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Regulation of Sleep and Circadian Rhythms by Metabolic Neuropeptides

Abstract

The increasing prevalence of metabolic disease in modern society has accelerated our need to understand factors that may be contributing to its development. Both circadian disruption and sleep deprivation are associated with metabolic dysfunction. Thus, for my dissertation I sought to gain insight into this association by studying the genetic and neural basis of interactions between circadian rhythms, sleep and metabolism. The relative simplicity of fly neuroanatomy and physiology, vast array of available genetic tools, and conservation across many organisms, makes Drosophila melanogaster an ideal model to dissect complex interactions between physiological systems. Through our studies we identified a novel role for a molecule that regulates feeding behavior, Neuropeptide F (NPF), in the circadian system. We found that NPF drives circadian gene expression of the detoxification gene sex-specific enzyme 1 in a peripheral metabolic tissue, possibly to coordinate consumption of toxins with their removal. Our results support a role for NPF in synchronizing behavior and metabolism to ensure circadian coherence and promote survival. In addition, we studied the interaction between sleep and metabolism by evaluating whether alterations in sleep cause metabolic dysfunction or are the result of shared molecular pathways. The insect equivalent of norepinephrine, octopamine, promotes wake in flies by signaling through insulin-producing neurons. We determined that although sleep and metabolic neural circuitry intersect, the octopamine signaling pathway regulates sleep and metabolism independently. This dissertation highlights the great power of Drosophila as a model organism to investigate complex interactions between different biological systems.

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REGULATION OF SLEEP AND CIRCADIAN RHYTHMS BY METABOLIC NEUROPEPTIDES

Renske Erion

A DISSERTATION

in

Cell and Molecular Biology

Presented to the Faculties of the University of Pennsylvania

in

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Degree of Doctor of Philosophy

2014

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REGULATION OF SLEEP AND CIRCADIAN RHYTHMS BY METABOLIC NEUROPEPTIDES

2014

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ABSTRACT

REGULATION OF SLEEP AND CIRCADIAN RHYTHMS BY METABOLIC NEUROPEPTIDES

Renske Erion

Amita Sehgal

The increasing prevalence of metabolic disease in modern society has accelerated our need to understand factors that may be contributing to its development. Both circadian disruption and sleep deprivation are associated with metabolic dysfunction. Thus, for my dissertation I sought to gain insight into this association by studying the genetic and neural basis of interactions between circadian rhythms, sleep and metabolism. The relative simplicity of fly neuroanatomy and physiology, vast array of available genetic tools, and conservation across many organisms, makes *Drosophila melanogaster* an ideal model to dissect complex interactions between physiological systems. Through our studies we identified a novel role for a molecule that regulates feeding behavior, Neuropeptide F (NPF), in the circadian system. We found that NPF drives circadian gene expression of the detoxification gene *sex-specific enzyme 1* in a peripheral metabolic tissue, possibly to coordinate consumption of toxins with their removal. Our results support a role for NPF in synchronizing behavior and metabolism to ensure circadian coherence and promote survival. In addition, we studied the interaction between sleep and metabolism by evaluating whether alterations in sleep cause

metabolic dysfunction or are the result of shared molecular pathways. The insect equivalent of norepinephrine, octopamine, promotes wake in flies by signaling through insulin-producing neurons. We determined that although sleep and metabolic neural circuitry intersect, the octopamine signaling pathway regulates sleep and metabolism independently. This dissertation highlights the great power of *Drosophila* as a model organism to investigate complex interactions between different biological systems.

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CHAPTER 1: Introduction

Circadian clocks synchronize daily variations in behavior and physiology with changes in the physical environment (i.e. light and temperature) imposed by the 24-hour rotation of the Earth. Circadian rhythms are exhibited throughout the animal kingdom, from primitive organisms like cyanobacteria to complex organisms like mammals. Conservation of circadian rhythms suggests they serve an important role in biology and confer a selective advantage. In addition, circadian clock disruption is associated with negative health consequences including sleep disorders, cancer and metabolic dysfunction (Marcheva et al., 2013).

Understanding the relationship between circadian rhythms and metabolism is important given the increasing prevalence of metabolic dysfunction in modern society. The association between circadian system disruption and metabolic syndrome suggests the circadian system plays an important role in maintaining metabolic homeostasis (Bass & Takahashi, 2010). For example, mutant mice lacking functional circadian clocks display impaired glucose tolerance and reduced insulin secretion among other metabolic defects (Marcheva et al., 2010; Rudic et al., 2004; Turek et al., 2005). The circadian system is responsible for coordinating cycles of rest:activity, fasting:feeding and energy storage:utilization. Desynchrony between these cycles, caused by increased feeding during normal periods of rest, can also lead to metabolic dysfunction as demonstrated by restricted feeding experiments in mice. Mice fed a high fat diet during their normal rest period gain significantly more weight compared to mice fed a high fat diet during their normal active period (Arble, Bass, Laposky, Vitaterna, & Turek, 2009). Analogous

findings have been reported in humans; for example, cross-sectional studies of night shift workers found that they have an increased prevalence of obesity, diabetes, and cardiovascular disease (Ellingsen, Bener, & Gehani, 2007; Karlsson, Knutsson, & Lindahl, 2001). Insight into the relationship between circadian and metabolic systems can be gained by understanding circadian regulation of metabolic genes and tissues.

The Circadian Clock in *Drosophila*

Components of the molecular clock were first elucidated through genetic screens using the fruit fly *Drosophila Melanogaster* (Konopka & Benzer, 1971). Since then, *Drosophila* has become a widely used model to study circadian rhythms. The core molecular oscillator consists of two basic helix-loop-helix (bHLH) transcription factors, CLOCK (CLK) and CYCLE (CYC), which heterodimerize to drive the expression of the genes *period* (*per*) and *timeless* (*tim*) (Zheng & Sehgal, 2012). Expression levels of *per* and *tim* transcripts peak during the early night and are translated into proteins in the cytoplasm. During the late night, PER and TIM proteins heterodimerize and translocate into the nucleus to inhibit the activity of CLK/CYC thereby repressing their own expression. At dawn PER and TIM proteins undergo degradation, relieving inhibition of CLK/CYC, allowing the cycle of transcription to begin again (Nitabach & Taghert, 2008). This transcriptional-translational feedback loop generates rhythms in the transcript levels of clock-controlled genes including *per* and *tim*, with a specific phase of peak expression and a period of roughly 24 hours.

There are roughly 150 clock neurons, identified by their expression of clock components, in the adult fly brain, with a well-established anatomical and functional organization. Clock neurons are divided into clusters according to their location and

circadian function in the brain. These clusters include the small and large ventrolateral neurons (sLNvs and lLNvs respectively), dorsal lateral neurons (LN_ds), lateral posterior neurons (LPN), and three groups of dorsal neurons (DN1, 2 and 3) (Figure 1-1) (Ravi Allada & Chung, 2010). The sLNvs are referred to as the master circadian regulator in *Drosophila* because they are necessary and sufficient to drive locomotor activity rhythms in the absence of any external cues (i.e. constant darkness or DD) (Grima, Chélot, Xia, & Rouyer, 2004; Renn, Park, Rosbash, Hall, & Taghert, 1999; Stoleru, Peng, Agosto, & Rosbash, 2004).

