
- Calm1
- Calm3
- Camk2a
- Camk2b
- Gabbr2
- Gabra3
- Gria1
- Grin1
- PSD-93
- PSD-95

C. Calcium Channel Subunits

Legend
- Cacna1a
- Cacna1b
- Cacna1c
- Cacna1g
- Cacna2d2

D. Nelf Junctions

Legend
- Nelf 1st junction
- Nelf 2nd junction
- Nelf 3rd junction
- Nelf 4th junction

E. Prokineticin 2

Legend
- Prok2

F. Sodium Channel Subunits

Legend
- Scn3a
- Scn3b
- Scn9a
Figure 2.4: Circadian Regulation of Synaptic Transmission. A) Circadian expression profiles for synaptic vesicle release genes. Snap25 (light aquamarine), Stbx1 (aquamarine), Syn2 (dark aquamarine), Syn3 (black). B) Transcripts responding to neurotransmitters cycle in phase: Calm1 (dark red), Calm3 (red), Camk2a (dark pink), Camk2b (pink), Gabbr2 (orange), Gabra3 (yellow), Gria1 (green), Grin1 (dark green), PSD-93 (light blue), PSD-95 (dark blue) C) Calcium channel subunits are plotted as Cacna1a (light pink), Cacna1b (dark pink), Cacna1c (red). Cacna1g (dark red), and Cacna2d2 (black). D) Nelf splice junctions are plotted in order of starting nearest to the transcriptional start site of the gene (junction nearest to TSS is splice junction 1, furthest is 4), (tan1, tan2, tan3, tan4). E) Sodium channel subunits are plotted as Scn3a (light blue), Scn3b (blue), Scn9a (dark blue). F) Prokineticin 2 transcript is plotted in dark purple. All non-SCN data are from Zhang and Lahens et al., (2014)[1].
Figure 2.5 Intracellular Signaling

A

INTRACELLULAR,SIGNALING,CASCADE

1

PDPK1, SLC20A1, SRPK1, ARHGA1, ABR, SIK1, SIK3, TNIK, PIK2, NR2C2, RND3, MAPK8IP, ACER, DUSP4

Phospho–Signaling

Inositol Metabolism

Pice1

Legend

Dusp4
Map4k2
Map4k3
Mapk8ip2
Mapk8ip3
Pik2
Sik1
Sik3
Tnik
Figure 2.5: Intracellular Signaling. A) Phase-set enrichment analysis finds “Intracellular Signaling Cascade” from the Biological Process GO Term gene set (C5: GO gene sets, Broad Institute MSigDBCollection), Kuiper’s q value < 0.001166667 (vs. uniform) (Zhang et al. In Review). B) Expression profile for the phosphate transporter Slc20a1 (purple). C) Cycling kinase signaling cascade, expressed following phosphate importer peak expression; Dusp4 (black), Mapk8ip2 (dark red), Mapk8ip3 (red), Map4k2 (pink), Plk2 (orange), Sik1 (yellow), Sik3 (green), Tnik (blue). D) Inositol signaling cascade including inositol transporter Slc5a3 (dark pink), Pip5k1a (orange), 3-phosphoinositide dependent kinase, Pdk1/Pdpk1 (black), and Rps6ka6 (blue). E) Cycling of Plce1 (red), a downstream effector of mapkinase and inositol signaling.
Figure 2.6 Cycling Transcripts Regulating RNA and Protein Metabolism and Aggregation

A

Plce1

B

Calr

C

Hsp90b1

D

Ahsa, Prnp, Stip1

Legend

U2af1

U2af2

Legend

Hnrnpa1

Hnrnpa1−minor

Hnrnpa2b1

Hnrnpa3

Hnrnpc

Hnrnph3

Hnrnpm

Legend

Ahsa1

Prnp

Stip1

Legend

Caprin1

Drosha

G3bp2

Rbm4b

Srpk1

Srpk2

TDP−43

Legend

Lsm14b

Lsm6

Legend

Hnmpa1−major

Hnmpa1−minor

Hnmpa2b1

Hnmpa3

Hnmpc

Hnmpf3

Hnmpm

SCN

Brain tissues

Peripheral Tissues

66
Figure 2.6: Cycling Transcripts Regulating RNA and Protein Metabolism and Aggregation. Plots of A) Plce1 B) Calreticulin, and C) Hsp90b1 transcript expression in the SCN (red) and other brain tissues (blue). Data plotted as fraction of peak transcript expression. D) Ahsa1 (orange), Prnp (brown), and Stip1 (yellow). E) Stip1 transcript expression in the SCN (red), other brain tissues (blue), and the periphery (black). F) Transcripts regulating RNA stability, splicing, and metabolism are plotted as Caprin1 (purple), Drosha (pink), G3bp2 (orange), Rbm4b (yellow), Srpk1 (green) Srpk2 (blue), TDP-43 (brown). G) U2af splice junctions are plotted for U2af1 (yellow), and U2af2 (purple). H) Lsm splice junctions are plotted as Lsm14b (dark aquamarine) and Lsm6 (orange). I) Hnrnp splice junctions are plotted as Hnrnpa1 major splice junction (green), Hnrnpa1 minor splice junction (yellow), Hnrnpa2b1 (light pink), Hnrnpa3 (dark aquamarine), Hnrnpc (light blue), Hnrnph3 (pink), Hnrnpm (brown). J) Hsph1 transcript expression in the SCN (red), other brain tissues (blue), and in the periphery (black). Data plotted as fraction of peak transcript expression. Non-SCN Data replotted from Zhang and Lahens et al. (2014)[1].
Figure 2.7 Phase delay between mRNA and protein for clock regulated genes
Figure 2.7: Phase delay between mRNA and protein for clock regulated genes:

Phase of peak expression cycling RNA transcripts (red) and Protein (blue) in the SCN. Transcript data are from the SCN 1.0 ST arrays data, and phase of peak expression of proteins identified in the Time of Day proteome from Chiang et al. (2014) (see Methods for details of JTK-CYCLE [113] analysis).
2.6 Supplemental Information

Supplemental Files:

**Supplementary_digital_File 1:** JTK-CYCLE Analysis of SCN 1.0 ST Data:

**Supplemental_digital_File 2:** Counts of Sense Genes in SCN RNA-Seq Data, 6 Hour Resolution

Supplemental_File_2_FINAL_master_list_of_genes_counts_MIN.sense.SCN_6hour_resolution_JTK.csv

**Supplemental_digital_File 3:** Counts of Antisense Genes in SCN RNA-Seq Data, 6 Hour Resolution

Supplemental_File_3_FINAL_master_list_of_genes_counts_MIN.antisense.SCN_6hour_resolution_JTK.csv

**Supplemental_digital_File 4:** Counts of Sense Strand Exons in SCN RNA-Seq Data, 6 Hour Resolution

Supplemental_File_4_FINAL_master_list_of_exons_counts_MIN.sense.SCN_6hour_resolution_JTK.csv

**Supplemental_digital_File 5:** Counts of Antisense Strand Exons in SCN RNA-Seq Data, 6 Hour Resolution

Supplemental_File_5_FINAL_master_list_of_exons_counts_MIN.antisense.SCN_6hour_resolution_JTK.csv

**Supplemental_digital_File 6:** Counts of Sense Strand Introns in SCN RNA-Seq Data, 6 Hour Resolution

Supplemental_File_6_FINAL_master_list_of_introns_counts_MIN.sense.SCN_6hour_resolution_JTK.csv

**Supplemental_digital_File 7:** Counts of Antisense Strand Introns in SCN RNA-Seq Data, 6 Hour Resolution

Supplemental_File_7_FINAL_master_list_of_introns_counts_MIN.antisense.SCN_6hour_resolution_JTK.csv

70
**Supplemental_digital_File_8**: Counts of Junctions in SCN RNA-Seq Data, 6 Hour Resolution

Supplemental_File_8_FINAL_master_list_of_junctions_counts_MIN SCN_6hour_resolution_JTK.csv

**Supplemental_digital_File_9**: Junction Table With Ranked Spliceforms

Supplementary_Fig_9_SCN_junction_table_with_ranked_spliceforms

**Supplementary_digital_File_10**: JTK-CYCLE Analysis of SCN MOE 430 Time Course from Hatori et al. (2014)

Supplementary_Table_10_Hatori_RMA_JTK

**Supplemental_digital_File_11**: SCN PSEA GO BP Analysis

Supplemental File 11 SCN PSEA GO BP Analysis .zip

**Supplemental_digital_File_12**: JTK-CYCLE Analysis of (Chiang et al. 2014) Time of Day Proteome

Supplementary_Figure_12_JTK_CYCLE_Chiang_et_al_2014.csv
Supplementary Figure S2.1: Comparison of \(-\log_{10}\) of BH.Q Values Between SCN 1.0 ST data and Hatori et al. (2014) SCN Data

Supplementary Figure 1: Comparison of \(-\log_{10}\) of BH.Q Values Between SCN 1.0 ST data and Hatori et al. (2014) SCN Data

A)

Supplementary Figure S2.1: Comparison of \(-\log_{10}\) of BH.Q Values Between SCN 1.0 ST data and Hatori et al. (2014) SCN Data. A) Negative \(\log_{10}\) of BH.Q values from SCN 1.0 ST data with BH.Q <0.05 are compared with corresponding negative \(\log_{10}\) of BH.Q values from the Hatori et al. (2014) SCN time course as analyzed by JTK-CYCLE (see Materials and Methods section). The size of the marker for each gene is scaled and colored according to the respective gene’s BH.Q value in the SCN 1.0 ST data.
Supplementary Figure S2.2: Comparison of Phase in SCN 1.0 ST data and Hatori et al. (2014) SCN Data.

**Supplementary Figure 2: Comparison of Phase in SCN 1.0 ST data and Hatori et al. (2014) SCN Data**

**A)** Phases of genes from SCN 1.0 ST data with BH.Q <0.05 are compared with corresponding Phase values from the Hatori et al. (2014) SCN time course as analyzed by JTK-CYCLE (see Materials and Methods section). Size of marker for each gene is scaled and colored according to the respective gene’s BH.Q value in the SCN 1.0 ST data.

**B)** Boxplot of phase differences between SCN 1.0 ST data and Hatori et al. (2014) SCN data (BHQ<0.05 in SCN 1.0 ST data).
2.7 Acknowledgements

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CHAPTER 3: The Effect of In Utero and Adult Diet on Adult Circadian Phenotype

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3.1 Abstract

The impact of circadian rhythms on metabolism has been a focus of recent efforts in the scientific community due to its effects at the behavioral, tissue, and molecular levels. Here we investigate the effects on in utero diet on adult circadian phenotypes in male and female mice, along with phenotype resulting from adult control or high fat diet following different in utero diets. Our data show that mouse circadian activity rhythms are to a great extent resistant to perturbation by in utero diet, and confirm the effect of high fat diet in lengthening circadian period of locomotor activity. Male and female mice show different locomotor activity patterns regardless of in utero or adult diet, namely greater error onset of activity in female mice, as well as greater alpha hour in constant darkness. PER2::LUCIFERASE expression in the SCN and arcuate nucleus showed opposite trends between the sexes, with male mice having higher amplitude and baseline of expression than females in the SCN, but females having higher baseline and amplitude of PER2::LUC expression in the arcuate nucleus. In utero and adult dietary perturbations showed trends of dampening baseline and amplitude in
both sexes in the SCN. In the arcuate nucleus, males and females showed sex differences in response to in utero and adult diet conditions in baseline and amplitude of PER2::LUC expression. Overall, we identify effects of in utero and adult diet on adult circadian rhythms at the behavioral level and at the level of PER2::LUC expression in hypothalamic nuclei that regulate circadian rhythms and metabolism.

### 3.2 Introduction

Metabolic and circadian homeostasis are both key health concerns for all life on earth. In Western society, metabolic conditions affected by the circadian clock such as obesity, diabetes, and metabolic syndrome are increasing health concerns in modern society [20,103]. Metabolism and the circadian clock interact on many levels, including behavioral, tissue, and molecular levels [100,217]. Clock disruption such as the ClockΔ19 mutation produces obesity and metabolic syndrome [13]. Furthermore, there is increasing evidence that circadian disruptions such as high levels of light in modern society, shift work, and other perturbations of the circadian clock may also affect metabolism [20]. Given the variety of possible threats to metabolic health, it is crucial to understand how different environmental conditions interact to affect the homeostasis and overall health of an animal. Here we look at the effect of in utero diet, and their effects on circadian phenotypes in adult male and female mice.
In utero effects on both circadian rhythms and metabolism are known to be powerful, long lasting, and impact the life of adult animals. Mouse pups are imprinted with the circadian light and dark schedules the mother mouse receives during gestation [218]. Furthermore, the light schedule imprinted during gestation affects the adult animal’s circadian period at both the behavioral and SCN gene expression levels [218].

In utero diet has similarly long lasting and powerful effects. Conditions of in utero dietary perturbation caused by a low protein (LP) diet, high fat diet (HF) or other conditions, affect feeding behavior and sucrose preference of adult mice, as well as the growth of the hypothalamus [219–221]. Furthermore, in utero diet causes long lasting epigenetic changes [220, 221]. There is some evidence that in utero diet affects epigenetic modifications in clock genes. For example, in utero diet has been shown to alter methylation in the primate periphery [104].

However, despite the powerful interaction of metabolism and circadian rhythms, and the long lasting effects of in utero diet, very few studies have looked at the effect of in utero diet on circadian rhythms. Sutton et al. (2010), show that 8 week old male mice that received a LP in utero diet have increased feeding and increased wheel running during the light phase compared with controls, and lower activity during the dark phase. In adult male rats that received a LP in utero diet, feeding is higher both during the light and dark phases when compared with controls [101]. Adult diet has the effect of dampening circadian expression of some genes [100]. Studies have also shown changes
in core clock components and circadian signaling molecules in the hypothalamus of adult male rats fed a LP in utero diet [102,222].

There is also evidence that in utero diet affects development of the SCN, with altered SCN structure in adult male mice that received a LP in utero diet. These finding are important, since the development of the circadian clock and the master mammalian pacemaker, the suprachiasmatic nucleus of the hypothalamus, is not completely understood [223]. Likewise, development of other areas of the hypothalamus that regulate hunger and satiety, and feeding and fasting, are similarly still under investigation. In the SCN, VIP and VP reactivity were both reduced in male rats fed a LP in utero diet compared with controls [222]. Development of other areas of the hypothalamus is also affected by in utero diet. Particularly intriguing data show that a LP in utero diet affects circadian expression of transcripts such as NPY and POMC, which regulate feeding and satiety, respectively, in the hypothalamus of 17 day old rats [101]. NPY is differentially expressed even at 35 days in the hypothalamus of LP and control rats [101]. These data are interesting from a circadian perspective since NPY is able to phase shift the SCN, although how levels of NPY altered by in utero diet may affect the SCN is not known [224].

Despite evidence that in utero diet may affect adult circadian rhythms, the effect of in utero diet on adult circadian period length has not been studied. Additionally, changes in circadian gene expression in adult animals in response to in utero diet has been incompletely studied at the level of the SCN [102]. To address these questions, we
have adapted models of in utero dietary perturbation where pregnant female mice are fed a control, low protein (LP), or high fat (HF) in utero diet to study circadian rhythms [220,221]. We also investigated the effect of interaction of control or LP in utero diet with control or adult high fat (HF) diet. We measured circadian period of locomotor activity and other activity parameters in constant darkness in adult male and female mice from these dietary conditions. Finally, we examine period, baseline, and period of PER2::LUC expression in the SCN and arcuate nuclei of adult mice that received these in utero and adult diets [23].

3.3 Materials and Methods

Animals:

C57/BL6J naïve females of 6-8 weeks of age were ordered from Jackson laboratories[225]. Upon arrival in the animal facility, females were allowed to acclimate for seven days. Females were then briefly mated with C57/BL6J sires [225]. Sires were removed prior to birth, so that paternal behavior did not affect pup development. Mice received control (Test Diet #5755), low protein (Test Diet # 5769), or a high fat diet (Test Diet #58G9) from the time of mating [226]. Dams continued receiving respective diets through gestation and weaning. Offspring were toe-clipped for identification purposes
prior to postnatal day 10. Offspring were weaned to standard chow (5010) or a high fat
diet (Test Diet #58G9) at four weeks of age [227,228]. All experiments were carried out
on adult animals of 2-4 months of age. All experiments were carried out in accordance
with regulations of the IACUC at the University of Pennsylvania.

**Circadian locomotor activity:**

Adult mice at 2-4 months of age were singly housed in running wheel cages in an
LD box. Mice were acclimated to the LD box and running wheel data were collected
during at least 10 days of 12/12 light/dark. Mice were then released into constant
darkness for at least 10 days while running wheel data were collected [100]. Running
wheel data were analyzed with ClockLab software (Actimetrics), and the results were
exported for statistical analysis. All data are expressed as mean ± (Standard Error of
the Mean)[34,229].

**Bilateral SCN and arc dissection, culture, and lumicycle analysis:**

Homozygous PER2::LUC mice were obtained from Jackson Laboratories, and a
homozygous PER2::LUC line was established and maintained (Jackson Laboratory Stock
No: 006852)[23,225]. Homozygous PER2::LUC males from this line were mated with WT
C57/BL6J naïve females using the same methods as described above. Offspring were
PER2::LUC heterozygotes from each of the in utero and adult dietary conditions described previously.

Adult 2-4 month old PER2::LUCIFERASE heterozygous male and female mice were sacked by cervical dislocation and decapitated. Brains were removed as described in Savelyev et al. (2011) and cultured in ice cold HBSS (Gibco/Life Technologies, Ref. 14175-079)[230]. 300 µm sections of the SCN and arcuate nucleus were dissected by vibratome (NVSLM1, World Precision Instruments) as described in Savelyev et al. (2011) with the aid of a mouse brain atlas [87]. The main change to the protocol from Savelyev et al. (2011) was that the bilateral SCN were left intact and not separated bilaterally. The arcuate nucleus was also left bilaterally intact. Bilateral SCN and arcuate nuclei from each animal were separately cultured on membranes (EMD Millipore Millicell Culture Plate Inserts; PICM0RG50). Each membrane was placed in 1200 µL of medium in a 35mm x 10 mm cell culture dish (Corning LS Part # 430165) as described in Savelyev et al (2011)[230]. Lumicycle recordings (36°C) were then recorded for seven days from each culture[231]. Data were quantified using Lumicycle analysis software. The first day of recording was removed, and the remaining data were and exported as unsubtracted counts of luciferase expression (Actimetrics)[231].

**Analysis of PER2::LUC recordings:**
Data were quantified using LumiCycle analysis software [231]. The first day of recording was removed, and the remaining data were exported as un-subtracted counts of luciferase expression [231]. Recordings were normalized to include the same number of measurements, starting at least one day after the start of recording, and continuing for at least five days of recording time. Data were then analyzed for period, amplitude, and baseline of PER2::LUC expression for recordings with period lengths between 20 and 28 hours using Wavelets () (https://cran.r-project.org/web/packages/wavelets/), and Waveclock(), (https://cran.r-project.org/src/contrib/Archive/waveclock/) software in R [232–234].

**Statistical analysis:**

Data were analyzed for difference between median values in different in utero diet groups in each sex by the Kruskal-Wallis test and p-values were adjusted for multiple comparisons using Kruskal-Wallis rank sum test. using Prism software in Graphpad and the Asbio() program in R [232,235–237]. Results were considered significant if they met the criterion of Kruskal-Wallis rank sum test adj. p<0.05. Results are reported as Kruskal-Wallis rank sum test adj. p-value in the text. Complete Kruskal-Wallis and Kruskal-Wallis rank sum test analysis can be found in Supplemental Table 3[238].
3.4 Results

Introduction:

The aim of our experiments was to test the hypothesis that in utero diet would affect adult circadian phenotype. We furthermore investigated if in utero diet interacted with adult diet in affecting circadian phenotype. We investigated circadian phenotype at the behavioral level by testing circadian period length of locomotor activity on running wheels. These experiments were conducted in constant darkness, to measure locomotor activity as a readout of the circadian clock, without the masking effects of the light dark cycle [239].

Locomotor activity in 12:12 light dark:

Mice were singly housed in running wheel compartments with a 12:12 light/dark cycle (LD) for at least 10 days to acclimate to the wheel running compartment. Activity recordings were assessed by visual inspection to ensure that mice from all dietary conditions entrained to the LD cycle. Male and female mice from all studied in utero diet conditions, and from all combinations of in utero diet and adult diet conditions successfully entrained to light. Total activity counts during LD showed that there were no significant differences in total activity between male mice from different in utero diet
conditions, or between female mice from different in utero diet conditions (Supplementary Figure 3.S1A and 3.S1B, Supplemental Tables 3.S1A and 3.S1B). There was a trend of greater activity in female mice than in male mice, that has been previously noted in the literature [52]. Overall, the LD activity data showed that in utero diet and adult diet conditions did not affect animals’ overall activity levels in LD. Therefore, any changes in locomotor activity observed in constant darkness would not be attributable to gross physical effects of in utero or adult diet.

**Tau of locomotor activity of in utero diet mice in constant darkness:**

Mice were released into constant darkness to measure circadian locomotor activity without the masking effects of the light/dark cycle. Circadian locomotor activity was measured for at least 10 days beginning at least 72 hours following release into constant darkness. Circadian locomotor activity period (tau) was measured for each animal (see Materials and Methods). Con-Con (In utero diet-Adult diet) males had an average tau of 23.6± 0.0389  (N = 25) in constant darkness. HF-Con males had an average period length of 23.7± 0.0361  (N = 26). Interestingly, LP-Con males had a longer tau than HF-Con males, with an average of 23.8± 0.0582  (N = 11), although this result did not reach statistical significance. Weaning to a high fat diet lengthened period following a control in utero diet; Con-HF males had an average period length of 23.8± 0.0326  (N = 17). The difference in tau between Con-HF and Con-Con males had a
Dunn’s adjusted p < 0.015948. The combination of an LP in utero diet followed by weaning to a high fat adult diet did not increase tau in LP-HF males, and in fact the average tau was 23.7± 0.0692 (N = 5), which is shorter than that of LP-Con males (Fig. 3.1A, Table 3.1B).

Con-Con females had an average tau of 23.7± 0.0304 (N=19) in constant darkness, which is slightly longer than that of control males. HF-Con females had an average tau of 23.8± 0.0309 (N=27), which was longer than the tau of Con-Con females, as well as longer than the tau of HF-Con males. Weaning to an adult high fat diet following a control in utero diet lengthened tau to 24.0± 0.0739 (N=5) in Con-HF females. LP in utero diet followed by a control adult diet did not result in a lengthened tau as it did in males, and LP-Con female mice had an average tau of 23.7± 0.0290 (N=10). Weaning to a high fat adult diet following a LP in utero diet resulted in a similar lengthening of tau similar to that seen in control females weaned to an adult HF diet.

The difference in tau between LP-Con females and LP-HF females had a Dunn’s adjusted p <0.015891. LP-HF females had an average tau in DD of 23.9± 0.0852 (N=8). The difference in tau between LP-Con females and LP-HF females was had a Dunn’s adjusted p <0.015891. Furthermore, the difference in tau between LP-Con females and Con-HF females had a Dunn’s adjusted p<0.00739, further highlighting the lengthening effect of HF adult diet on tau (Fig. 3.1B, Table 3.1B).

We note that the differences in tau observed in different dietary perturbations was at least partially caused by factors other than the weight of the animals while on
the wheels. This type of independence of weight and tau has been noted previously in comparisons of tau in adult male mice fed a control or HF adult diet [100]. In our data, the most notable independence of weight and tau is seen in the LP-Con male mice, which have a long tau in DD, yet have the lowest average weight of any of the groups of male mice (Supplemental Fig. 3.S2A, Supplemental Table 3.S2A). Patterns of average weight on wheels were similar in female mice, with mice that received a HF in utero diet having slightly higher weights than controls, with highest average weight in mice given an adult HF diet, and mice given a LP in utero diet had lower average weights regardless of adult diet (Supplemental Fig. 3.S2B, Supplemental Table 3.S2B).

Error of activity onset for in utero diet animals in constant darkness:

The error of onset of activity in constant darkness was 0.734 hours ±0.196 (N=25) in Con-Con male mice. Male mice receiving a high fat diet either in utero or as adults had less error of activity onset; HF-Con males had an average error of activity onset of 0.411 hours ± 0.0399 (N=26), and Con-HF males had an error of activity onset average of 0.497 hours ± 0.236 (N=17). An in utero LP diet increased error of activity onset, but did not show an interaction with weaning to an adult high fat diet. LP-Con males and LP-HF males had average errors of activity onset of 1.04 hours ± 0.605 (N=11), and 1.05 hours ± 0.724 (N=5), respectively (Fig. 3.2A, Table 3.2A).
Error of onset of activity was greater in female mice than in male mice in all in utero and adult diet groups, with the exception of LP-Con animals, where error of onset of activity was higher in males. Con-Con females had an average error of onset of activity of 0.893 hours ± 0.265 (N=19). A high fat diet given either in utero or as an adult diet did not greatly change error of onset of activity. HF-Con females had an error of onset of activity of 0.820 hours ±0.220 (N=27), while Con-HF females had an error of onset of activity of 0.780 hours ± 0.418 (N=5). LP-Con females had an average error of activity onset of 0.685± 0.104 (N=10), which is lower than the error of activity onset of LP-Con male mice, as previously noted. Intriguingly, the LP-HF females had an average error of activity onset of 2.14± 1.16 (N=8) (Fig. 3.2B, Table 3.2B). We note that this trend in higher average error of activity onset in LP-HF females must be interpreted with caution, since this group has a low N compared with controls, and error of activity onset may be a measurement that is susceptible to the effects of outliers or near outliers. Error of onset of activity measurements show a fairly wide range between animals in the same in utero diet conditions. The S.E.M. is sometimes more than half of the value of the average, as can bee seen in the Con-HF and LP-HF females.

**Alpha hour in in utero diet animals in constant darkness:**

Con-Con male mice had an average alpha hour (time from activity onset to activity offset (Actimetrics)) of 6.94 hours ± 0.924 (N=25). HF diet given in utero or
following weaning resulted in a slight increase in alpha hour of 7.17± 0.590 (N=26, HF-Con-M), and 7.30± 1.09 (N=17, Con-HF-M), respectively. LP in utero diet decreased alpha hour, and showed a trend of interaction with HF adult diet as well. LP-Con males had an average alpha hour of 6.61± 0.862 (N=11), and LP-HF males had an average alpha hour of 4.37± 0.982 (N=5) (Fig. 3.3A, Table 3.3A).

Female mice had longer alpha hours than male mice, in accord with previous accounts in the literature [52]. Con-Con females had an average alpha hour of 9.22 hours ± 0.754 (N=19). An in utero HF diet did not change the alpha hour, as HF-Con females had an average alpha hour of 9.29 hours ± 0.583 (N=27). A control in utero diet followed by weaning to an adult HF diet increased alpha hour in females to 11.3± 1.73 (N=5) in Con-HF females. An in utero LP diet decreased alpha hour in females to 6.23± 1.11 (N=10) and to in 8.11± 1.99 (N=8) LP-HF-F females (Fig 3.3B, Table 3.3B).

**PER2::LUC expression in the SCN**

**Period of PER2::LUC expression in the SCN**

Period length of PER2::LUC expression in the SCN of male mice was similar between in utero diet groups, with an average period of 24.6 ± 0.471 (N = 4) for Con-Con males, 24.1 ± 0.115 (N = 9) for HF-Con males, and 24.6 ± 0.119 (N=3) for LP-Con Males. Furthermore, period length of PER2::LUC expression in the SCN was similar in
male control and LP mice weaned to a high fat diet. The average period was 24.3 ± 0.187 (N = 8) for Con-HF males, and 24.3 ± 0.176 (N = 2) for LP-HF males (Fig. 3.4A, Table 3.4A).

PER2::LUC period length was likewise similar between female mice receiving control, HF, or LP in utero diets. The average period length of PER2::LUC expression was 24.1±0.583 (N=4) in Con-Con female mice, 25.0±0.0 (N=1) in HF-Con female mice, and 24.5±0.869(N=5) in LP-Con female mice. Additionally, period length of PER2::LUC expression in the SCN was similar in female mice weaned to a high fat diet, with an average period length of 24.6± 0.127 (N=6) in Con-HF female mice, and 24.1±0.101(N=3) in LP-HF female mice (Fig. 3.4B, Table 3.4B).

**Baseline PER2::LUC expression**

Baseline PER2::LUC expression showed more variation in male mice receiving different in utero diets. Baseline expression of PER2::LUC expression was 97.3± 23.8 (N=4) for Con-Con males. A reduction in baseline was seen in HF-Con male mice with an average of 56.0±9.92 (N=9), while LP-Con male mice showed a baseline much closer to controls, with an average of 94.1±28.3 (N=3). Weaning to a high fat diet had a smaller effect in male mice that received a control in utero diet compared to male mice that received a LP in utero diet. Con-HF males had an average baseline of 83.9±14.8 (N=8) in the SCN, while the average for LP-HF males was 37.2±6.32 (N=2) (Fig. 3.5A, Table 3.5A).
Baseline PER2::LUC expression was lower in female mice when compared with male mice that had received the same diet. Baseline PER2::LUC expression was 79.9±15.5 (N=4) in Con-Con Females, showed a decrease to 51.5±0.0 (N=1) in HF-Con females, and an even lower baseline of 30.8± 6.79 (N=5) LP-Con females. Con-HF females had a baseline of 59.1±16.4  (N=6)(Fig. 3.5B, Table 3.5B). Since both a LP in utero diet followed by weaning to a control adult diet, and a control in utero diet followed by weaning to a HF adult diet both resulted in a decrease in baseline expression in the SCN, it is not clear why the combination of LP in utero diet and adult HF diet results in an increase compared to LP-Con in female mice, although this difference could be further investigated as described in the discussion.