Under 12hr light: 12 hr dark laboratory conditions set to mimic the solar day/night cycle (LD), fly locomotor activity is bimodal with a peak of activity during the morning and evening (Dubruille & Emery, 2008). Different clock neurons control the morning and evening bouts of activity. The identity of the morning and evening oscillators were discovered through cell-specific ablation and rescue and found to be the sLNvs and a combination of the LN_ds and the 5th sLNv, respectively (Grima et al., 2004; Stoleru et al., 2004). Thus the regulation of locomotor rhythms is complex and sensitive to environmental conditions.

The functions and molecular compositions of the dorsal clock neurons are not as well understood as the lateral neurons. Multiple studies suggest the DN1s integrate environmental and peptidergic signals to modulate rest:activity rhythms (Lear, Zhang, & Allada, 2009; L. Zhang et al., 2010; Y. Zhang, Liu, Bilodeau-Wentworth, Hardin, & Emery, 2010). For example, PDF-negative clock neurons (which include the DN1s and LNds) are capable of driving rest:activity rhythms under constant light but not constant darkness (Stoleru et al., 2007). In contrast to the DN1 cluster, the DN2 cluster does not appear to be important for rest:activity rhythms, but is sufficient for rhythms in temperature preference (H. Kaneko et al., 2012). Little is known about the molecular

identity of the DN3s beyond limited evidence that a subset of DN3s are glutamatergic (Hamasaka et al., 2007). Currently the function of the DN3 cluster in circadian rhythms is entirely unknown. Understanding the functions and molecular compositions of individual clock neuron clusters will be informative for probing interactions between different clusters. Ultimately, dissecting connections between clusters will illuminate how clock neurons form complex neural circuits capable of integrating environmental and internal inputs to generate coordinated outputs.

Regulation of Circadian Rhythms by Neuropeptides

Neuropeptides regulate physiology and behavior in the adult fly and act to integrate information regarding the flies' internal state with external sensory cues (Taghert & Nitabach, 2012). In the insect central nervous system, neuropeptides have been shown to act as circulating neurohormones, local neuromodulators, and local cotransmitters of fast-acting neurotransmitters (Nässel, 2009). With respect to the regulation of circadian rhythms, several neuropeptides are implicated including pigment-dispersing factor (PDF), neuropeptide F (NPF; long NPF), short neuropeptide F (sNPF), IPNamide, ion transport peptide (ITP), diuretic hormone 44 (DH44) and Drosophila insulin-like peptides (DILPs) (Cavanaugh et al., 2014; Hermann-Luibl, Yoshii, Senthilan, Dircksen, & Helfrich-Forster, 2014; Johard et al., 2009; G. Lee, Bahn, & Park, 2006; Nässel & Winther, 2010; Renn et al., 1999; Zheng, Yang, Yue, Alvarez, & Sehgal, 2007).

For many of these neuropeptides, the role they play in the circadian system is not clear. Furthermore, there are likely additional neuropeptides that have not yet been identified. These circadian-relevant neuropeptides are expressed in clock neurons as follows: PDF is expressed in the LNvs; NPF is expressed in 3 out of the 6 LNds

(specifically in males); sNPF is expressed in the LNvs and 2 of the NPF-negative LNds; IPNamide is expressed in a subset of DN1s; ITP is expressed in the sLNvs, including the 5th sLNv, 2 sNPF-positive LNds and one NPF positive LNd. DH44 and DILPs are expressed in non-clock neurons known as the DH44-positive neurons and Insulin-Producing Cells (IPCs) respectively (Nässel & Winther, 2010). Refer to Figure 1-1 for a detailed depiction of the expression of these neuropeptides in the adult fly brain.

PDF Serves as a Synchronizing Signal in the Circadian System

Of the circadian-relevant neuropeptides, PDF has the most extensively studied role with respect to circadian circuits. PDF is involved in the regulation of rest:activity rhythms in the fly. Mutants lacking PDF display an altered activity pattern in LD; the morning peak of activity is reduced or absent while the phase of the evening peak is advanced by 1-2 hours (Renn et al., 1999). Furthermore, *Pdf* mutants become arrhythmic after several days in constant darkness (Renn et al., 1999). PDF is expressed in both the small and large LNvs; however its expression in the sLNvs is likely responsible for its regulation of rest:activity rhythms in DD because oscillations in core clock proteins are severely dampened or absent in the large LNvs in DD (Yang & Sehgal, 2001). Additionally, an increasing body of evidence suggests that PDF secretion synchronizes oscillation patterns between clock neurons, particularly the morning and evening oscillators, to create a coherent clock network (Lin, Stormo, & Taghert, 2004; Stoleru, Peng, Nawathean, & Rosbash, 2005; Yoshii et al., 2009).

The mechanisms through which PDF synchronizes the clock network are not well understood. The overall levels of PDF RNA and protein do not oscillate in a circadian manner, but PDF protein does rhythmically accumulate in the dorsal projections of the small LNvs. It is thought that this accumulation in the sLNv projections reflects rhythmic

release of PDF in the dorsal region of the brain. Intriguingly, it has been suggested that rhythmic accumulation of PDF in the sLNv axons may not be necessary for rhythmic locomotor activity, but regardless PDF release in the dorsal region of the fly brain is essential for generating rest:activity rhythms under constant conditions (Helfrich-Förster et al., 2000). PDF binds to the PDF receptor (PDFR), a G-Protein Coupled Receptor (GPCR), which functions as the sole receptor of PDF in *Drosophila* (Hyun et al., 2005; Lear et al., 2005; Mertens et al., 2005). PDFR is expressed in many neurons including the morning and evening oscillators and signals through adenylate cyclase and the second messenger cAMP. Interestingly, PDFR couples to different adenylate cyclases in different clock neuron clusters, supporting the hypothesis that PDF signaling components are sequestered into “circadian signalosomes” and explains why different clock neurons respond differently to PDF (Duvall & Taghert, 2012).

Role of Neuropeptide F is not Well-Established in Circadian Rhythms

Neuropeptide F (NPF) and short NPF (sNPF) are both associated with the circadian system; however, compared to PDF their roles are much less clear. Despite their similar names, NPF and sNPF belong to two distinctly different invertebrate neuropeptide families (Nässel & Wegener, 2011). Sequence analysis suggests that NPF is closely related to neuropeptide Y (NPY) in mammals, whereas sNPF is not. Although NPF and sNPF have similar functions to NPY, they signal through different pathways and do not play redundant roles. NPF is implicated in a variety of behavioral and physiological processes including feeding, courtship, and aggression (Dierick & Greenspan, 2007; C He et al., 2013; Lingo, Zhao, & Shen, 2007; Q. Wu, Zhao, & Shen, 2005). NPF is expressed in neurons in the brain and endocrine cells in the midgut, although the role of NPF in endocrine cells in the midgut is not understood (Brown et al.,

1999; Nässel & Wegener, 2011). NPF was first implicated in the circadian system by the discovery that it is expressed in 3 LNds specifically in males (G. Lee et al., 2006). NPF is expressed in roughly 26 neurons in males and 20 neurons in females (additional 6 neurons in males represent the 3 LNds in each brain hemisphere) (Nässel & Wegener, 2011). More recently it has been reported that NPF is also expressed in the sLNvs, some lLNvs and the 5th small LNv (Hermann, Yoshii, Dusik, & Helfrich-Förster, 2012). Although NPF expression in clock neurons is established, much remains to be elucidated regarding the functional role of NPF in circadian rhythms.