**Amplitude of PER2::LUC expression in the SCN**

The amplitude of PER2::LUC expression in the SCN of male mice was highest in controls, with Con-Con male mice having an average amplitude of 7.88± 2.88 (N=4). HF in utero or adult diet resulted in decreased amplitude of PER2::LUC expression: HF-Con male mice had an average PER2::LUC expression amplitude of 5.52± 1.23 (N=9), and Con-HF male mice had an average PER2::LUC expression amplitude of 6.93± 1.45 (N=8). LP-Con male mice had an amplitude similar to normal mice, with an average of 7.24± 2.13 (N=3). LP-HF male mice, on the other hand had an average of 4.06± 0.262 (N=2). The lack of an effect of the LP-Con diet on SCN amplitude, while LP-HF diet results in a
lower amplitude suggests that HF diet leads to lowered amplitude of SCN PER2::LUC expression, and that these changes can be long-lasting (Fig. 3.6A, Table 3.6A).

Con-Con female mice had an average PER2::LUC expression amplitude of 4.50±1.19 (N=4) in the SCN. This is much less than the average amplitude of Con-Con male mice of 7.88±2.88 (N=4). In contrast to the male PER2::LUC mice, where HF in utero or adult diet resulted in decreased amplitude of PER2::LUC expression, in female mice in utero or adult HF diet increased amplitude of PER2::LUC expression when compared with controls. HF-Con female mice had an average PER2::LUC expression level of 5.83±0.0 (N=1), and Con-HF female mice had an average amplitude of 7.40±3.18 (N=6). LP in utero diet resulted in lower amplitude, with LP-Con female mice having an amplitude of 2.01±0.398 (N=5). HF adult diet once again resulted in an increase of amplitude, with LP-HF Female mice having an amplitude of 4.08±0.866 (N=3). Overall, LP in utero diet lowered amplitude of PER2::LUC expression in female mice, while HF diet increased amplitude (Fig. 3.6B, Table 3.6B).

**PER2::LUC expression in the arcuate nucleus**

**Period length of PER2::LUC expression in the arcuate nucleus:**

Period length of PER2::LUC expression in the arcuate nucleus of Con-Con Male mice was 23.5±0.0 (N=1), while Con-Con Male mice had an average period length of
23.0± 0.576 (N=3). Male mice receiving an LP in utero diet had a slightly longer period length of PER2::LUC expression in the arcuate nucleus. LP-Con Male mice had an average period length of 24.1± 0.434 (N=2), while LP-HF Male mice had an average period length of 24.3± 0.101 (N=3) (Fig. 3.7A, Table 3.7A).

The average period length of PER2::LUC expression in the arcuate nucleus of Con-Con Female mice was 23.6±0.0985 (N=3). Period lengths were similar in other in utero diet groups; Con-HF Females had a period length of 24.2± 0.604 (N=3), LP-Con Females had an average period length of 22.9± 0.997 (N=3), and 23.5± 0.425 (N=2) was the average period length in LP-HF Females. In the case of LP-Con Females, the lower average of 22.9 was mainly due to a short period in the arcuate nucleus from one animal, and not from an overall difference in the period length of the LP-Con Female group (Fig. 3.7B, Table 3.7B).

**Baseline of PER2::LUC expression in the arcuate nucleus:**

Con-Con-Male mice had an average PER2::LUC expression of 30.7± 0.0 (N=1) in the arcuate nucleus. PER2::LUC expression in the arcuate nucleus of Con-HF-Male mice was slightly higher, with an average of 34.9± 2.50 (N=3). LP in utero diet resulted in a higher baseline in the arcuate nucleus of both LP-Con-Male mice, with an average baseline of 45.0± 8.47 (N=2), and in LP-HF-Male mice with an average of 45.8± 2.49 (N=3) (Fig. 3.8A, Table 3.8A).
In contrast to the increase in baseline of PER2::LUC expression in the arc of male mice following dietary perturbation, baseline was decreased in the arc of female mice following dietary perturbation. Con-Con-Female mice showed an average baseline of 64.6± 9.45 (N=3) in the arcuate nucleus. Con-HF-Female mice had an average baseline of 42.9± 6.80 (N=3), a modest decrease compared with Con-Con-Female mice. LP-Con-Female mice had the lowest baseline, with an average of 35.3± 1.52 (N=3). LP-HF-Female mice also had low baseline in the arcuate nucleus compared with controls, with an average of 43.2± 5.07 (N=2) (Fig. 3.8B, Table 3.8B).

**Amplitude of PER2::LUC expression in the arcuate nucleus:**

The pattern of amplitude of PER2::LUC expression was similar to the pattern of baseline expression in the arcuate nucleus of male mice. Con-Con-Male mice had an average amplitude of PER2::LUC expression of 1.46± 0.0 (N=1) in the arcuate nucleus. PER2::LUC expression in the arcuate nucleus of Con-HF-Male mice was slightly higher, with an average of 2.16± 1.23 (N=3). LP in utero diet resulted in an increase of amplitude in the arcuate nucleus of both LP-Con-Male mice, with an average amplitude of 4.00± 2.57 (N=2) and in LP-HF-Male mice with an average amplitude of 5.29± 0.689 (N=3) when compared with Con-Con-Male mice(Fig. 3.9A, Table 3.9A).

The pattern of PER2::LUC amplitude in female mice was similar to the pattern of baseline expression in the arcuate nucleus. Further more, the pattern of amplitude
expression in female mice was lower in response to dietary perturbation compared with increased amplitude in males with similar dietary perturbation, the same pattern as seen with baseline of PER2::LUC expression. Con-Con-Female mice showed an average amplitude of 9.63± 3.02 (N=3) in the arcuate nucleus. Con-HF-Female mice had an average amplitude of 3.44± 1.62 (N=3), a modest decrease compared with Con-Con-Female mice. LP-Con-Female mice had the lowest amplitude, with an average of 1.59± 0.536 (N=3). LP-HF-Female mice also had low amplitude in the arcuate nucleus compared with controls, with an average of 2.98± 1.21 (N=2) (Fig. 3.9B, Table 3.9B).

Summary:

Overall, the most significant results were seen in changes in circadian period length of locomotor activity in constant darkness. HF adult diet increased circadian period length more than in utero HF diet in both males and females. A LP in utero diet produced a lengthened circadian period in male mice, while not affecting female locomotor period. This sex difference was only evident when mice were weaned to a control adult diet. When male and female mice from the LP in utero diet condition were weaned to an adult high fat diet, both groups of animals exhibited a lengthening of tau (Fig. 1, Table 1). Period length of PER2::LUC expression in the SCN and arcuate was similar across in utero and adult diet, as well as the sexes of mice. This shows the robustness of circadian period length in response to perturbation. This effect has
previously been shown in response to genetic perturbations of the circadian clock in the SCN, although to our knowledge this effect has not previously been shown in the arcuate nucleus [47]. Different in utero diet conditions showed trends of lowering baseline and amplitude of PER2::LUC expression in the SCN of male and female mice, and having sex specific trends on baseline and amplitude in the arcuate nucleus.

### 3.5 Discussion

**Introduction:**

Our data show that adult circadian rhythms in male and female mice are largely robust following perturbations by in utero diet. We show that the effect of high fat diet in lengthening circadian period of locomotor activity in male mice reported in previous studies by Kohsaka et al. (2007) is also seen in female mice [100]. Male and female mice show sex differences in locomotor activity that are consistently different under different dietary conditions. Female mice have greater error in onset of activity and longer alpha hours in constant darkness compared with male mice (Fig. 3.2A-B and Fig. 3.3A-B). PER2::LUC expression in the SCN and arcuate nuclei of male and female mice did not show significant differences in response to in utero or adult dietary perturbation (Fig. 3.4A-B, Fig. 3.7A-B). Male and female mice showed trends of sex differences in PER2::LUCIFERASE, amplitude, and baseline expression in the SCN and arcuate nucleus (Fig. 3.5A-B, Fig. 3.6A-B, Fig. 3.8A-B, Fig. 3.9A-B).
Effects of HF in utero and HF adult diet on circadian locomotor activity:

Previous data have shown that a HF diet lengthens circadian period in adult male mice [100]. There are also reports that a LP in utero diet alters circadian rhythms at the molecular level, although the effect of an LP in utero diet on circadian locomotor activity in mice has not been previously studied, to our knowledge[222,240]. Our research investigated the hypothesis that in utero diet would affect adult circadian period of locomotor activity. We also investigated the possible interaction between in utero diet and adult diet on effects of adult circadian rhythms. We furthermore hypothesized that there might be sex-specific differences in circadian rhythms as a result of in utero diet, and that these differences might also be affected by weaning to adult HF diet. Our research shows that the effects of in utero diet on adult circadian rhythms are subtle, although there are some sex-specific trends. The data also show interaction between in utero diet and weaning to an adult HF diet, along with sex-specific differences in these responses.

HF in utero diet showed a trend of lengthened circadian period in locomotor activity compared with a control in utero diet in both male and female mice. In both sexes, this trend was less than the effect of an adult HF diet in lengthening circadian period (tau). These effects are consistent with data from Borengasser et al. (2014) showing that circadian gene expression in the livers of adult mice is more affected by
adult HF diet than by gestation by an obese dam followed by weaning to a control diet [241]. Furthermore, Borengasser et al. showed that gestation by an obese dam followed by weaning to an adult high fat diet had a greater effect than gestation by an obese dam followed by weaning to a control diet. Results from our lab and others suggest that the circadian clock is able to adapt to a HF in utero diet at both the levels of circadian locomotor activity and in terms of gene expression. Borengasser et al. (2014) have seen similar patterns in gene expression in the liver. Our specific data showing a greater lengthening of tau in mice given an adult HF diet compared with those that received a HF diet in utero may mean that the SCN is able to adapt to in utero diet, as the SCN is thought to have a role in determining circadian locomotor activity period [47].

Male mice that received an in utero LP diet showed a trend of lengthened adult circadian rhythm, but this effect was not shown in female mice. Both male and female mice showed a trend of lengthened circadian period when weaned to an adult HF diet following a LP in utero diet. These data show that LP in utero diet differentially effects circadian locomotor activity period in male and female mice.

It may be that under conditions of nutritional growth restrictions that male and female mice are affected differently, and that these differences affect the core circadian clock. It is possible that levels of sex-related hormones are altered by in utero diet, and some of these have previously been shown to affect period length of circadian locomotor activity [52,242]. Furthermore, there may be sex differences in the response levels of
feeding and satiety signals in mice exposed to LP in utero diets. For example, levels of
Npy in the hypothalamus differ over the circadian cycles of adult male mice fed a control
or LP in utero diet, and Npy levels vary between male mice fed a control or HF adult diet
[100,240]. NPY has also been shown to phase shift the SCN. It would be interesting to
use a high through-put method to measure RNA expression of clock genes, androgens,
estrogens, and transcripts for phase shifting-peptides related to metabolism such as NPY
in the SCN and arcuate nucleus from animals subjected to different in utero and adult
diets. Changes in levels of expression in these transcripts could help identify the specific
molecules affected by diet that may contribute to circadian changes. Once these genes
are identified, their protein expression could be studied through Western blots or
tissue-slice immunohistochemistry [109,222].

**PER2::LUCIFERASE expression in the SCN:**

The period length of PER2::LUC was similar between male and female mice in
conditions of both in utero and adult diet perturbations. There are several possibilities
to explain this result. Period length of the SCN may be resistant to in utero dietary
perturbations, just as period length in the SCN is resistant to loss of function of some
core molecular clock genes [47]. It may be that the changes in circadian locomotor
period, which were in all cases less than an hour, were not sufficient enough to be
accompanied by changes in period length of PER2::LUC expression.
Another possibility is that the lengthening of circadian period of locomotor activity in some dietary conditions is the result of changes in the SCN that do not affect PER2::LUC expression. It may be that expression of a secreted signal is altered that affects locomotor activity, and PER2::LUC period length is not affected. This possibility could be tested by collecting the SCN region from mice in each in utero diet and adult diet condition, and measuring expression of RNA and proteins that correspond to core molecular clock components and secreted signals such as Prokineticin2, which codes for PROK2, a key secreted cue for circadian locomotor output in mice [21].

It is also possible that in utero diet does not affect the structure of the SCN itself, but affects the SCN’s connections with other areas of the brain and periphery. Mice that receive a control in utero diet during gestation, but are suckled by dams receiving high fat diet, show deficits in connectivity between brain regions, and specifically between hypothalamic nuclei [243]. To our knowledge, this effect has not been investigated for neuronal connections between the SCN and other brain regions. It would be interesting to investigate changes in neuronal connectivity between the SCN and arcuate nuclei, as well as between the SCN and other brain regions in male and female mice from different dietary conditions, to see if these connections are altered.

Another possibility is that period length is not the primary property of the SCN that is altered in response to in utero diet. Indeed, both baseline and amplitude of PER2::LUC expression in the SCN trended toward being affected by in utero and adult diet, while period length did not. These data are consistent with a trend in the data
from Pendergrast et al. (2013) showing SCN amplitude is decreased in adult male mice given a high fat diet, while SCN period length is not affected [244]. It may be that baseline, amplitude, and other SCN characteristics change more in response to metabolic or environmental conditions, such as food availability. Properties such as baseline and amplitude of SCN firing may be more malleable than period, since environmental conditions that affect SCN amplitude and baseline such as metabolic or environmental conditions, such as food availability may change quickly. On the other hand, the main variables that affect period, which are the length of times for day-light and darkness, change very slowly over the course of the year in the typical life of a wild type mouse. It therefore seems that there is more adaptive advantage in having robust period length in the SCN, to preserve time keeping, while having more adaptable baseline and amplitude in the SCN in response to changing food conditions.

How baseline and amplitude of PER2::LUC expression may be changed in the SCN remains an open question. It may be that expression of core clock genes or genes that can alter SCN function are altered by in utero or adult diet, or the combination thereof. Indeed, expression of some RNA transcripts in the hypothalamus has been shown to be changed by an adult high fat diet in male mice [100]. Furthermore, the amplitude of circadian RNA expression for some genes in these mice is shown to be dampened in the hypothalamus, which is consistent with dampened amplitude of the SCN. It is also possible that dampened amplitude is not only related to dampened baseline, but may be caused by it. Dampened amplitude caused by weakening of the clock may coincide
with lower clock gene expression [215]. These conjectures could be tested by experiments similar to those proposed to examine possible changes in SCN output signals mentioned previously.

**PER2::LUCIFERASE expression in the arcuate nucleus:**

The period of PER2::LUC expression in the arcuate nuclei did not show significant differences between dietary conditions, similar to what was seen in the SCN. Like the SCN, trends in PER2::LUC expression in the arcuate nucleus occurred in terms of baseline and amplitude. Intriguingly, baseline and amplitude of PER2::LUC expression in the arcuate nucleus showed opposite sex specific trends of expression in response to in utero and adult diet perturbation. Specifically, male mice showed a trend toward increased baseline and amplitude in the arcuate nuclei of all in utero and adult diet groups compared with controls. Female mice, however, showed the opposite trend, with baseline and amplitude of PER2::LUC expression in the arcuate nucleus having a lower average in each in utero and adult diet group than controls. We note that the trends in males is speculative, as the N for arcuate nuclei studied in males is too low for trends to be conclusive.

These sex differences in the arcuate nucleus are particularly interesting given the circadian locomotor periods of the animals. The lengthened period of PER2::LUC expression in the arcuate nucleus of all male dietary groups compared to controls is
interesting given that circadian period of locomotor activity is longer in all dietary groups compared to controls. Although interesting, these results must be interpreted with caution. It may be that there is a correlational relationship between lengthened period of locomotor activity and period of PER2::LUC expression, and the two effects are caused by a third, unknown variable.

The relationship between sex differences in PER2::LUC expression and circadian locomotor activity:

Finally, we note that baseline and amplitude of PER2::LUC in the SCN is generally lower in female mice than in male mice from the same in utero diet or adult diet group. The lower baseline of PER2::LUC expression is consistent with lower firing of the SCN during the day in female mice when compared with male mice as observed by Kuljis et al. (2013). Furthermore, Kuljis et al. (2013) observed a lower amplitude of SCN firing over the LD cycle in females than in males, which is consistent with our data [52]. The molecular underpinnings of these differences are not known, but may relate to sexually dimorphic expression of VIP and AVP in male and females [245]. We note that it will be useful to use immunohistochemistry or other techniques to characterize changes in the SCN that accompany changes in PER2::LUC expression in dietary conditions [222]. These experiments would act as a control for PER2::LUC expression differences due to dissection variations. These experiments would also serve to uncover subtle
morphological changes that may affect the SCN aside from overall size and cell population.

The lowered baseline and amplitude of PER2::LUC expression in the SCN of females is consistent with the greater statistical significance of circadian period length in DD in response to in utero diet, as well as greater error of activity onset. Although not proven, it is frequently hypothesized in the circadian field that lower amplitude of circadian rhythms facilitates resetting of the clock [24]. Lower baseline may also facilitate resetting of the clock, since there is less gene expression to be shifted. Lower initial amplitude of the SCN in female mice is therefore consistent with greater changes in period length and error onset in response to in utero and adult dietary perturbations. This may mean that females are more responsive to environmental changes [52]. Interestingly, females exhibit more running activity than males, as shown be the trend of longer alpha hour in DD in females than males across dietary conditions. This trend is interesting given the higher amplitude of PER2::LUCIFERASE expression in the SCN and arcuate nuclei of male mice. It may be that male mice have greater amplitude changes in the SCN and produce bouts of activity at more specific, but shorter circadian time intervals, corresponding to more precise onset, and shorter alpha hours. Female mice, on the other hand, have less amplitude of PER2::LUC expression in the SCN and exhibit more activity over a less specific range of time, shown by greater error in onset of activity in DD, along with longer alpha hours.
Intriguingly, the opposite trend of baseline and amplitude was seen in the arcuate nucleus. PER2::LUC expression in terms of baseline expression and amplitude was generally higher in female mice than in male mice. It would be interesting to continue investigating these effects by characterizing the specific clock genes that are involved in these changes, as well as expression levels of sex-related hormones and feeding signals such as NPY known to affect the clock. Experiments such as those described previously could be performed to see if expression of these genes are circadianly altered by diet in the arcuate nucleus in a sex-specific manner.

**Future Directions:**

The long lasting effects of in utero diet on adult PER2::LUC expression in the SCN and arcuate nucleus are intriguing, and must be further characterized to understand their molecular and developmental underpinnings, as well as their relationship to circadian period length of locomotor activity. Studies of changes in gene expression of molecular clock and circadian signaling genes in the SCN and other brain regions could be expanded to include in utero and adult dietary conditions as described previously. Furthermore, epigenetic changes in the promoters of clock genes or related signaling molecules could be investigated. This is a particularly interesting avenue for future investigation, as LP or HF in utero diet has been shown to affect DNA methylation patterns in the hypothalamus and other brain regions of adult mice [220,221].
Furthermore, known and predicted molecular clock components are thought to affect methylation levels and other epigenetic modifications [34,246].

**Summary:**

Overall, our data show that the effects of in utero diet on adult circadian rhythms are less potent than the effects of adult diet on circadian rhythms. A HF in utero diet has less effect on circadian locomotor period than an adult HF diet, and this effect is seen in both male and female mice. A LP in utero diet has sex-specific effects on adult circadian phenotype; male mice have a longer period length in DD, while female DD period length is similar to controls. SCN period length is not significantly affected by in utero or adult diet, although trends of lowered baseline and amplitude of PER2::LUC expression were seen in both sexes. The arcuate nucleus shows sex specific response to diet conditions. Males showed a trend of increased baseline and amplitude of PER2::LUC in dietary conditions, while females showed the reverse trend. Overall, we characterized differences of circadian locomotor activity and PER2::LUC expression in the SCN and arcuate nucleus of male and female mice in a variety of in utero and adult diet conditions.
3.6 Figures

Fig. 3. 1: Tau (DD) In Male and Female Mice

**Table 3.1A:** Tau (DD) of Male Mice

<table>
<thead>
<tr>
<th>Diet</th>
<th>Con-Con-M</th>
<th>HF-Con-M</th>
<th>Con-HF-M</th>
<th>LP-Con-M</th>
<th>LP-HF-M</th>
</tr>
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<tbody>
<tr>
<td>Mean</td>
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<td>23.7</td>
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<tr>
<td>Std. Error of Mean</td>
<td>±0.0389</td>
<td>±0.0361</td>
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<td>±0.0582</td>
<td>±0.0692</td>
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<tr>
<td>Number of values</td>
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<td>26</td>
<td>17</td>
<td>11</td>
<td>5</td>
</tr>
</tbody>
</table>

**Table 1B:** Tau (DD) of Female Mice

<table>
<thead>
<tr>
<th>Diet</th>
<th>Con-Con-F</th>
<th>HF-Con-F</th>
<th>Con-HF-F</th>
<th>LP-Con-F</th>
<th>LP-HF-F</th>
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</thead>
<tbody>
<tr>
<td>Mean</td>
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<td>23.8</td>
<td>24</td>
<td>23.7</td>
<td>23.9</td>
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<tr>
<td>Std. Error of Mean</td>
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<td>±0.0852</td>
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<td>Number of values</td>
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<td>10</td>
<td>8</td>
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Fig. 3. 1: Tau (DD) In Male and Female Mice. Locomotor activity of A) male and B) female mice singly-housed with running wheels was measured as wheel revolutions for at least ten days in constant darkness was recorded. Circadian period length of activity was then calculated for each animal using the ClockLab program for MathLab from Actimetrics (see Methods section for further details). Period length in hours is shown as mean (hours) with error bars representing ± standard error of the mean (S.E.M.). A) Tau (DD) in male mice. B) Tau (DD) in female mice.
Table 3.2A: Error Onset (DD) in Male Mice

<table>
<thead>
<tr>
<th>Diet</th>
<th>Con-Con-M</th>
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<th>Con-HF-M</th>
<th>LP-Con-M</th>
<th>LP-HF-M</th>
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<tr>
<td>Mean</td>
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<td>Std. Error of Mean</td>
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<td>Number of values</td>
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Table 3.2B: Error Onset (DD) in Female Mice

<table>
<thead>
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<th>Diet</th>
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<th>HF-Con-F</th>
<th>Con-HF-F</th>
<th>LP-Con-F</th>
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<tbody>
<tr>
<td>Mean</td>
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<td>Std. Error of Mean</td>
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</table>

* Kruskal-Wallis rank sum test Adj. p<0.05
Fig. 3.2: Error Onset (DD) In Male and Female Mice. A-B) Locomotor activity of mice singly-housed with running wheels was measured as wheel revolutions for at least ten days in constant darkness was recorded. Error of onset of activity and alpha hour was then calculated for each animal using the ClockLab program for MathLab from Actimetrics (see Methods section for further details). A-B) Error of onset in hours is shown as mean (hours) with error bars representing ± standard error of the mean (S.E.M.). A) Error of onset of activity (DD) in male mice. B) Error of onset of activity (DD) in female mice.
Fig. 3.3: Alpha Hour (DD) In Male and Female Mice

**Fig. 3.3A**
Alpha Hour in In Utero Diet Male Mice

**Fig. 3.3B**
Alpha Hour in In Utero Diet Female Mice

### Table 3.3A: Alpha Hour (DD) Male Mice

<table>
<thead>
<tr>
<th>Diet</th>
<th>Con-Con-M</th>
<th>HF-Con-M</th>
<th>Con-HF-M</th>
<th>LP-Con-M</th>
<th>LP-HF-M</th>
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</thead>
<tbody>
<tr>
<td>Mean</td>
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<td>6.61</td>
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<tr>
<td>Std. Error of Mean</td>
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<td>±0.982</td>
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<td>5</td>
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</tbody>
</table>

Data expressed as Average ±S.E.M.

### Table 3.3B: Alpha Hour (DD) Female Mice

<table>
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<tr>
<th>Diet</th>
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<th>HF-Con-F</th>
<th>Con-HF-F</th>
<th>LP-Con-F</th>
<th>LP-HF-F</th>
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</thead>
<tbody>
<tr>
<td>Mean</td>
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<td>Std. Error of Mean</td>
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</tr>
</tbody>
</table>

Data expressed as Average ±S.E.M.
Fig. 3.3: Alpha Hour (DD) In Male and Female Mice. A-B) Locomotor activity of mice singly-housed with running wheels was measured as wheel revolutions for at least ten days in constant darkness was recorded. Error of onset of activity and alpha hour was then calculated for each animal using the ClockLab program for MATLAB from Actimetrics (see Methods section for further details)[229,247]. A-B) Alpha hour in hours is shown as mean (hours) with error bars representing ± standard error of the mean (S.E.M.). A) Alpha hour (DD) of activity in male mice. B) Alpha hour (DD) of activity in female mice.
Fig. 3.4: Period of PER2::LUC Expression in the SCN of Male and Female Mice

**Fig. 3.4A**

Period Length of PER2::LUC Expression in SCN of Female Mice

Data Expressed as Average, ± SEM

**Fig. 3.4B**

Period Length of PER2::LUC Expression in SCN of Male Mice

Data Expressed As Average, ± SEM

---

**Table 3.4A: Period of PER2::LUC Expression in the SCN of Male Mice**

<table>
<thead>
<tr>
<th>Diet</th>
<th>Con-Con-M</th>
<th>HF-Con-M</th>
<th>Con-HF-M</th>
<th>LP-Con-M</th>
<th>LP-HF-M</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mean</td>
<td>24.6</td>
<td>24.1</td>
<td>24.3</td>
<td>24.6</td>
<td>24.3</td>
</tr>
<tr>
<td>Std. Error of Mean</td>
<td>±0.471</td>
<td>±0.115</td>
<td>±0.187</td>
<td>±0.119</td>
<td>±0.176</td>
</tr>
<tr>
<td>Number of values</td>
<td>4</td>
<td>9</td>
<td>8</td>
<td>3</td>
<td>2</td>
</tr>
</tbody>
</table>

**Table 3.4B: Period of PER2::LUC Expression in the SCN of Female Mice**

<table>
<thead>
<tr>
<th>Diet</th>
<th>Con-Con-F</th>
<th>HF-Con-F</th>
<th>Con-HF-F</th>
<th>LP-Con-F</th>
<th>LP-HF-F</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mean</td>
<td>24.1</td>
<td>25</td>
<td>24.6</td>
<td>24.5</td>
<td>24.1</td>
</tr>
<tr>
<td>Std. Error of Mean</td>
<td>±0.583</td>
<td>±0</td>
<td>±0.127</td>
<td>±0.869</td>
<td>±0.101</td>
</tr>
<tr>
<td>Number of values</td>
<td>4</td>
<td>1</td>
<td>6</td>
<td>5</td>
<td>3</td>
</tr>
</tbody>
</table>
Fig. 3.4: Period of PER2::LUC Expression in the SCN of Male and Female Mice. A-B)
Bilateral suprachiasmatic nucleus explants were cultured from adult mice of 10-12 weeks of age. After initial twenty-four hours of recording (not used), at least three days of subsequent recording were used for Wavelet analysis of period length (See Methods section for further details). Period length in hours in SCN explants is shown as mean with error bars representing ± standard error of the mean (S.E.M.). A) Period length in hours in SCN explants from male mice. B) Period length in hours in SCN explants from female mice.
Fig. 3.5: Baseline of PER2::LUC Expression in the SCN of Male and Female Mice

Table 3.5A: Baseline of PER2::LUC Expression in the SCN of Male Mice

<table>
<thead>
<tr>
<th>Diet</th>
<th>Con-Con-M</th>
<th>HF-Con-M</th>
<th>Con-HF-M</th>
<th>LP-Con-M</th>
<th>LP-HF-M</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mean</td>
<td>97.3</td>
<td>56</td>
<td>83.9</td>
<td>94.1</td>
<td>37.2</td>
</tr>
<tr>
<td>Std. Error of Mean</td>
<td>±23.8</td>
<td>±9.92</td>
<td>±14.8</td>
<td>±28.3</td>
<td>±6.32</td>
</tr>
<tr>
<td>Number of values</td>
<td>4</td>
<td>9</td>
<td>8</td>
<td>3</td>
<td>2</td>
</tr>
</tbody>
</table>

Table 3.5B: Baseline of PER2::LUC Expression in the SCN of Female Mice

<table>
<thead>
<tr>
<th>Diet</th>
<th>Con-Con-F</th>
<th>HF-Con-F</th>
<th>Con-HF-F</th>
<th>LP-Con-F</th>
<th>LP-HF-F</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mean</td>
<td>79.9</td>
<td>51.5</td>
<td>59.1</td>
<td>30.8</td>
<td>41.3</td>
</tr>
<tr>
<td>Std. Error of Mean</td>
<td>±15.5</td>
<td>±0</td>
<td>±16.4</td>
<td>±6.79</td>
<td>±7.37</td>
</tr>
<tr>
<td>Number of values</td>
<td>4</td>
<td>1</td>
<td>6</td>
<td>5</td>
<td>3</td>
</tr>
</tbody>
</table>
Fig. 3.5: Baseline of PER2::LUC Expression in the SCN of Male and Female Mice. A-B)

Bilateral suprachiasmatic nucleus explants were cultured from adult mice of 10-12 weeks of age. After initial twenty-four hours of recording (not used), at least three days of subsequent recording were used for Wavelet analysis of baseline (See Methods section for further details). A-B) Baseline PER2::LUC bioluminescence is shown as mean ± with error bars representing standard error of the mean (S.E.M.). A) Baseline PER2::LUC bioluminescence in SCN explants from male mice. B) Baseline PER2::LUC bioluminescence in SCN explants from female mice.
Fig. 3.6: Amplitude of PER2::LUC Expression in the SCN of Male and Female Mice