NPF has been proposed to regulate some sexually dimorphic aspect of circadian rhythms due to its male-specific expression in the LNds (G. Lee et al., 2006). Downregulation of *npf* or the NPF receptor (*npfr*) reduces the evening peak of activity in LD conditions in both males and females; indicating the role of NPF in locomotor rhythms is not sexually dimorphic (C He et al., 2013; Hermann et al., 2012). In contrast, overexpression of *npf* or *npfr* increases sleep in male flies but not female flies suggesting NPF regulates sleep in a sexually dimorphic manner (Chunxia He, Yang, Zhang, Price, & Zhao, 2013). NPF has been implicated in the regulation of energy homeostasis; however, it is not known whether NPF mediates circadian control of energy homeostasis in the fly. Additional studies are required to clarify the function of NPF in the circadian system.

Neuropeptides in the Pars Intercerabalis Function in Circadian Outputs

The pars intercerabalis (PI), the fly equivalent of the mammalian hypothalamus, is implicated in the control of circadian rest:activity rhythms (Cavanaugh et al., 2014; de Velasco et al., 2007; Helfrich-Forster et al., 2000). The projections of multiple clock neuron clusters are in close proximity to the PI, raising the possibility that the circadian

clock network directly modulates cells in this region (M. Kaneko & Hall, 2000). The PI contains a variety of neurosecretory cells that express different neuropeptides including Diuretic hormone 44 (DH44), SIFamide and Drosophila insulin-like peptides (DILPs). The DH44-positive neurons, SIFamide neurons and DILP-positive neurons are located adjacent to each other in the PI, however they are three distinct populations (Cavanaugh et al., 2014). The DH44-positive neurons and SIFamide-positive neurons have definitively been shown to be part of the circadian rest:activity output circuit.

Unlike the DH44 neurons, the DILP-expressing IPCs do not appear to regulate rhythmic rest:activity behavior. In both vertebrates and invertebrates, insulin is a key metabolic hormone that modulates carbohydrate and lipid metabolism in response to an organisms' nutritional state. Dysregulation of insulin production, release, or downstream signaling leads to metabolic disease, including diabetes and obesity (Baker & Thummel, 2007; Hoffmann, Romey, Fink, & Roeder, 2013). The role of insulin in metabolic homeostasis as well as development, fertility and lifespan is well established, whereas the function of insulin in the brain and behavior is not as well understood (Britton, Lockwood, Li, Cohen, & Edgar, 2002; S. Broughton & Partridge, 2009; Ikeya, Galic, Belawat, Nairz, & Hafen, 2002; Rulifson, Kim, & Nusse, 2002). In mammals, insulin is known to act on the brain to modulate behaviors relating to reproduction, feeding, and memory (Gerozissis & Kyriaki, 2003). *Drosophila* provides an excellent opportunity to elucidate the role of insulin signaling in neuronal function and behavior given how complex mammals are (Teleman, 2010).

Studying the role of insulin signaling in behavior became more feasible by the identification of eight insulin-like genes in the genome of the fruit fly, *Drosophila melanogaster* (Brogiolo et al., 2001; Colombani, Andersen, & Léopold, 2012; Grönke, Clarke, Broughton, Andrews, & Partridge, 2010). In mammals, insulin and insulin-like

growth factors signal through several different receptors whereas in *Drosophila* all insulin-like peptides (DILPs) signal through a single insulin receptor (dInR) (Brogiolo et al., 2001; Fernandez, Tabarini, Azpiazu, Frasch, & Schlessinger, 1995; Yamaguchi, Fernandez, & Roth, 1995). In the adult fly, three of these ILPs (2, 3 and 5), are expressed IPCs. The IPC axons project to other regions in the brain and to the fly heart (Rulifson et al., 2002). Secreted ILPs can activate insulin signaling in the brain or head or enter the fly circulatory system to activate systemic insulin signaling in peripheral tissues. The regulation of ILP production and release has recently been reviewed by Nässel and colleagues (Nässel, Kubrak, Liu, Luo, & Lushchak, 2013).

Although DILPs do not act as circadian signals to drive rhythmic behavior, IPCs appear to receive circadian input since Slowpoke binding protein (SLOB) is rhythmically expressed in these cells (Jaramillo et al., 2004). SLOB binds to and modulates the activity of the calcium-dependent potassium channel Slowpoke to alter membrane excitability and synaptic transmission (Jaramillo et al., 2004; Ma, Zhang, & Levitan, 2011; Schopperle et al., 1998; Shahidullah, Reddy, Fei, & Levitan, 2009). *Slob* was originally identified as one of the most robustly cycling transcripts by multiple independent genome-wide analyses of circadian gene expression in fly heads (Ceriani et al., 2002; Claridge-Chang et al., 2001; M. J. McDonald & Rosbash, 2001). *Pdf* mutants and the clock mutant Clk^{jrk} do not rhythmically express SLOB in the IPCs, suggesting that daily oscillations in SLOB expression require the circadian clock and PDF (Jaramillo et al., 2004). *Slob* mutants have normal locomotor rhythms but display metabolic defects supporting the idea that the IPCs do not regulate locomotor rhythms but may regulate some other physiologic or metabolic rhythm (Sheldon, Zhang, Fei, & Levitan, 2011).

Circadian regulation of the endocrine system has not been extensively studied in insects (Bloch, Hazan, & Rafaeli, 2013). It is not known whether DILPs are expressed or

released in a circadian manner, although there is some evidence that insulin-like peptides are regulated by the circadian system in other insects (Vafopoulou & Steel, 2002). Microarray studies of *Drosophila* heads reported circadian expression of *susi*, which is a negative regulator of Phosphatidylinositol-3 kinase (PI3K) activity, a component of the downstream insulin signaling pathway (Claridge-Chang et al., 2001; M. J. McDonald & Rosbash, 2001; Ueda et al., 2002; Wittwer et al., 2005). Interestingly, *slob* mutants have altered insulin signaling, however it is unclear whether SLOB cycling regulates the rhythmic release of insulin (Sheldon et al., 2011). Currently, the tools needed to measure rhythmic release of insulin in flies are not available. Circadian regulation of the fly endocrine system remains an important area of future investigation.

Regulation of Behavior via the Insulin-Signaling Pathway

This section was part of a published review (Erion & Sehgal, 2013a). Regulation of behavior via the insulin-signaling pathway is graphically summarized in Figure 1-2.

Sexually Dimorphic Locomotor Behavior

Although the IPCs do not directly regulate locomotor rhythms, they have been implicated in other aspects of locomotor behavior. Compared to females, the activity pattern of males is more consolidated, as indicated by fewer activity/inactivity periods (or start/stop bouts) over the same total distance traveled (Belgacem & Martin, 2002; Gatti, Ferveur, & Martin, 2000). Restricted expression of the sex-determination transcription factor, *transformer* (tra), identified a subset of neurons in the PI, henceforth called feminizing cells (FCs), that are capable of feminizing the locomotor activity pattern of male flies (Gatti et al., 2000). The axonal projections of both FCs and IPCs terminate at the corpus cardiacum/corpus allatum (cc/ca) (Gatti et al., 2000; Ikeya et al., 2002). This

gland synthesizes juvenile hormone (JH) which regulates many important processes in insects including metamorphosis, reproduction and aging (Tu, Yin, & Tatar, 2005).