Table 3.6A: Amplitude of PER2::LUC Expression in the SCN of Male Mice

<table>
<thead>
<tr>
<th>Diet</th>
<th>Con-Con-M</th>
<th>HF-Con-M</th>
<th>Con-HF-M</th>
<th>LP-Con-M</th>
<th>LP-HF-M</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mean</td>
<td>7.88</td>
<td>5.52</td>
<td>6.93</td>
<td>7.24</td>
<td>4.06</td>
</tr>
<tr>
<td>Std. Error of Mean</td>
<td>±2.88</td>
<td>±1.23</td>
<td>±1.45</td>
<td>±2.13</td>
<td>±0.262</td>
</tr>
<tr>
<td>Number of values</td>
<td>4</td>
<td>9</td>
<td>8</td>
<td>3</td>
<td>2</td>
</tr>
</tbody>
</table>

Table 3.6B: Amplitude of PER2::LUC Expression in the SCN of Female Mice

<table>
<thead>
<tr>
<th>Diet</th>
<th>Con-Con-F</th>
<th>HF-Con-F</th>
<th>Con-HF-F</th>
<th>LP-Con-F</th>
<th>LP-HF-F</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mean</td>
<td>4.5</td>
<td>5.83</td>
<td>7.4</td>
<td>2.01</td>
<td>4.08</td>
</tr>
<tr>
<td>Std. Error of Mean</td>
<td>±1.19</td>
<td>±0</td>
<td>±3.18</td>
<td>±0.398</td>
<td>±0.866</td>
</tr>
<tr>
<td>Number of values</td>
<td>4</td>
<td>1</td>
<td>6</td>
<td>5</td>
<td>3</td>
</tr>
</tbody>
</table>
Fig. 3.6: Amplitude of PER2::LUC Expression in the SCN of Male and Female Mice. A-B)

Bilateral suprachiasmatic nucleus explants were cultured from adult mice of 10-12 weeks of age. After initial twenty-four hours of recording (not used), at least three days of subsequent recording were used for Wavelet analysis of baseline and amplitude (See Methods section for further details). A-B) Amplitude of PER2::LUC bioluminescence is shown as mean with error bars representing ± standard error of the mean (S.E.M.). A) Amplitude of PER2::LUC bioluminescence in SCN explants from male mice. B) Amplitude of PER2::LUC bioluminescence in SCN explants from female mice.
Table 3.7A: Period of PER2::LUC Expression in the Arcuate Nucleus of Male Mice

<table>
<thead>
<tr>
<th>Diet</th>
<th>Con-Con-M</th>
<th>Con-HF-M</th>
<th>LP-Con-M</th>
<th>LP-HF-M</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mean (Hours)</td>
<td>23.5</td>
<td>23.0</td>
<td>24.1</td>
<td>24.3</td>
</tr>
<tr>
<td>Std. Error of Mean (Hours)</td>
<td>±0.00</td>
<td>±0.576</td>
<td>±0.434</td>
<td>±0.101</td>
</tr>
<tr>
<td>Number of values</td>
<td>1</td>
<td>3</td>
<td>2</td>
<td>3</td>
</tr>
</tbody>
</table>

Table 3.7B: Period of PER2::LUC Expression in the Arcuate Nucleus of Female Mice

<table>
<thead>
<tr>
<th>Diet</th>
<th>Con-Con-F</th>
<th>Con-HF-F</th>
<th>LP-Con-F</th>
<th>LP-HF-F</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mean (Hours)</td>
<td>23.6</td>
<td>24.2</td>
<td>22.9</td>
<td>23.5</td>
</tr>
<tr>
<td>Std. Error of Mean (Hours)</td>
<td>±0.0985</td>
<td>±0.604</td>
<td>±0.997</td>
<td>±0.425</td>
</tr>
<tr>
<td>Number of values</td>
<td>3</td>
<td>3</td>
<td>3</td>
<td>2</td>
</tr>
</tbody>
</table>
Fig. 3.7: Period of PER2::LUC Expression in the Arcuate Nucleus of Male and Female Mice. A-B) Bilateral arcuate nucleus explants were cultured from adult mice of 10-12 weeks of age. After initial twenty-four hours of recording (not used), at least three days of subsequent recording were used for Wavelet analysis of period length (See Methods section for further details). Period length in hours in AN explants is shown as mean with error bars representing ± standard error of the mean (S.E.M.). A) Period length in hours in arcuate nucleus explants from male mice. B) Period length in hours in arcuate nucleus explants from female mice.
Fig. 3.8: Baseline of PER2::LUC Expression in the Arcuate Nucleus of Male and Female Mice

Table 3.8A: Baseline of PER2::LUC Expression in the Arcuate Nucleus of Male Mice

<table>
<thead>
<tr>
<th>Diet</th>
<th>Con-Con-M</th>
<th>Con-HF-M</th>
<th>LP-Con-M</th>
<th>LP-HF-M</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mean</td>
<td>30.7</td>
<td>34.9</td>
<td>45</td>
<td>45.8</td>
</tr>
<tr>
<td>Std. Error of Mean</td>
<td>±0</td>
<td>±2.5</td>
<td>±8.47</td>
<td>±2.49</td>
</tr>
<tr>
<td>Number of values</td>
<td>1</td>
<td>3</td>
<td>2</td>
<td>3</td>
</tr>
</tbody>
</table>

Table 3.8B: Baseline of PER2::LUC Expression in the Arcuate Nucleus of Female Mice

<table>
<thead>
<tr>
<th>Diet</th>
<th>Con-Con-F</th>
<th>Con-HF-F</th>
<th>LP-Con-F</th>
<th>LP-HF-F</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mean</td>
<td>64.6</td>
<td>42.9</td>
<td>35.3</td>
<td>43.2</td>
</tr>
<tr>
<td>Std. Error of Mean</td>
<td>±9.45</td>
<td>±6.8</td>
<td>±1.52</td>
<td>±5.07</td>
</tr>
<tr>
<td>Number of values</td>
<td>3</td>
<td>3</td>
<td>3</td>
<td>2</td>
</tr>
</tbody>
</table>
Fig. 3.8: Baseline of PER2::LUC Expression in the Arcuate Nucleus of Male and Female Mice. A-D) Bilateral arcuate nucleus explants were cultured from adult mice of 10-12 weeks of age. After initial twenty-four hours of recording (not used), at least three days of subsequent recording were used for Wavelet analysis of baseline (See Methods section for further details). A-B) Baseline PER2::LUC bioluminescence is shown as mean with error bars representing ± standard error of the mean (S.E.M.). A) Baseline PER2::LUC bioluminescence in arcuate nucleus explants from male mice. B) Baseline PER2::LUC bioluminescence in arcuate nucleus explants from female mice.
Fig. 3.9: Amplitude of PER2::LUC Expression in the Arcuate Nucleus of Male and Female Mice

Table 3.9A: Amplitude of PER2::LUC Expression in the Arcuate Nucleus of Male Mice

<table>
<thead>
<tr>
<th>Diet</th>
<th>Con-Con-M</th>
<th>Con-HF-M</th>
<th>LP-Con-M</th>
<th>LP-HF-M</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mean</td>
<td>1.46</td>
<td>2.16</td>
<td>4</td>
<td>5.29</td>
</tr>
<tr>
<td>Std. Error of Mean</td>
<td>±0</td>
<td>±1.23</td>
<td>±2.57</td>
<td>±0.689</td>
</tr>
<tr>
<td>Number of values</td>
<td>1</td>
<td>3</td>
<td>2</td>
<td>3</td>
</tr>
</tbody>
</table>

Table 3.9B: Amplitude of PER2::LUC Expression in the Arcuate Nucleus of Female Mice

<table>
<thead>
<tr>
<th>Diet</th>
<th>Con-Con-F</th>
<th>Con-HF-F</th>
<th>LP-Con-F</th>
<th>LP-HF-F</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mean</td>
<td>9.63</td>
<td>3.44</td>
<td>1.59</td>
<td>2.98</td>
</tr>
<tr>
<td>Std. Error of Mean</td>
<td>±3.02</td>
<td>±1.62</td>
<td>±0.536</td>
<td>±1.21</td>
</tr>
<tr>
<td>Number of values</td>
<td>3</td>
<td>3</td>
<td>3</td>
<td>2</td>
</tr>
</tbody>
</table>
Fig. 3.9: Amplitude of PER2::LUC Expression in the Arcuate Nucleus of Male and Female Mice. A-B) Bilateral arcuate nucleus explants were cultured from adult mice of 10-12 weeks of age. After initial twenty-four hours of recording (not used), at least three days of subsequent recording were used for Wavelet analysis of amplitude (See Methods section for further details). A-B)) Amplitude of PER2::LUC bioluminescence is shown as mean ± with error bars representing standard error of the mean (S.E.M.). A) Amplitude of PER2::LUC bioluminescence in AN explants from male mice. B) Amplitude of PER2::LUC bioluminescence in AN explants from female mice.
3.7 Supplementary Information

Supplemental Figure 3.1: Total Activity in LD in Male and Female Mice

Table S3.1A: Total Activity Counts (LD) in Male Mice

<table>
<thead>
<tr>
<th>Diet</th>
<th>Con-Con-M</th>
<th>HF-CON-M</th>
<th>Con-HF-M</th>
<th>LP-Con-M</th>
<th>LP-HF-M</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mean</td>
<td>30801</td>
<td>31416</td>
<td>30804</td>
<td>30487</td>
<td>27151</td>
</tr>
<tr>
<td>Std. Error of Mean</td>
<td>±1588</td>
<td>±1618</td>
<td>±1347</td>
<td>±1045</td>
<td>±4379</td>
</tr>
<tr>
<td>Number of values</td>
<td>24</td>
<td>25</td>
<td>17</td>
<td>11</td>
<td>6</td>
</tr>
</tbody>
</table>

Table S3.1B: Total Activity Counts (LD) in Female Mice

<table>
<thead>
<tr>
<th>Diet</th>
<th>Con-Con-F</th>
<th>HF-CON-F</th>
<th>Con-HF-F</th>
<th>LP-Con-F</th>
<th>LP-HF-F</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mean</td>
<td>33968</td>
<td>34388</td>
<td>35010</td>
<td>29577</td>
<td>32719</td>
</tr>
<tr>
<td>Std. Error of Mean</td>
<td>±1468</td>
<td>±1559</td>
<td>±1603</td>
<td>±2438</td>
<td>±2688</td>
</tr>
<tr>
<td>Number of values</td>
<td>14</td>
<td>26</td>
<td>4</td>
<td>10</td>
<td>8</td>
</tr>
</tbody>
</table>
Supplemental Figure S3.1: Total Activity in LD in Male and Female Mice. A-B) Total activity counts per day were measured in male and female mice entrained to a 12:12 hr. light/dark cycle (See Methods section for further details). A-B)) Average total activity of mice on wheels in LD is shown as mean (counts per day) with error bars representing ± standard error of the mean (S.E.M.). A) Average total activity per day (LD) of male mice from in utero and adult diet conditions while on running wheels. B) Average total activity per day (LD) of female mice from in utero and adult diet conditions while on running wheels.
Supplemental Figure S3.2 Average Weight of Male and Female Mice on Running Wheels

**Table S3.2A: Average Weight on Wheels of Male Mice**

<table>
<thead>
<tr>
<th>Diet</th>
<th>Con-Con-M</th>
<th>HF-Con-M</th>
<th>Con-HF-M</th>
<th>LP-Con-M</th>
<th>LP-HF-M</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mean</td>
<td>24.4</td>
<td>24.1</td>
<td>25</td>
<td>22.3</td>
<td>22.5</td>
</tr>
<tr>
<td>Std. Error of Mean</td>
<td>±0.307</td>
<td>±0.308</td>
<td>±0.457</td>
<td>±0.271</td>
<td>±1.08</td>
</tr>
<tr>
<td>Number of values</td>
<td>24</td>
<td>28</td>
<td>17</td>
<td>12</td>
<td>5</td>
</tr>
</tbody>
</table>

**Table S3.2B: Average Weight on Wheels of Female Mice**

<table>
<thead>
<tr>
<th>Diet</th>
<th>Con-Con-F</th>
<th>HF-Con-F</th>
<th>Con-HF-F</th>
<th>LP-Con-F</th>
<th>LP-HF-F</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mean (g)</td>
<td>19.7</td>
<td>19.4</td>
<td>19.5</td>
<td>17.5</td>
<td>17.9</td>
</tr>
<tr>
<td>Std. Error of Mean</td>
<td>±0.226</td>
<td>±0.204</td>
<td>±0.771</td>
<td>±0.307</td>
<td>±0.859</td>
</tr>
<tr>
<td>Number of values</td>
<td>18</td>
<td>28</td>
<td>5</td>
<td>11</td>
<td>8</td>
</tr>
</tbody>
</table>
Supplemental Figure S3.2 Average Weight of Male and Female Mice on Running Wheels. A-B) Mice were weighed when put on running wheels and when removed (See Methods section for further details). A-B) Average weight of mice on wheels is shown as mean (grams) with error bars representing ± standard error of the mean (S.E.M.). A) Average weight of male mice from in utero and adult diet conditions while on running wheels. B) Average weight of female mice from in utero and adult diet conditions while on running wheels.
Supplemental Table S3.1  Kruskal-Wallis Test With Kruskal-Wallis rank sum test

**Tau Male (DD)**

Kruskal-Wallis test

| P value | 0.0264 |

Exact or approximate P value? Approximate

P value summary *

Do the medians vary signif. (P < 0.05) Yes

Number of groups 5

Kruskal-Wallis statistic 11.01

Data summary

| Number of treatments (columns) | 5 |
| Number of values (total)       | 84 |

**TauMDD Male DD**

Kruskal-Wallis rank sum test

data: Tau by Diet

Kruskal-Wallis chi-squared = 11.011, df = 4, p-value = 0.02644

95% Confidence intervals for Kruskal-Wallis comparisons

<table>
<thead>
<tr>
<th>Diff</th>
<th>Lower</th>
<th>Upper</th>
<th>Decision</th>
<th>Adj. P-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Avg.rankConCon-Avg.rankConHF</td>
<td>-24.17765</td>
<td>-45.67603</td>
<td>2.67926</td>
<td>Reject H0</td>
</tr>
<tr>
<td>Avg.rankConCon-Avg.rankHFCon</td>
<td>-4.40615</td>
<td>-23.56212</td>
<td>14.74981</td>
<td>FTR H0</td>
</tr>
<tr>
<td>Avg.rankConHF-Avg.rankHFCon</td>
<td>19.77149</td>
<td>-1.55889</td>
<td>41.10188</td>
<td>FTR H0</td>
</tr>
<tr>
<td>Avg.rankConCon-Avg.rankLPCon</td>
<td>-11.28727</td>
<td>-36.03074</td>
<td>13.45619</td>
<td>FTR H0</td>
</tr>
<tr>
<td>Avg.rankConHF-Avg.rankLPCon</td>
<td>12.89037</td>
<td>-13.57231</td>
<td>39.35306</td>
<td>FTR H0</td>
</tr>
<tr>
<td>Avg.rankHFCon-Avg.rankLPCon</td>
<td>-6.88112</td>
<td>-31.47876</td>
<td>17.71652</td>
<td>FTR H0</td>
</tr>
<tr>
<td>Avg.rankConCon-Avg.rankLPHF</td>
<td>-5.46</td>
<td>-38.96281</td>
<td>28.04281</td>
<td>FTR H0</td>
</tr>
<tr>
<td>Avg.rankConHF-Avg.rankLPHF</td>
<td>18.71765</td>
<td>-16.0742</td>
<td>53.5095</td>
<td>FTR H0</td>
</tr>
<tr>
<td>Avg.rankHFCon-Avg.rankLPHF</td>
<td>-1.05385</td>
<td>-34.4491</td>
<td>32.34141</td>
<td>FTR H0</td>
</tr>
<tr>
<td>Avg.rankLPCon-Avg.rankLPHF</td>
<td>5.82727</td>
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Tau Female (DD)

Kruskal-Wallis test
P value 0.0015
Exact or approximate P value? Approximate
P value summary **
Do the medians vary signif. (P < 0.05) Yes
Number of groups 5
Kruskal-Wallis statistic 17.54

Data summary
Number of treatments (columns) 5
Number of values (total) 69

Kruskal-Wallis rank sum test

data: TauF and DietF
Kruskal-Wallis chi-squared = 17.539, df = 4, p-value = 0.001518

95% Confidence intervals for Kruskal-Wallis comparisons

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**Error Onset Male (DD)**

Kruskal-Wallis test

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Exact or approximate P value? Approximate

P value summary *

Do the medians vary signif. (P < 0.05) Yes

Number of groups 5

Kruskal-Wallis statistic 11.69

Data summary

Number of treatments (columns) 5

Number of values (total) 84

95% Confidence intervals for Kruskal-Wallis comparisons

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Error Onset Female (DD)

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Number of groups     5
Kruskal-Wallis statistic    2.884

Data summary
Number of treatments (columns)  5
Number of values (total)       69

Alpha Hour Male (DD)

Kruskal-Wallis test
P value   0.6056
Exact or approximate P value?  Approximate
P value summary      ns
Do the medians vary signif. (P < 0.05)  No
Number of groups     5
Kruskal-Wallis statistic    2.721

Data summary
Number of treatments (columns)  5
Number of values (total)       84

Alpha Hour Female (DD)

Kruskal-Wallis test
P value   0.0972
Exact or approximate P value?  Approximate
P value summary      ns
Do the medians vary signif. (P < 0.05)  No
Number of groups     5
Kruskal-Wallis statistic    7.851

Data summary
Number of treatments (columns)  5
Number of values (total)       69

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3.8 Acknowledgements

We thank Robert George and Dr. Teresa Reyes from the Reyes Lab, University of Pennsylvania for the animal mating protocol, and for information on the following diets: high fat, low protein, and basal in utero diets, and adult high fat diet. We also thank all members of the Reyes lab for discussion of protocols, data, and scientific principles. We thank Dr. Yool Lee and Dr. Anand Venkataraman for assistance with wheel running experiments, discussion of protocols, discussion of data and scientific principles.
CHAPTER 4: General Discussion and Future Directions

4.1 Discussion of general conclusions from Chapter 2

Introduction:

Data from the RNA profile of the mouse SCN discussed in Chapter 2 show that the SCN is similar to other brain regions in terms of general organization of rhythmic transcript expression, yet still retains SCN specific circadian functions, as shown by the large number of transcripts that only cycle in the SCN (Fig. 2.2A). The mouse SCN expresses several hundred rhythmic transcripts when profiled with Affymetrix 1.0ST arrays (Fig. 2.1A-C). The number of cyclic RNAs is similar to other profiled brain regions, including the whole hypothalamus, brainstem, and cerebellum, each of which also expresses several hundred rhythmic transcripts. The number of cycling transcripts in the SCN and other brain regions is low compared to peripheral tissues, which frequently express ~1,000 transcripts. Some organs, including lung, kidney and liver, express over 2,000 cycling transcripts [1].

The reasons for the comparatively low number of cyclers in brain tissues compared to peripheral tissues are not known. Brain tissues contain heterogeneous populations of cells, and it is possible that specific sub-populations contain cycling transcripts that are not highly expressed enough to be detectable by arrays, or the signal may be cancelled out because the transcripts are constitutively expressed in
some cell types but rhythmic in others. Furthermore, transcripts may be rhythmic but have different peak phases in different cells, and therefore appear to be arrhythmic by array analysis.

Furthermore, there are few overlapping rhythmic transcripts between the SCN and other brain regions, as well as between the SCN and peripheral tissues. This is similar to the results of previous studies, which show that cycling transcripts are largely tissue specific, with the exception of a few cyclers that are part of the circadian clock, or relate to cross-tissue clock function [1,32]. Core clock components that cycle across the SCN and other tissues include \( Dbp, Nr1d1, Nr1d2, Per1-3 \) [2,3] (Chapter 2, Figure 2.2).

Expression of transcripts for core molecular clock components is more similar between the SCN and other brain regions than between the SCN and peripheral tissues. As discussed in Chapter 2, transcripts for transcriptional activators \( Arntl \) and \( Npas2 \) show low amplitude in the SCN and other brain tissues compared with peripheral tissues, although they are largely similar in phase. Low amplitude may be necessary to facilitate resetting of the clock [24]. Most other core clock components show similar amplitude in the SCN, other brain regions and the periphery, as shown in Chapter 2 (Chapter 2, Figure 2.2). Transcripts for \( Per1-3 \) are the exception, and peak earlier in the SCN than in other brain regions and the periphery. These data are in agreement with experiments using the PER2::LUCIFERASE mouse model, that show PER2::LUC expression peaking in the SCN prior to peak expression in other tissues [23]. Early expression of Per transcript and protein in the SCN compared with other tissues may
mean PERs, especially PER2, “lead” circadian rhythms in the periphery. Alternatively, PERs may respond to the periphery and hence be out of phase. How PER2 may or respond to the periphery remains an open question in circadian biology (Chapter 2, Fig. 2.2F-H).

**Cross Brain Cyclers – What do they mean?**

In addition to components of the molecular clock, transcripts that cycle in the SCN and other brain tissues shed light on functions of the circadian clock. The ~two dozen cyclers identified in Chapter 2 that cycle in the SCN and other brain regions are particularly intriguing (Chapter 2, Fig. 2.2A-B). Cross tissue cyclers have roles in the core clock, neuronal connectivity, calcium homeostasis, and heat shock response related proteins [1]. These transcripts all have functions in relationship to the three functions of the circadian clock; self-sustained oscillation, adaptation to the environment, temperature compensation, and may be involved in the entrainment of peripheral tissues [2,22,45]. The identification of specific cyclers that carry out these functions across tissues will be useful for further study.

**Neuronal Connectivity Factors: Importance for Clock Function**
How the mammalian central pacemaker in the SCN transmits timing information is largely unknown, aside from the secretion of PROK2, CLC, and TGF-α [48]. Here we identify cross brain cyclers that may modulate neuronal connectivity across the brain at different circadian time points. Importantly, these cyclers not only peak at different times, but may also affect neuronal connections in different ways. *Ahnak* is a tight junction molecule, and peaks during the dark phase [130]. *Mmp14* codes for a matrix metalloproteinase that regulates neuronal connectivity through interaction with and cleavage of molecules such as L1CAM or NCAM [133,135,137]. *Mmp14* peaks at the beginning of the light phase (Fig2.3A-D).

In addition to neuronal connectivity factors, we identify a number of cyclers in the SCN that regulate synaptic transmission (Fig. 2.3 G-H, Fig. 2.4A-F). Briefly, these cyclers regulate synaptic vesicle release, neurotransmitter reception, post-synaptic scaffolding, and down-stream signal transduction. Several transcripts are known to be involved in long term-potentiation of neurons, such as *Calm1*, *Camk2a*, and *PSD-95* [248]. Furthermore, several of these transcripts cycle in phase with homologous transcripts, specifically *Calm3*, *Camk2b*, and *PSD-93*. These transcripts may be involved in the phenomenon of iterative meta-plasticity, which is circadianly regulated changes in long term potentiation or depression (LTP or LTD, respectively)[159]. Furthermore, homologous cyclers such as *PSD-93* and PSD-95 may have divergent functions in regulating synaptic activity. Ratios of amplitude or phase of the two transcripts relative to each other may be modulated by environmental stimulation to change the LTP/LTD
of neurons in the SCN, and hence the excitation level of some neuronal populations in the SCN at certain times of day. Furthermore, these homologous transcripts may also mediate cell-type specificity in the SCN, such as the different period lengths of PER2::LUCIFERASE expression in SCN neurons cultured separately [47,60].

Our data furthermore identify a panoply of kinases, phosphatases, and ion channels that may signal in conjunction with neuronal connectivity and synaptic transmission factors to regulate period length, phase, and amplitude of SCN excitability, response to light, and network properties (Fig. 2.4A-F, Fig. 2.5A-E). In Section 4.2, we discuss methods for future study of these transcripts.

**Cycling Transcripts for Axonal Guidance Signaling:**

The presence of cycling transcripts related to axon guidance in the adult mouse SCN is intriguing. Axon guidance has mostly been studied in nervous system development. Briefly, neurons express repressive or attractive membrane bound or secreted signals. Axons with receptors or other ligands for these signals respond by avoiding or contacting the neurons secreting these signals. In some cases, axon guidance cues or receptors form gradients for guiding growth of axons expressing the corresponding receptor or cue. These interactions determine the routes axons take during the development, as well as their synaptic contacts [249].
The role of secreted axon guidance factors in circadian rhythms is a little studied area, although circadian regulation of these factors has been noted in data from a few studies [1,114,142]. Our data in Chapter 2 show the cycling of several transcripts for both signals and receptors in the adult mouse SCN. Since little is known about the function of these molecules in adult circadian rhythms, we sought information to guide future studies by researching evidence for involvement of these molecules in development of the SCN, and adjacent areas of the hypothalamus and optic chiasm.

Cycling transcripts from our data from Chapter 2 related to CNS development include *Sema3e, Sema6c, Nrp2, PlxnA4, PlxnB1, PlxnB3, Slit1*, and *Robo4* (Fig. 2.3, G-J). The function of these cyclers in adult SCN function is unknown, although there is some evidence that these cyclers’ genes participate in the development of the mammalian diencephalon, a region that includes the SCN [250,251].

*NRP2* participates in mouse hypothalamic development, particularly through migration of GnRH (Gonadotrophin Releasing Hormone) neurons from the vomero-nasal bulb to the median eminence of the hypothalamus [252]. SEMA3E participates in hypothalamus development by preventing the apoptosis of GnRH neurons, as does PLEXIND1 [253]. Knock out of PLEXIND1 or SEMA3E results in fewer GnRH positive neurons in the mouse hypothalamus [253]. *PlexinA4* transcript is detected in the embryonic mouse hypothalamus [254]. *Nrp2, Sema3e, Sema6c, PlxnA4, PlxnB1*, and *PlxnB3* were found to be differentially expressed at different points in mouse embryonic hypothalamus development [250].
SLIT is secreted by midline glia, and Slit1 RNA is present in the region near the optic chiasm during mouse diencephalon development, as are Robo1 and Robo2, transcripts [251]. SLITs and ROBOs are thought to provide guidance cues for the axons of RGCs (Retinal Ganglionic Cells), specifically where they form the optic chiasm. Furthermore, knock out of the Slit1 gene in mice leads to aberrant development of the optic chiasm [255]. We are unaware of data describing a role for Robo4 in hypothalamus development. There is some evidence that ROBOs may have roles in the circadian clock, namely that expression of a hypomorphic form of ROBO in Drosophila leads to shortened period length of locomotor activity [142]. To our knowledge, a circadian function of ROBOs in mammals has not been studied.

Our data show the cycling transcripts of axon guidance cues and receptors that play roles in development of a functioning adult hypothalamus in mammals. Further study of these cyclers may involve in situ hybridization and IHC studies to show when these molecules localize to the SCN during development, as was previously done for L1CAM and NCAM1 [137]. Furthermore, much knowledge could be gained by the creation of an RNA expression atlas of the SCN over embryonic and postnatal development, similar to the atlas created by Shimogori et al. (2010) for the whole murine hypothalamus [250]. In addition, axon guidance cyclers could be investigated by standard knock out and other studies for characterizing the roles of molecules in circadian rhythms, as discussed in a later section of Chapter 4.
RNA and Protein Homeostasis

A number of transcripts involved in RNA metabolism cycle, peaking near dawn. These include Drosha, a regulator of miRNAs, and many splicing factors [194,203]. Since these transcripts peak in phase slightly prior to dawn, they may be part of a circadian rush hour of RNA transcription and splicing. Additionally, some splicing factors may have specific roles in regulation of the circadian clock in the SCN. Rbm4b is a mammalian homologue of the Drosophila gene Lark. LARK modulates period length in Drosophila [213,214,256]. An additional cycler from the RNA regulation rush hour is Caprin1, which is a predicted core clock component, and has been shown to regulate translation of CamkIIα [34,207]. Some regulators, such as TDP-43, CAPRIN1, G3bBP, RBM4B, and SRPK1, are known to localize to aggregations of stalled RNA translation known as stress granules that form during some forms of cellular stress [205,206,208,209].

The latter possibility is particularly intriguing, since cycling transcripts for a variety of splicing regulators peak in phase with transcripts for heat shock proteins. The peak in splicing regulators may represent a rush hour of splicing activity. The clock is known to regulate cycles of RNA transcription and translation, and the peak of cycling splice factors occurs at ~ CT 0, while the peak number of cycling transcripts occurs later, at ~CT 4. Splicing and translation have overlapping regulation, so translation of RNA may increase during this phase as well [204]. Heat shock proteins may peak in
expression during this phase to control for an increase in misfolded proteins that may occur with at the same time as an increase in translation[211].

A number of cycling transcripts code for heat shock chaperones that are critical for ER homeostasis, and response to misfolded proteins (Fig.2.6 B-D,J)[185,212]. Furthermore, many of these transcripts cycle across the brain. The heat shock proteins coded for by these transcripts may protect the brain from misfolded proteins that may be more likely to occur at certain circadian time points, due to an animal’s activity, neuronal activity, or circadian times when translation of RNA into protein is heightened. Further evidence for circadian regulation of protein homeostasis are the cycling transcript Stip1, and the transcript for its ligand, Prnp. STIP1 stimulation of PRNP has been shown to increase protein synthesis in an AKT, PI3K, MTOR dependent manner [186].

Relationship of Phase of Peak RNA and Peak Protein Expression:

An important and unanswered question in characterizing circadian rhythms in the SCN is how rhythmic expression of RNA relates to rhythmic expression of protein. To this end, we compared our microarray data of cycling transcripts in the SCN with proteins found by Chiang et al. (2014) to have differential expression at different times of day [109]. The Chiang et al. (2014) data set consists of mass-spectrometry identification and quantification of proteins of mouse SCN samples at six time points over twenty-four hours, with four replicates at each time point [109]. 421 proteins were found to vary expression by time of day in this data set, referred to as the TOD (Time of
Day) data set. 28 proteins from the time of day data set had transcripts identified as cyclers in our SCN microarray data in Chapter 2. We then examined the phase difference between cycling transcripts and TOD proteins as described in Chapter 2. We found that 15 genes had a phase difference of 6 or more hours between peak RNA and peak protein expression. 13 genes had less than 6 hours between RNA and protein peaks. This distribution is similar to differences seen by Robles et al. (2014) between peak RNA and protein expression in circadianly expressed genes. In the Robles et al. (2014) data, 40% of genes had peak protein expression more than six hours following peak RNA transcript expression, while ~50% of genes showed two to six hours between peak RNA and protein expression [33].