JH and insulin have both been implicated in the sexual dimorphism of locomotor behavior. Feeding male flies an inhibitor of 3-Hydroxy-3-Methylgluaryl CoA Reductase (HMGCR), a key JH biosynthesis enzyme, feminizes their locomotor activity (Belgacem & Martin, 2002). HMGCR mutants and targeted reduction of HMGCR in the ca both abolish sexual dimorphism of locomotor behavior. Male mutants of *takeout*, a putative JH binding protein and known circadian output gene, also display feminization of their locomotor activity (Meunier, Belgacem, & Martin, 2007). Similarly, sexual dimorphism of locomotor activity is eliminated in insulin receptor mutants and flies with ablated IPCs. Interestingly, the insulin receptor is expressed in the ca and insulin receptor mutants have altered JH levels suggesting that there is a link between the insulin and JH pathways (Belgacem & Martin, 2006; M Tatar et al., 2001). Furthermore, targeted reduction of the insulin receptor in the ca suppresses HMGCR expression in this tissue (Belgacem & Martin, 2007). Thus it appears that insulin, JH, and *takeout* may interact to regulate sexual dimorphism of locomotor behavior in flies; however their exact relationship remains unclear.

Feeding Behavior

Insulin-like peptides have also been shown to play an important role at several stages of feeding behavior in *Drosophila* (Itskov & Ribeiro, 2013). The stages of feeding begins with the motivation to eat, followed by a search for a suitable food source, which can be influenced by smell and taste among other factors and ends upon reaching a satiated state. Maintaining adequate energy stores is critical for animal survival and reproduction. As a result, neuropeptides have evolved to modulate feeding behavior in

response to an animal's internal physiological state as well as changes in food availability in the environment.

Hunger or deprivation due to limited food availability provokes animals to acquire food. Initiation of motivated foraging is crucial for survival so animals have developed mechanisms to ensure that this response occurs under appropriate conditions. The insulin signaling pathway links the fly's internal metabolic state with the initiation of feeding behavior. In larval IPCs, constitutive activation of a downstream effector of the InR, p70/S6 kinase (dS6K), reduces foraging motivation and food acquisition (Oldham & Hafen, 2003; Q. Wu, Zhang, Xu, & Shen, 2005). This attenuated feeding response may be mediated by increased ILP release since pan-neuronal overexpression of *ilp2* or *ilp4* also reduced motivated foraging (Q. Wu, Zhang, et al., 2005). Overall these data indicate that hunger normally downregulates S6K activity in the IPCs to reduce insulin release and drive deprived animals to search for and acquire food. Starvation-induced food acquisition is also modulated by insulin signaling in mushroom body neurons known as Kenyon cells (Zhao & Campos, 2012). Inhibition of insulin signaling in Kenyon cells throughout development reduces food intake following starvation. Interestingly, temporary suppression of synaptic transmission by these neurons also partially reduces food intake. Thus insulin signaling in Kenyon cells during development may modulate the synaptic activity of these neurons to ultimately regulate food acquisition.

Feeding and sleep are mutually exclusive behaviors that occur based on the integration of hunger, sleep drive and environmental cues (D. M. McDonald & Keene, 2010). The insect neurotransmitter octopamine promotes wake in flies by signaling through the IPCs. Flies with mutant octopamine receptors (*oamb* mutants) have an increased drive to sleep compared to wildtype animals (Crocker, Shahidullah, Levitan, & Sehgal, 2010). We wondered how *oamb* mutants would react to starvation given their

high drive for sleep. Would they choose to forgo food for sleep, or forgo sleep to eat? In this dissertation I will address the role of octopamine and the IPCs in the balance between food intake and sleep.

After assuming motivated foraging, animals must utilize their sense of smell in order to find an adequate food source. Starvation decreases the amount of time a fly takes to find food by heightening their sense of smell, or in more technical terms, by enhancing odor representation in neurons that process olfactory input, known as odorant receptor neurons (ORNs) (Root, Ko, Jafari, & Wang, 2011). Enhancement of odor representation is facilitated by the expression of short neuropeptide F (sNPF) and its receptor (sNPFR) in specific ORNs (Carlsson, Diesner, Schachtner, & Nässel, 2010; Root et al., 2011). sNPFR is related to the mammalian NPY receptor; however, NPY is actually not closely related to sNPF but is more closely related to neuropeptide F (NPF) (Nässel & Wegener, 2011). Regardless, sNPF does function similarly to NPY in that both are known to promote feeding behavior (Kageyama et al., 2012; K.-S. Lee, You, Choo, Han, & Yu, 2004). Intriguingly, Root and colleagues found that insulin interacts with the sNPF pathway by acting as a satiety signal to decrease sNPFR expression in ORNs and in turn decrease motivated feeding (Root et al., 2011). Hence during starvation when insulin signaling is low, sNPFR levels are high to mediate increased ORN sensitivity to odors and to encourage food acquisition.

The gustatory system also plays a role in feeding behavior primarily by influencing food choice. When determining what to eat, animals must assess both the nutritional content and palatability of a food source. A hungry fly initially decides what to eat based on taste; choosing the sweeter option over the more calorie dense option. However over time, this preference shifts towards the more calorie rich option (Stafford, Lynd, Jung, & Gordon, 2012). This change in preference is at least partially mediated by

a decrease in insulin. *Ilp2* and *ilp3* mutants and decreased insulin signaling throughout the brain all show an increased initial preference for the more caloric food source instead of the sweeter option (Stafford et al., 2012). This suggests that insulin contributes to calorie sensing and food source preference by acting directly on neurons in feeding circuits.

In the absence of a preferred food source, animals will feed on less palatable food sources. Drosophila larvae prefer to feed on rich liquid food as opposed to solid food. This preference is partially mediated by insulin signaling in cells that promote food intake through signaling downstream of the NPF receptor, which is distinct from the sNPF receptor but is also related to the NPY receptor. Downregulating insulin signaling in NPFR neurons causes fed larvae to be hungry and also increases their consumption of the less preferred solid food (Q. Wu, Zhao, et al., 2005). Conversely, upregulation of the insulin pathway in NPFR neurons of deprived animals elicits attenuated feeding of solid food (Q. Wu, Zhao, et al., 2005).

In states of starvation, animals adapt by undertaking risky behaviors including searching for food under less than optimal conditions and consuming normally aversive noxious food. Under unfavorable conditions like cold temperature, the NPF/IIS signaling cascade enables starving *Drosophila* larvae to adapt and continue searching for food in order to survive (Lingo et al., 2007). This pathway also regulates risk-sensitive food acquisition with respect to noxious or bitter compounds. Overexpression of NPFR as well as down regulation of insulin signaling in NPFR neurons both increase consumption of noxious or bitter compounds in nondeprived larvae (Q. Wu, Zhao, et al., 2005). These results indicate that the NPF and ILP signaling mediate foraging responses under adverse conditions presumably to promote survival. In this dissertation, I identify a novel

role for NPF in the circadian system related to the expression of an enzyme involved in xenobiotic detoxification.

After determining what to eat, animals must also determine how much they will eat. Under very poor nutrient conditions flies will compensate by eating more. Interestingly this change in feeding behavior does not occur in IPC ablated flies (S. J. Broughton et al., 2010). Similarly, inhibition of IPCs also results in attenuation of food intake under poor nutrient conditions (Cognigni, Bailey, & Miguel-Aliaga, 2011). However, given that IPC ablation or inhibition presumably affects the expression levels of ILPs as well as other peptides expressed in these cells, alterations in feeding behavior may not simply be due to changes in insulin signaling. In addition to ILPs, the IPCs also express the cholecystokinin-like peptide, drosulfakinin (DSK) (Park, Veenstra, Park, & Taghert, 2008; Söderberg, Carlsson, & Nässel, 2012). IPC specific reduction of *dsk* increases food consumption and alters the ability of the fly to discriminate between food choices. However, this effect is not independent of insulin because reduction of *dsk* also alters *ilp* transcript levels (Söderberg et al., 2012). Nonetheless, DSK likely modulates the amount of food a fly consumes by acting in conjunction with DILPs to convey fullness to the animal.

In addition to what to eat and how much to eat, flies have a preference for when to eat. Previous work done in our lab found that flies have a daily feeding rhythm; under LD conditions, flies eat during the day, primarily in the morning, and this rhythmic pattern persists in DD (Xu, Zheng, & Sehgal, 2008). The authors determined that this daily rhythm in fly feeding is controlled by the circadian clock and light. Clock control was demonstrated by the absence of feeding rhythms in clock mutants in DD. Regulation of rhythmic feeding by light was indicated by the presence of a feeding rhythm, albeit with peak feeding shifted to the late afternoon, in the clock mutant Clk^{jrk} under LD conditions

(Xu et al., 2008). Interestingly, it appears multiple clocks contribute to the fly feeding rhythm including a clock located in a peripheral metabolic tissue.

Regulation of Peripheral Clocks

Circadian rhythms are regulated by a multi-clock system comprised of clocks in both the brain and in peripheral tissues. Circadian clocks in the brain drive rhythmic behavior while peripheral clocks generally drive rhythmic outputs associated with the function of the tissue in which they are located (Tomioka, Uryu, Kamae, Umezaki, & Yoshii, 2012). Peripheral clocks act in a coordinated manner to generate coherent outputs and establish homeostasis. The mechanisms that ensure this coordination are largely unknown. In mammals, neuronal clocks play an important role in maintaining stable phase relationships within and between clocks located in peripheral tissues (Dibner, Schibler, & Albrecht, 2010). The central clock, or suprachiasmatic nucleus (SCN), does this indirectly through regulation of behavioral feeding:fasting cycles, which lead to cycles in nutrient availability, or directly through autonomic innervation and/or humoral signals such as glucocorticoids (Maury, Ramsey, & Bass, 2010). There are likely additional unidentified molecules and mechanisms involved in synchronizing individual clocks with each other. The genetic tractability and relative simplicity of *Drosophila* provides a promising tool for identifying new molecules and mechanisms important in this process.

In *Drosophila,* clocks have been identified in numerous peripheral tissues including the compound eye, gustatory and olfactory sensilla, malpighian tubules, prothoracic gland, and reproductive system (Beaver et al., 2002; Chatterjee, Tanoue, Houl, & Hardin, 2010; Hege, Stanewsky, Hall, & Giebultowicz, 1997; Ito, Goto, Shiga,

Tomioka, & Numata, 2008; Myers, Yu, & Sehgal, 2003; Tanoue, Krishnan, Krishnan, Dryer, & Hardin, 2004). Some organs can maintain circadian rhythmicity in the absence of the central clock located in the brain and are therefore referred to as autonomous clocks (Plautz, Kaneko, Hall, & Kay, 1997). These organs possess a complete entrainment mechanism in addition to the core clock components. There are however peripheral clocks that depend on the central clock. For example, the clock in the prothorasic gland regulates rhythms in eclosion, the emergence of the adult fly from its pupal case. Ablating the LNvs abolishes rhythmic expression of core clock genes in the prothorasic gland resulting in arrhythmic eclosion (Myers et al., 2003).

The Fat Body Clock

Fat body (FB) tissue is analogous to mammalian liver and adipose tissue in that it functions as a major site of energy storage and utilization in the fly (Figure 1-3) (Arrese & Soulages, 2010). In addition to its role in energy homeostasis, the FB plays important roles in immunity and detoxification (Ferrandon, Imler, Hetru, & Hoffmann, 2007; Misra, Horner, Lam, & Thummel, 2011). Like other peripheral tissues in *Drosophila*, the FB expresses core clock genes and possesses a functional clock that controls tissue-specific cyclic gene expression (Xu et al., 2008). The clock drives rhythmic expression of 137 genes involved in carbohydrate and lipid metabolism, immune defense, xenobiotic detoxification and other FB functions. Disruption of the FB clock abolishes oscillatory expression of the majority of circadian FB genes; however 56 out of 137 genes continue to cycle (Xu, DiAngelo, Hughes, Hogenesch, & Sehgal, 2011). One aspect of my thesis work was to determine what drives the rhythmic expression of these genes.

Disrupting the FB clock shifts the phase of the feeding rhythm but does not eliminate it suggesting the contribution of other unidentified clocks. Disruption of the FB

clock also results in increased sensitivity to starvation and decreased glycogen and lipid stores in FB tissue (Xu et al., 2008). Interestingly, disruption of neuronal clocks has the opposite effect; these flies display increased resistance to starvation and increased glycogen and lipid stores. Concurrent disruption of both the neuronal and fat body clocks results in normal glycogen and lipid levels and starvation response (Xu et al., 2008). This indicates clocks in the brain and fat body interact and that such interactions are important for regulating fly metabolism. Together these data suggest that the fat body may receive circadian input from neuronal clocks. As part of my thesis work I sought to dissect circadian-related communication between neuronal clocks and the fat body in order to better understand how the circadian system influences metabolic homeostasis.

Intersection of Sleep and Metabolism in *Drosophila*

In addition to probing interactions between the circadian system and metabolism, I wanted to investigate the interaction between metabolism and another important behavioral system, sleep. Timing of sleep, sleep duration and sleep quality are determined by the circadian clock and sleep homeostat. The opportunity to study the interaction between sleep and metabolism arose fortuitously through the discovery that octopamine, the insect equivalent of norepinephrine, promotes wakefulness in the fly by binding to octopamine receptors on the cell membranes of IPCs (Crocker & Sehgal, 2008; Crocker et al., 2010). Given that the major output of the IPCs is ILPs, I asked whether octopamine signaling in the IPCs promotes wakefulness by modulating the insulin-signaling pathway. I also wanted to evaluate whether octopamine signaling affects metabolism by modulating energy stores in the fat body. Our work highlights the

usefulness of *Drosophila* to study complex interactions between metabolism and physiological systems, like circadian rhythms and sleep.