These data show that differences between peak RNA transcript expression and peak protein expression vary widely between genes in the SCN and in the liver. These results highlight the complex nature of the relationship between circadian RNA and protein expression. Recent data from a comparison of circadian transcript expression and circadian expression measured by ribosomal profiling in U2OS cells, Jang and Lahens et al. (2015), offers insight into this matter [257]. These authors showed that for genes with both circadian transcript and circadian translation, there is very little lag time between peak expression of RNA and peak ribosomal fraction presence (translation) of the RNA. Jang and Lahens et al. (2015) postulate that the short lag between peak transcript and peak translation, followed by a longer lag (~6 hours) until protein accumulation may mean that circadian protein accumulation is largely regulated by
post-translational modifications or other mechanisms [257]. Although investigations into circadian translation in the SCN have not been carried out using ribosomal profiling, it is possible that a similar short lag between transcription and translation takes place in the SCN, followed by a lag time until protein-accumulation. Circadian ribosomal profiling could therefore shed further light on circadian gene expression patterns in the SCN.

We note that only 28 genes overlap between cycling genes in our microarray data and the TOD data set, while each data set contains hundreds of genes (523 cycling transcripts in our data, and 421 genes in TOD data set). Therefore, most cycling transcripts do not correspond to TOD proteins, and most TOD proteins do not correspond to cycling transcripts.

In the case of the majority of cycling transcripts in the SCN not having a corresponding protein in the TOD, this may be due to the resolution of the Chiang et al. (2014) study, as well as the current limitations of mass spectrometry [109]. The Chiang et al. (2014) study had 4 hour resolution over one day [109]. It is possible that a similar proteomics study of the SCN with higher resolution over 2 days would discover more proteins that reached statistical significance for variation over time. Another issue is that many proteins important for the circadian clock have comparatively low expression rates compared to other proteins. For this reason, proteomics studies of the SCN have not detected the cycling of core clock proteins, even though some core clock proteins have been shown to have circadian expression in the SCN through PER2::LUC studies [24,109].
In the case of proteins in the TOD dataset that do not correspond to cycling RNA transcripts in the SCN, this phenomenon was also seen in the Robles et al. (2014) paper, where ~20% of cycling proteins correspond to non-cycling RNA transcripts in liver. It may be that many non-cycling transcripts are circadianly translated in the SCN and liver in a similar manner to circadian translation in U2OS cells as described by Jang and Lahens et al. (2015).

Overall, we found 28 transcripts that cycle in the SCN that have corresponding proteins in the TOD data set from Chiang et al. (2014). Our analysis showed that the lag between peak phase of expression of RNA and peak expression of protein varies widely. However, roughly half of genes have a lag less than six hours, with the other half having a lag greater than six hours. This variation in lag reflects lag differences seen between peak circadian RNA and protein expression in the liver found by Robles et al. (2014). The majority of cycling transcripts in the SCN do not have corresponding proteins in the Chiang et al. (2014) TOD dataset. More complete characterization of the relationship between circadian transcription and protein expression will depend on experiments performing ribosomal profiling in the SCN, as well proteomics time courses with advances in mass spectrometry, and increased resolution spanning at least 48 hours.

4.2 Future Directions from Chapter 2

Given the number of cycling transcripts in the SCN, it would be interesting to investigate the roles of the proteins they encode. There are a number of transcripts
mediating neuronal connectivity, synaptic function, RNA splicing, and heat shock factors that may reveal important roles in regulation of the circadian clock and clock output upon further investigation. A variety of techniques are available to determine cell type specificity and localization in the SCN, as well as mouse models of gene down regulation or deletion to determine effects on function in the circadian clock.

The SCN contains a highly heterogeneous cell population. An interesting first step for investigating cycling transcripts would be in situ hybridizations to locate cycling transcripts in specific regions and/or cell types in the SCN. Immunohistochemistry could be used in addition to, or instead of in situ hybridization, to determine if and when proteins translated from cycling RNA transcripts are circadianly expressed. Given the number of neuronal connectivity and synapse related transcripts, it is likely that some cyclers are highly cell type specific. Localizing these transcripts to particular subregions and cell types is the first step in determining how they function. Determination of localization and cell type specificity techniques could also be used to further characterize transcripts coding for heat shock factors and aggregation prone proteins.

Given the highly heterogeneous nature of cell type in the SCN, it would be advantageous to develop fluorescent proteins to mark cell types in the SCN that could then be used for FACS sorting prior to further experiments characterizing cell type-specific gene expression. FACS sorting has already been successfully used to characterize circadian rhythms in the brain, although it has not yet been used in the SCN to our knowledge [258]. Use of FACS sorting in the SCN would greatly increase the speed
and extent of characterization of gene expression in the SCN, as less high-throughput methods may not be efficacious in investigating the many cycling genes in the SCN in the context of cell-type specific roles.

**Distinguishing Between Circadian and Sleep Contributions:**

Some cyclers, particularly those involved in neuronal connectivity and synaptic transmission, may exhibit twenty-four hour cycles of expression due to wake and sleep cycles, and may not be directly driven by the molecular circadian clock. For example, Maret *et al.* (2007) conducted microarray analysis of whole brain from mice sleep deprived for six hours starting at ZT 0, 6, 12, 18, and then sacrificed, and from non-sleep deprived control mice sacrificed at the same time points [259]. In control mice, ~2,000 transcripts showed significant variation in expression for at least one time of day. In sleep deprived mice, fewer than four hundred transcripts showed time of day variation. The authors concluded that a large amount of time of day variation in RNA expression in the whole brain is due to the wake/rest cycle, not to circadian variation [259]. A similar protocol could be followed to determine whether or not the wake/rest cycle is responsible for some of the variation in SCN gene expression discussed in Chapter 2. The protocol from Maret *et al.* (2007) could be modified so that mice were released into constant darkness for at least twenty-four hours before tissue collection, so that light would not be a confounding factor. Control and sleep deprived mice could then be
sacrificed at each of four time points as in the protocol described by Maret et al. (2007), and their SCNs could be collected and analyzed by microarray as was done in Chapter 2. Transcripts that showed time of day variation in controls could be used to confirm variation in expression over time of day that was seen in the data from Chapter 2. Transcripts that cycled in Chapter 2 that were confirmed as having time of day variation could be compared with transcript expression in the sleep deprived mice. Transcripts that no longer showed time of day variation in SCNs from mice that are sleep deprived may show variation over time of day in response to the wake/activity cycle. However, it is possible that transcripts that are truly regulated by the circadian clock change response to sleep deprivation. Indeed, Maret et al. (2007) found that some components of the core molecular clock showed changes in expression in response to sleep deprivation [259].

Other types of experiments, such as desynchrony protocols, can be used to attempt to dissociate sleep and circadian rhythms, or at least dissociate some components of sleep and circadian rhythms. Some desynchrony protocols employ non-circadian cycles of light and darkness, lack of light and dark cycles or other manipulations of zeitgeibers to separate the circadian clock from the sleep cycle. In humans, desynchrony protocols result in the separation of circadian rhythm (as measured by circadian body temperature (CBT), and wake/rest activity cycles [260]. Intriguingly, NREM sleep syncs with the wake/rest cycle, and REM sleep syncs with the free running circadian cycle, as measured by CBT [260]. Rodent protocols that also lead
to dissociation of REM and NREM sleep have been developed. Notably, these protocols also cause desynchrony between the ventrolateral (core) and dorsomedial (shell) SCN regions [260]. Furthermore, molecular studies of the desynchrony between the core and shell suggest that NREM sleep follows the same rhythm as the core, while NREM sleep follows the same rhythm as the shell [260,261].

There are many examples of desynchrony protocols. We will adapt the protocol for desynchrony in rats from Cambras et al. (2008) for use in mice. This desynchrony will be used due to its relatively mild perturbation of the circadian clock, its success in dissociating NREM and REM sleep in rats, and successful dissociation of SCN core and shell gene expression in rats [261]. This protocol consists of a 22 hour cycle of light and darkness, with 11 hours of light alternating with 11 hours of darkness. Cambras et al. (2008) found that this protocol dissociated locomotor activity to a 22 hour pattern of locomotor activity regulated by the light cycle (T \(_{22h}\)), and a free running period of greater than 24 hours (T \(_{>24h}\)). Intriguingly, slow-wave sleep cycles synced with the 22 hour locomotor activity rhythm regulated by the LD cycle, while paradoxical sleep (REM sleep) synced with the free running pattern of locomotor activity [261].

Due to the presence of locomotor activity cycles with differing time periods, a T \(_{22h}\) and a T \(_{>24h}\) cycle, at some points the peak of the T \(_{22h}\) hour cycle will coincide with the peak of the T \(_{>24h}\) cycle, and sometimes these cycles will be anti-phase. Collecting core and shell tissue from time points when it is subjective day in the T \(_{22h}\) cycle, and subjective night in the T \(_{>24h}\) cycle, and when it is subjective night in the T \(_{22h}\) cycle and
subjective day in the $T_{>24h}$ hour cycle, has previously been successfully in showing
differential expression of core clock genes in these two subdivisions of the SCN during
desynchrony [262]. Furthermore, collection of the core and shell at times that are
subjective day in both the $T_{22h}$ and $T_{>24h}$ cycles shows that expression of core clock genes
is similar in these regions when compared with expression of these genes when it is
subjective night in both regions [262]. Importantly, these desynchronized rhythms
partially persist in constant darkness following the 22 hour LD cycle, at least in rats
[262]. This is evidence that the $T_{22h}$ rhythm represents an entrained oscillator, and not
“masking” by light.[261,262].

These experimental designs could be performed in mice to investigate gene
expression associated with REM vs. NREM sleep, and the relationship of this expression
to the core and shell of the SCN. Adult C57 BL/6J mice would be subjected to 22 hour
cycles of 11 hours of light and 11 hours of darkness as in Cambras et al. (2008), for at
least two weeks. Locomotor activity, CBT, NREM sleep, and REM sleep would be
measured as in de la Iglesia et al. (2004), to confirm that the $T_{22h}$ and $T_{>24h}$ cycles occur
in this LD cycle in mice, and that NREM follows the $T_{22h}$ cycle and that REM sleep syncs
with the $T_{>24h}$ cycle. If these patterns do not follow the same pattern in mice, the
experiment would be adjusted to whatever pattern emerged, as necessary. If the $T_{22h}$
and $T_{>24h}$ cycles occur in mice, the mice would then be released into constant darkness,
and mice would be sacked at times when the projected $T_{22h}$ and $T_{>24h}$ cycles would
coincide for subjective day, coincide for subjective night, be subjective day in the $T_{22h}$
cycle and subjective night in the T_{>24h} cycle, and be subjective night in the T_{22h} cycle and subjective day in the T_{>24h} cycle [261,262]. Brains would be collected at each of these time points, and the SCN core and shell will then be dissected on a cryostat. RNA would then be pooled with an N of three of each region in each condition, and examined by microarray analysis, similar to experiments described in Chapter 2.

Gene expression analysis would consist of determining which genes were differentially expressed when the T_{22h} cycle and the T_{>24h} hours cycles were anti-phase, compared to when the two cycles were both either subjective day or subjective night. Further analysis would include determining which transcripts were in phase with either the activators or repressor arms of the core clock in the core and shell, and hence may connect the core and shell with NREM and REM sleep, respectively. Genes showing differential regulation between the core and shell could also be analyzed for functional annotation by a variety of means, and for mechanisms of regulation by different transcription factors through investigation of transcription factor binding sites using data from published ChIP-seq experiment and similar studies [263].

RNA Metabolism:

Several cycling RNA splice factor transcripts may have specific clock function, such as Lark homologue Rbm4b, and predicted core clock component Caprin1 (Fig. 2.6F)[34,213,214,256]. It would be interesting to test knock out mouse models of these
genes for circadian phenotype. If circadian rhythms are affected in knockout models, it would be interesting to perform follow up studies to further understand how regulation of RNA splicing in the SCN is related to circadian output at the molecular, tissue, and behavioral levels.

A number of cyclers related to RNA metabolism in baseline conditions are known to localize to complexes of translationally stalled RNAs known as stress granules during times of cellular stress [264]. It would be interesting to characterize stress granule formation in the SCN, to see if stress granules are more easily induced or differentially localized at some circadian times versus others.

**Stip1, Prnp, and Protein Synthesis**

Transcripts for *Stip1* and *Prnp* cycling in phase are a particularly intriguing avenue for further investigation (Fig. 2.6D-E). Previous research has shown that STIP1 stimulation of neurons induces protein translation through PRNP and an AKT, PI3K, and MTOR dependent pathway. Furthermore, PRNP deficient mice exhibit lengthened circadian period of locomotor activity in constant darkness, although the molecular mechanism for this phenotype is unknown. It would be interesting to compare levels of protein expression in PRNP deficient mice and wild-types, and see if protein expression is altered in PRNP deficient mice. A high-throughput method such as mass spectrometry should be used, as the most efficient method of determining which clock related
proteins are affected. Furthermore, it would be interesting to see if pharmacological manipulation of STIP1, AKT, PI3K, or MTOR affects circadian phenotype, particularly in a PRNP dependent manner [186,265]. Effect of pharmacological manipulation of AKT, PI3K, or MTOR and the effect on circadian phenotype could be examined in WT and PRNP deficient mice [186].

**Standard Circadian Tests of Knock-out Mice**

Cycling transcripts in the SCN RNA profile can be examined for circadian phenotypes using knockout mice. Knockout mice and wild type littermates could have their circadian locomotor activity period measured in constant darkness. Furthermore, phase-response curves for knockout mice compared with wild-type controls could determine if any of these mouse lines have altered responses to light at specific circadian times. If these mice are found to have circadian phenotypes in locomotor activity assays and PRCs, knock-out mouse lines can be mated with PER2::LUC lines. PER2::LUC expression could then be examined in the SCN and other tissues of knockout mice and wild type controls[23,239,266]. Previous studies of this type have shown that period of PER2::LUC expression in the SCN of knock-out mice frequently gives insight to roles of these genes in generating network properties of the SCN and circadian locomotor activity [47,267].
If knock out mice are unavailable for some animals, siRNA can be infused to mice through cannulation, and observation of circadian locomotor activity under gene knock-down can be observed. In some cases, cycling transcripts of interest may code for proteins that are known drug targets. It would be interesting to see if pharmacological manipulation of circadian period length is possible in these cases, and how phase-response curves are affected. Pharmacological modulation of the circadian clock is an intriguing avenue for further study, with implications for medical intervention in jet lag, shift-work disorders, and other cases of circadian perturbation [19,20].