Figures

Figure 1-1

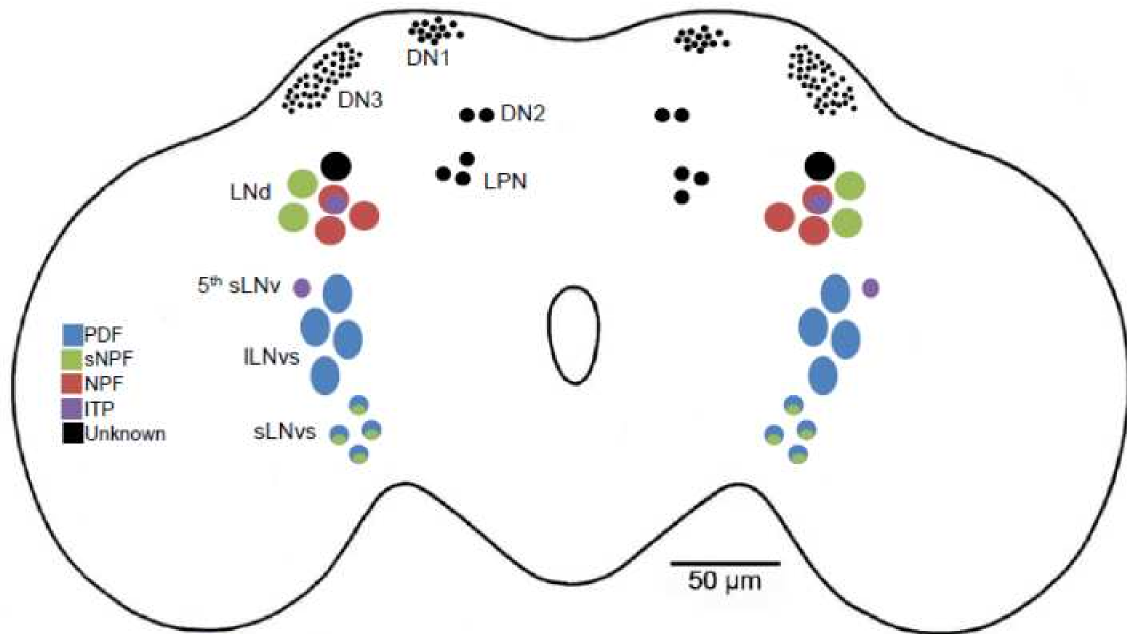


Figure 1-1. Circadian neurons in the *Drosophila* brain express a variety of peptides.

The roughly 150 circadian neurons in the adult fly brain are defined by their location and function. This schematic depicts the known expression of various peptides in subsets of circadian neurons. Abbreviations- small and large ventrolateral neurons (sLNvs and lLNvs respectively), dorsal lateral neurons (LN_ds), lateral posterior neurons (LPN), and three groups of dorsal neurons (DN1, 2 and 3), pigment dispersing factor (PDF), short Neuropeptide F (sNPF), Neuropeptide F (NPF), and ion transport peptide (ITP). This figure was adapted from the following review in Journal of Neuroscience-"The circadian system: plasticity at many levels." (Muraro, Pírez, & Ceriani, 2013)

Figure 1-2

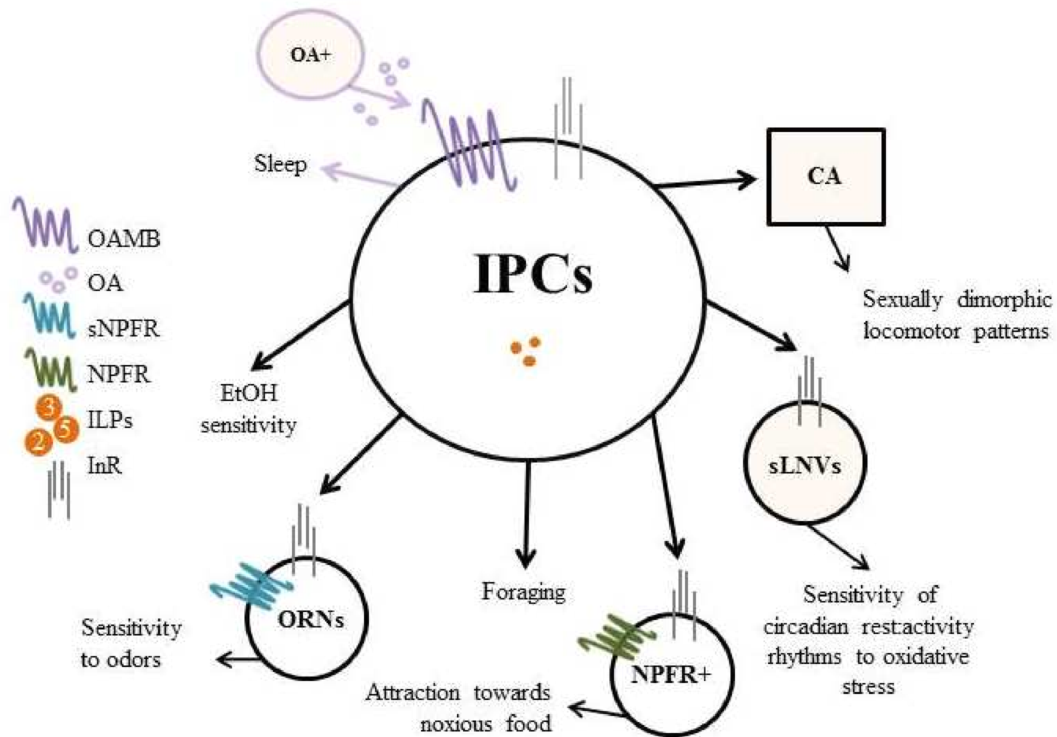


Figure 1-2. Regulation of behavior by the Insulin-Producing Cells (IPCs) in *Drosophila*.

The IPCs modulate feeding and locomotor (shaded) behavior through the insulin pathway. Decreased insulin production/release from the brain IPCs results in decreased downstream insulin signaling and leads to increased ethanol (EtOH) sensitivity and motivated foraging. Decreased insulin signaling in sNPFR (short Neuropeptide F Receptor) expressing odor receptor neurons (ORNs) and in NPFR (Neuropeptide F Receptor) expressing neurons, increases the sensitivity of ORNs to odors and increases the attraction of flies towards normally aversive or noxious food sources, respectively. With respect to locomotion, octopaminergic neurons signal through the IPCs to promote wake; however, this effect is independent of insulin signaling. Insulin signaling in the corpus allatum (CA), a non-neuronal endocrine gland (indicated by square), drives sexual dimorphism of locomotor patterns. Lastly, insulin signaling in the circadian small ventrolateral neurons (sLNVs) modifies the sensitivity of rest:activity rhythms to oxidative stress. (Erion & Sehgal, 2013b)

Figure 1-3

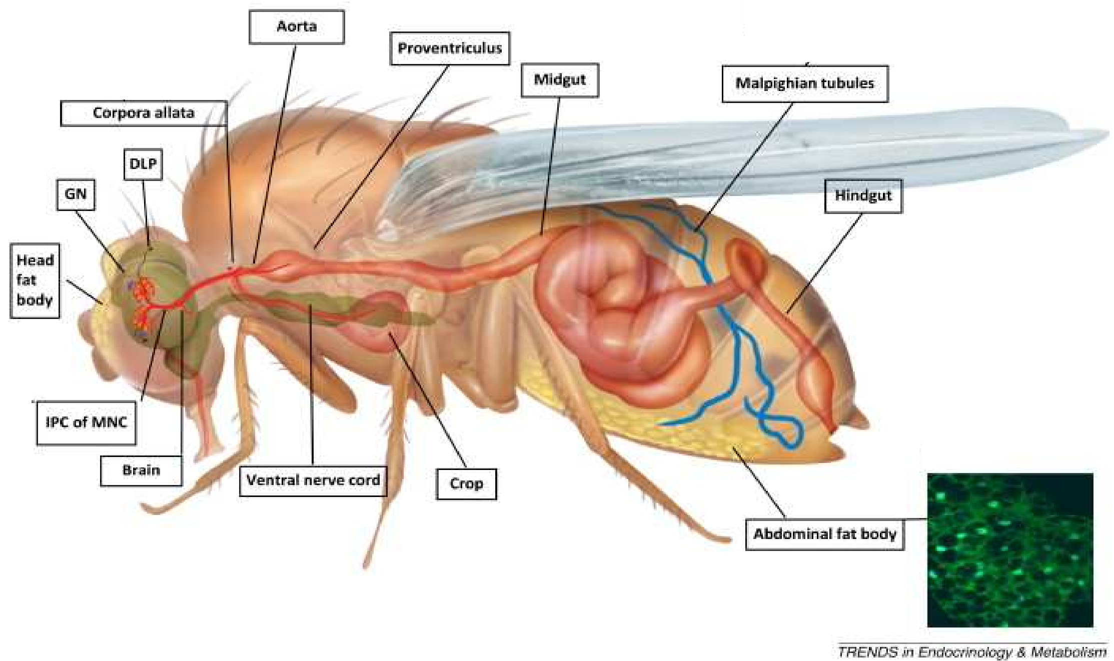


Figure 1-3 Anatomy of Adult Fly

Shown here is the localization of the abdominal fat body with respect to the brain and other internal organs. Abbreviations: DLP, Dorsal Lateral Peptidergic Neurons; GN, GABA secretory neurons; IPC, insulin-producing cells. This figure was adapted in accordance of journal copyright laws from the following review: Tatar *et al*. “Nutrient Control of Drosophila longevity.” *Trends Endocrinol Metab*. (2014). (Marc Tatar, Post, & Yu, 2014)

CHAPTER 2: Neuronal clocks and peptidergic signaling regulate circadian gene expression in a peripheral metabolic tissue

Abstract

In *Drosophila melanogaster*, disrupting the molecular clock in the brain or fat body, a peripheral metabolic tissue, leads to metabolic dysfunction, suggesting that metabolic homeostasis requires coordination between these clocks. Input of other rhythmic cues to the fat body is also indicated by the finding that some cyclically expressed fat body transcripts do not depend on the fat body clock for rhythmic expression. We show here that free-running oscillations of the molecular clock in the fat body require an intact molecular clock in the PDF-positive central clock cells in the fly brain. We also investigated the cycling of fat body clock-independent cycling transcripts and find that they do not oscillate in mutants lacking functional clocks in all tissues, implying that rhythmic expression of these transcripts is due to circadian input from other clocks. However, rhythmic expression of one such fat body clock-independent gene, *cyp4d21* or *sex-specific enzyme 1* (*sxe1*), is not controlled by PDF cells, but by clocks located in neurons expressing neuropeptide F (NPF). Abolishing NPF signaling also eliminates rhythmic *sxe1* expression suggesting that NPF can act as a circadian signal from the brain to drive circadian expression of metabolic transcripts in the fat body. These data indicate the importance of neuronal clocks in maintaining circadian gene expression in the periphery and identify a new role for NPF in the circadian system.

Introduction

Circadian clocks constitute an endogenous timekeeping system that synchronizes daily variations in behavior and physiology with changes in the physical environment, such as day and night, imposed by the 24 hour rotation of the Earth (Zheng & Sehgal, 2012). A cooperative network of tissue-specific circadian clocks ensures opposing biochemical and behavioral processes are temporally coordinated and compartmentalized (Wijnen & Young, 2006). Coherent circadian systems preserve organismal physiology and disruption of these systems is associated with deleterious health consequences including cancer, cardiovascular disease and metabolic syndrome (Marcheva et al., 2010, 2013; Turek et al., 2005).

Drosophila melanogaster is a well-established model of circadian rhythms and recently has become a more widely recognized tool for the study of energy metabolism (Ravi Allada & Chung, 2010; Padmanabha & Baker, 2014). In *Drosophila*, the core molecular oscillator consists of two transcription factors, CLOCK (CLK) and CYCLE (CYC), which heterodimerize to drive the expression of the genes *period* (per) and *timeless* (*tim*) (Zheng & Sehgal, 2012). During the late night, PER and TIM proteins translocate into the nucleus to inhibit the activity of CLK/CYC thereby repressing their own expression. Eventually PER and TIM proteins undergo degradation, relieving inhibition of CLK/CYC and allowing the cycle of transcription to begin again. This transcriptional-translational feedback loop generates 24 hour rhythms in *per* and *tim* transcript levels, as well as the transcript levels of other clock-controlled genes (Zheng & Sehgal, 2012). The neuronal clock network in the fly brain is comprised of roughly 150 circadian neurons, which are divided into clusters based on their function and location in the brain (Ravi Allada & Chung, 2010). The lateral neuron clusters include the small and large ventral lateral neurons (LNvs), the lateral posterior neurons (LPNs), and the lateral

dorsal neurons (LN_ds). The dorsal neuron clusters are divided into three groups, dorsal neuron (DN) 1, 2, and 3. The small LNvs (sLNvs) are referred to as the central clock because they are necessary and sufficient for behavioral rhythms in rest:activity in the absence of external cues (Grima et al., 2004; Stoleru et al., 2004). The sLNvs produce the neuropeptide pigment dispersing factor (PDF), which synchronizes circadian neurons in the fly brain and regulates rest:activity rhythms (Lin et al., 2004; Renn et al., 1999; Stoleru et al., 2005; Yoshii et al., 2009). The central clock and PDF are also implicated in the function of circadian clocks in some peripheral tissues (Krupp et al., 2013b; Myers et al., 2003).

The peripheral clock located in the fat body, a tissue analogous to mammalian liver and adipose tissue, regulates feeding behavior and nutrient storage and drives the rhythmic expression of genes involved in metabolism, detoxification, innate immunity, and reproduction (Arrese & Soulages, 2010; Xu et al., 2011, 2008). The fat body clock regulates oscillations in the levels of most, but not all, circadian transcripts in the fat body. Roughly 40% of rhythmically-expressed fat body transcripts continue to be expressed in a circadian manner following the disruption of the fat body clock (Xu et al., 2011). The clock or clocks responsible for driving rhythmic expression of these genes have not been identified. One hypothesis is that clocks located in the brain release circadian signals to regulate the expression of these genes. Abolishing molecular clocks in the brain alters metabolic homeostasis causing increased glycogen stores in the fat body. Neuronal clock regulation of rhythmic gene expression in the fat body could potentially mediate its control of metabolic homeostasis. Interestingly, abolishing the molecular clock in the fat body has the opposite effect on metabolic homeostasis in that this manipulation reduces glycogen stores in the fat body. Furthermore, abolishing the clock in both tissues concurrently causes no metabolic phenotypes, highlighting the

importance of interactions between the clocks in the brain and fat body to maintain metabolic homeostasis (Xu et al., 2008).