4.3 General Conclusions from Chapter 3

Understanding the molecular underpinnings of the circadian clock in the SCN is important in order to understand the clock’s response to perturbation. Circadian function has been shown to be perturbed in many disorders, including obesity, diabetes, and metabolic syndrome [13,14]. Overt circadian rhythms have also been shown to be perturbed in mouse models, such as lengthening of circadian locomotor period in DD in adult male mice fed a high fat diet [100].

To further study the effect of diet on the circadian clock, we developed a model of the effect of in utero diet on adult circadian rhythms, combined with studies of how in utero diet interacted with an adult HF in male and female mice [102,220,221]. We measured circadian locomotor activity of these mice as adults. We furthermore
characterized the expression of PER2::LUCIFERASE expression in the SCN and arcuate nuclei of PER2::LUC heterozygous male and female mice given the same in utero and adult diet conditions as those from our locomotor activity experiments [23].

**General Conclusions from Behavioral Data:**

Our behavioral data show an adult HF diet leads to a greater lengthening of Tau in DD, when compared to an in utero HF diet. The LP in utero diet leads to a lengthening effect of Tau in DD in male mice, but does not seem to affect female Tau in DD. However, both male and female mice that received and in utero LP diet have a trend of lengthened Tau in DD when weaned to an adult HF diet (Fig. 3.1A-B).

Additional circadian parameters were also altered by in utero diet and adult diet. Trends of greater error of activity onset in DD were seen in female mice compared to male mice in all in utero diet and adult diet combinations studied. Our data also showed a trend of lengthened alpha hour of activity in female mice compared to male mice in most in utero diet conditions (Fig. 3.2A-B, Fig. 3.3A-B).

**General Conclusions from PER2::LUCIFERASE Data:**

The circadian period of expression of PER2::LUC period in the SCN was robust in both male and female mice in response to both in utero diet and adult dietary
perturbation (Fig. 3.4A-B). These findings are similar to those demonstrating the ability of the circadian clock in the SCN to maintain a relatively robust period in response to some genetic perturbations to the molecular clock [47].

Other properties of the SCN were less robust in response to in utero dietary perturbation, with trends of decreased baseline and amplitude of PER2::LUC in the SCN of male and female mice in response to some dietary perturbations (Fig. 3.5, 3.6). The arcuate nucleus also had sex differences in baseline and amplitude. Arcuate nuclei from male mice showed increases in baseline and amplitude of PER2::LUC expression in all dietary conditions compared with controls, while female mice showed decrease in baseline and amplitude in all dietary conditions compared with controls (Fig. 3.8AB, 3.9A-B).

**Overall Conclusions from Chapter 3:**

Circadian period of locomotor activity was lengthened in both sexes of mice by an adult HF diet, while an in utero HF diet lengthened period length to a lesser extent in these groups. A LP in utero diet lengthened circadian locomotor activity period in male mice, while not affecting females. Both male and female mice exhibited lengthened circadian period when given a LP in utero diet followed by adult HF diet. Other parameters showed sex differences, such as trends of increased error of onset and increased alpha hour in female mice compared to males (Figs. 3.2A-B, 3.3A-B). This
robustness of period length may have evolved since there is probably not much of an adaptive value to shifting period length; day length changes slowly no matter where on earth an animal is. Other factors, such as diet and temperature, change more quickly and require that an animal react more quickly, and amplitude and baseline may adjust in response. Accordingly, parameters such as baseline and amplitude of PER2::LUC expression were more variable in response to in utero and adult dietary perturbations. Furthermore, sex specific differences in these data were observed. Baseline and amplitude of PER2::LUC expression were generally lower in female mice than in male mice, while the opposite trend was seen in PER2::LUC expression in the arcuate nucleus.

4.4 Future Directions from Chapter 3

At the behavioral level, our data show that period length is robust in response to perturbation by in utero diet, while an adult HF diet lengthens circadian period length, when the in utero diet was either control or LP. This robustness of the SCN and circadian period of locomotor activity has also been shown in mice that are deficient in some molecular clock components [47]. However, circadian period of locomotor activity is not the only important component of the physiological circadian clock. Additional important behavioral components include response to phase shifts, and response to light pulses.
It would be interesting to do phase response curves (PRCs) for male and female mice that have received an LP or HF in utero diet, and compare their adult PRCs with those of control animals. These experiments could also be performed for male and female mice that were weaned to a control or high fat diet. Other manipulations that take place before rodents reach adult hood have been shown to have long lasting effects on circadian parameters, such as length of light and darkness received during gestation [218,268]. It would be interesting to see if these lighting conditions interact with in utero or adult dietary conditions. These experiments may yield greater effects of in utero diet on adult circadian period, as it is likely that WT mice experience changes in food availability according to geographic location, which will also determine the light cycle an animal experiences [269]. Furthermore, if response to light is altered in in utero diet animals, it would be interesting to see if changes in clock-related gene expression are seen in the SCN from animals in 12:12 LD conditions when compared with in utero diet animals from DD conditions. These experiments would shed light on how the clock interacts with diet and light conditions.

Previous studies have shown that a LP in utero diet alters growth of the SCN [222]. Furthermore, the literature shows that in utero diet alters the growth of the arcuate nucleus, with effects lasting into adulthood [219,222]. Specifically, Aguilar-Roblero et al. (1997) have shown that LP diet in rats results in smaller size of the adult SCN, with the number of cells expressing both VIP and AVP being reduced in adult male rats that received a LP in utero diet [222]. It would be interesting to confirm these
changes in growth in our models of in utero diet, and see if these changes persist in utero diet perturbations combined with adult high fat diet. Furthermore, it would be interesting to see if changes in development of the SCN and arcuate nucleus were accompanied by changes in expression of genes for core circadian clock components, as well as expression of molecules known to phase shift circadian rhythms.

Furthermore, long lasting changes in gene expression and SCN or arcuate nucleus structure may be accompanied by epigenetic modifications. In utero diet has previously been shown to alter epigenetic modifications in the brain [220,221]. Core clock genes have been shown to be differentially methylated in the SCN compared to some peripheral tissues at different stages of normal development [270]. Although changes in methylation levels of circadian genes in response to dietary perturbations have not been extensively studied, a few experiments suggest epigenetic modifications may be affected by maternal diet [104]. Furthermore, genes associated with the molecular clock may affect the methylation by interaction with homocysteine metabolism, thereby affecting the availability of methyl donors and hence methylation levels [34,246].

Additional experiments may also address sex specific differences in circadian rhythms between male and female mice. Data from our lab discussed in Chapter 3 and from other labs shows that there are sex differences in circadian behavioral and gene expression patterns. Our PER2::LUC data and electrophysiological data from the Colwell group show differences in the SCN of male and female mice [52]. Kuljis et al. (2013)
show greater excitability in the SCN of male mice than in female mice during the day. Our PER2::LUC data from Chapter 3 (Fig. 3.2A-B, Fig. 3.3A-B) show a trend of decreased baseline and amplitude of expression of PER2::LUC in the SCN of female mice compared with male mice. Lower baseline and amplitude of the circadian clock in female mice may facilitate resetting of the clock, which may render female mice more adaptable to changes in the environment. Furthermore, female mice have differences in circadian behavior, such as increased error in onset of activity in LD shown by Kuljis et al. (2013), as well as increased error in onset of activity in DD that remains in different in utero dietary conditions as discussed in Chapter 3 (Fig. 3.2A-B). It would be interesting to investigate the molecular and structural characteristics of the SCN and other brain regions that underlie sex differences in circadian behavior and SCN excitability and amplitude. Our data from Chapter 2 offer interesting possibilities of cycling genes that may be responsible for sex differences in circadian rhythms [52].

4.5 Future Directions from Integration of Chapters 2 and 3

The overall significance of circadian function is its role in maintaining an organism’s homeostasis. Perturbation of circadian homeostasis plays a role in many pathological conditions including cancer, sleep disorders, obesity, diabetes, metabolic syndrome, and neurodegeneration [13,14,16,18,215]. Furthermore, circadian related genes are implicated in autism spectrum disorders and psychiatric disorders, although
how circadian genes are involved in these illnesses is not well understood [18,271]. Some of these conditions are more prevalent in males or females, and the influence of sex differences in circadian rhythms may be relevant to disease etiology and treatment [245].

The role of sex hormones in circadian rhythms is partially known. Testosterone and estrogen both play roles in determining circadian locomotor activity. Testosterone surges in males are thought to be related to the precise increase in activity in males at the beginning of the wake period, which is less precise in females and is reflected in females’ greater error of onset of activity. Indeed, gonadectomy lessens the precision and extent of locomotor activity in males at the start of the wake period. Furthermore, testosterone level is negatively correlated with length of activity, which is in agreement with the longer alpha hour observed in females versus male mice in Chapter 3 [245]. In females rats, stages of the estrus cycle are thought to modulate day to day variation in locomotor activity, and a similar phenomenon may play a role in the greater error of activity onset in female mice than in males in our data and others [52,272]. Sex differences in circadian rhythms extend to the level of the SCN. Androgen receptors are found in the SCN core, and levels are higher in males than in females. Estrogen Receptor β (ERβ) is found in the SCN shell, and levels are higher in females than in males. Furthermore, VIP levels are higher in females than in males [245].
In addition to circadian sex differences caused by gonadal hormones, some sex differences are thought to be due to sex chromosomes. These discoveries were made by Kuljis et al. (2013) using mice from the four core genotypes, XYM, XXM, XYF, and X XF. Precision of activity onset is at least partially regulated by gonadal hormones, as XYF and X XF mice had lower precision than gonadotypic male mice. However, X XM mice had precision levels higher than those of gonadal females, but lower than that of X YM mice, leaving room for the possibility of a sex chromosome component of precision of activity onset. Intriguingly, mice with two X chromosomes exhibited longer alpha hour of activity regardless of gonadal phenotype, i.e., alpha hour was longer in X XM and X XF mice than either X YM and XY F mice [52].

It would be interesting to investigate the relationship between sex chromosomes and sex differences in circadian rhythms. Some sex chromosome genes involved in these differences may be regulated by the circadian clock, and may be observable as cyclers at the RNA level. Interesting candidate genes from Chapters 2 may be useful in generating hypotheses for future study. Cycling transcripts from the SCN include transcripts coding for proteins that regulate neuronal connectivity and synaptic transmission. Some of these cyclers are located on the X chromosome, and therefore may have differential expression in male and female mice, due to the presence of a paternal and maternal allele of each gene in females, but only the maternal allele in males (Table 3.1). Although X-inactivation may lead to similar expression levels in males and females of genes with only one allele in males, some genes escape X-inactivation, and there are
varying degrees of escape from X-inactivation [273]. Furthermore, mosaic expression of genes on the X chromosome may mean that two alleles of X-chromosome located genes are expressed in females, while only one allele is expressed in males [273]. It would be interesting to see if mosaic expression of SCN-cyclers on the X chromosome are related to differences in circadian locomotor activity observed in male and female mice as discussed in Chapter 3. Cycling genes located on the X or Y chromosome, or on autosomal chromosomes may also be differentially expressed in male and female mice through imprinting or other epigenetic modification, mosaicism, or regulation by sex hormones [52,273].

It would be interesting to determine if cycling transcripts found in Chapter 2 are related to the differences in male and female mice described in Chapter 3. Data from our lab and from Kuljis et al. (2013) show that female mice have greater error in onset of activity of males in both LD and DD, and also have increased alpha hour of activity (Chapter 3)[52]. Furthermore, error onset is affected by some in utero diet conditions. It would be interesting to see if the greater error onset and/or alpha hour in females is due to differences in neuronal connectivity in the SCNs of male and female mice.

Intriguingly, our results show a cycling receptor on the X chromosome, Gabra3. Female mice may therefore express two alleles of this GABA receptor, while male mice express one. It would be interesting to determine which cells in the SCN express Gabra3 RNA and proteins, and if the type or number of these cells varies between male and female mice. It would also be interesting to see if excitation levels of the SCN over the
circadian cycle vary in a different way in male and female Gabra3 knock out mice. The main caveat for these types of studies is that GABA is sometimes excitatory in the SCN, so this possibility must be investigated [24]. If differences in SCN excitability are noted in these mice, they could be tested for circadian locomotor phenotypes, as previously described.

Interestingly, several cycling transcripts on the X chromosome have been identified as candidate disease genes for autism spectrum disorders. These include Rps6ka6, L1cam, Sh3kbp1, and Tspan7 [274–276]. Female mice may have less susceptibility to genetic perturbations of these genes, since they have two copies, while males have one. Interestingly, female mice that are heterozygous for L1cam have been shown to express autism-like behavioral characteristics. It would be interesting to see if mice from these models have different periods of circadian locomotor activity, alpha hour, responses to light pulses in constant darkness, or changes in SCN firing over the circadian cycle [52,137,149,239,266]. It would be interesting to perform similar experiments for other genes on the X chromosome that cycle in the SCN.

An alternative possibility is to examine the expression of cyclers on the X chromosome in the SCN of mice that are lacking one or more molecular circadian clock components, such as Bmal1 -/- or Per2 -/- knockout mice. It would be interesting to see if overall expression levels of L1cam, Rps6ka6, or other genes are lower in the SCN of these mice than in WT mice. It may be that the relationship of the circadian clock and
other diseases is related to clock gene involvement in overall expression levels, as opposed to having an effect at a specific time of day.

Overall, SCN cyclers on the X chromosome characterized in our research discussed in Chapter 2 offer intriguing possibilities for further study of circadian sex differences that we discussed in Chapter 3.

**Summary:**

Our data expand the horizon for circadian experimentation at the level of baseline gene expression in the SCN, behavioral response to in utero and adult diet, and sex differences in circadian rhythms. Gene expression data in the SCN discussed in Chapter 3 show hundreds of cycling transcripts as well as numerous circadianly regulated splice junctions. These cyclers represent areas key for both the core molecular clock and for the role of the clock in physiological homeostasis. These transcripts code for proteins that regulate neuronal connectivity, synaptic transmission, signaling, ion homeostasis, gene splicing, and protein quality control. Our behavioral *in vivo* experiments in Chapter 3 showed the response of the circadian clock to in utero and adult dietary perturbation, and identified sex specific effects on circadian locomotor activity. SCN and arcuate nucleus explants from PER2::LUC mice from different in utero and adult dietary combinations uncovered trends in sex specific response to diet in these hypothalamic nuclei. We then used our data from Chapter2 to identify cycling
genes in the SCN that may be of interest in continuing studies of sex differences in the SCN, as well as changes in SCN gene expression in response to environmental and genetic perturbations. Overall, our data show previously undiscovered circadian regulation of gene expression coordinated between the SCN and other brain clocks, as well as the response of these clocks to combinations of *in utero* and adult dietary perturbation.
4.6 SCN Cyclers With Genes Located on the X Chromosome

Table 4.1 SCN Cyclers With Genes Located on the X Chromosome

<table>
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<th>Adj. P</th>
<th>Chromosome</th>
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Chapter 2, Zhang and Lahens et al. (2014)

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