Neuropeptide F (NPF) is implicated in a variety of behavioral and physiological processes in *Drosophila* including feeding, courtship, and aggression (Dierick & Greenspan, 2007; C He et al., 2013; Lingo et al., 2007; Q. Wu, Zhao, et al., 2005). NPF is expressed in neurons in the brain and endocrine cells in the midgut, although the role of NPF in the midgut is not understood (Brown et al., 1999; Nässel & Wegener, 2011). In the brains of male flies, NPF is expressed in a subset of circadian neurons; however only recently has there been any evidence of a functional role for NPF in the circadian system (G. Lee et al., 2006). Ablation of NPF-positive neurons slightly lengthens the free-running circadian period and knockdown of *npf* or the *npf receptor* (*npfr*) modulates rest:activity rhythms under light:dark conditions (C He et al., 2013; Hermann et al., 2012). NPF is related to the vertebrate Neuropeptide Y (NPY), which has been associated with circadian rhythms as a mediator of non-photic circadian entrainment (Besing et al., 2012; Maywood, Okamura, & Hastings, 2002; Yannielli & Harrington, 2004). Mutant mice lacking NPY display rhythmic behavior, but do not adapt to phase shifts as efficiently as controls (Harrington et al., 2007). Although both NPY and NPF appear to play only minor roles in regulating rhythmic locomotor behavior, it is not known whether these peptides play important roles in other aspects of circadian rhythms, such as circadian control of energy homeostasis.

In this study, we used *Drosophila* to probe neuronal clock control of circadian gene expression in a peripheral metabolic tissue. We found that the fat body clock requires the central clock neurons, or sLNvs, to function in constant darkness. Interestingly, a different circadian neuron cluster, the LN_ds, is important for driving rhythmic expression of fat body clock-independent transcripts. Lastly, we identified a novel circadian role for NPF in the circadian system.

Methods

Fly Genetics

The following strains were used: Iso31 (isogenic *w1118* stock; (Ryder et al., 2004)), Pdf-Gal4, Npf-Gal4 (Bloomington #25681), 911-Gal4 (InSITE Library (del Valle Rodríguez, Didiano, & Desplan, 2012)), Dilp2-Gal4 (Rulifson et al., 2002), UAS-CLKΔ (Bloomington #36318), UAS-CYCΔ (Bloomington #36317), Clk^{jrk} (R Allada, White, So, Hall, & Rosbash, 1998), NPFR mutant (Bloomington #10747)

Adult Fat Body Collection

Flies (roughly 5-7 days old) were entrained for at least 3 days in 12 hour light: 12 hour dark cycles (LD) at 25℃ before they were harvested . The abdominal fat body was obtained by dissecting the fly abdomen to remove all internal organs; leaving the fat body attached to the cuticle, to be collected on dry ice for RNA extraction.

Mice Husbandry and Liver Collection

NPY knockout mice were obtained from The Jackson Laboratory (004545) along with their background strain for controls (002448). Genotyping primers listed on the Jackson website were used. Primers for cyp2b10 were as follows: F 5' GAC TTT GGG ATG GGA AAG AG3' R 5' CCA AAC ACA ATG GAG CAG AT3'. At either ZT10 or ZT22, livers from NPY knockouts and their background controls were collected and immediately frozen in liquid nitrogen. Kits and procedures to isolate RNA and cDNA are the same as described below for fly fat bodies.

Real-Time Quantitative PCR

For each time point, fat bodies from 12 male flies were collected for RNA preparation. Total RNA was extracted using Trizol reagent (Life Technologies, Grand Island, NY) according to manufacturer's protocol. All RNA samples were treated with RNase-free DNase (Qiagen). RNA was reversed transcribed to generate cDNA using a High Capacity cDNA Reverse Transcription kit (Life Technologies, Grand Island, NY). Quantitative PCR was performed on a 7900HT Fast-Real-Time PCR (ABI) using Sybr Green (Life Technologies, Grand Island, NY). The following primer sequences were used for qpcr: *alpha tubulin* (Forward 5' CGTCTGGACCACAAGTTCGA 3' and reverse 5' CCTCCATACCCTCACCAACGT 3'), *per* (Forward 5' CGTCAATCCATGGTCCCG 3' and reverse 5' CCTGAAAGACGCGATGGTG 3'), *cyp4d21/sxe1* (Forward 5' CTCCTTTGGTTTATCGCCGTT and reverse 5' TTATCAGCGGCTTGTAGGTGC), *sxe2* (Forward 5' TGCGGTACGATCTTTATACGCC 3' and reverse 5' CTAACTGGCCATTTCGGATTGA 3'). The level of alpha tubulin mRNA was used as a control for the total RNA content in each sample. The values of transcript levels for other RNAs were normalized to those of alpha tubulin. To analyze *cyp2b10* levels in mouse livers the following primer sequences were used for qpcr: *cyp2b10* (Forward 5' GACTTTGGGATGGGAAAGAG 3' and reverse 5' CCAAACACAATGGAGCAGAT 3') beta actin

Results

The Central Clock Regulates the Fat Body Clock in Constant Darkness

To investigate neuronal clock regulation of circadian gene expression in the abdominal fat body, we first sought to determine whether the PDF-positive central clock neurons are necessary for fat body clock function. Central clock control of peripheral clocks is not unprecedented in the fly. For example, the central clock is required for rhythmic expression of clock components in the prothoracic gland (PG), a peripheral

tissue that gates rhythmic eclosion (Myers et al., 2003). Expressing a pro-apoptotic gene, *head-involution defective* (*hid*), in the LNvs eliminates oscillations of TIM protein levels in the PG, causing eclosion to become arrhythmic (Myers et al., 2003). Rather than ablate the central clock cells by expressing *hid*, we sought to disrupt only the core molecular oscillator so that all non-circadian functions of these cells would remain intact. We expressed a dominant-negative version of CLK transcription factor (CLKΔ) specifically in the LNvs using the GAL4/UAS expression system. Accordingly, UAS-CLKΔ was expressed under control of *Pdf*-GAL4. CLKΔ lacks regions of the CLK DNA-binding domain which prevents CLK from binding DNA and activating gene transcription, while the protein interaction domain is left unaltered to allow CLK to heterodimerize with CYC (Tanoue et al., 2004). To confirm that expression of CLKΔ in the LNvs effectively abolished the LNv molecular clock, we evaluated a well-established circadian output regulated by the central clock, namely free-running rhythms in rest:activity under constant conditions. Under 12:12hr light:dark conditions (LD) the flies are rhythmic due to the masking effects of light; however as we expected, under conditions of constant darkness (DD), the vast majority of male flies became completely arrhythmic, verifying the ability of CLKΔ to disrupt the molecular clock and downstream behavior (data not shown).

To assess functionality of the core molecular oscillator in abdominal fat body tissue we measured *per* transcript levels in dissected fat bodies over the course of the day (Fig.2-1A). Fat body *per* rhythm was not altered in flies with disrupted central clocks (Pdf-Gal4>UAS- CLKΔ) under LD conditions (Fig.2-1B). Under this condition light may be directly driving *per* rhythms in the fat body preventing us from seeing the effects of ablating the central clock. Therefore we evaluated *per* rhythms in DD. Expression of core clock components quickly dampens in many peripheral tissues under DD conditions such that by the sixth day in constant darkness (DD6), rhythmic gene expression in the

fat body is essentially absent (Xu et al., 2011). We reasoned that on the first day in DD the fat body clock is still entrained directly to the external light/dark cues making it difficult to assess underlying regulation of the fat body clock by the central clock. Therefore we opted to test rhythmic expression of the core clock gene *per* on the second day in DD (DD2). *per* levels are rhythmic in the abdominal fat body of control flies on DD2; however, flies expressing CLKΔ in the LNvs no longer display *per* rhythms in the abdominal fat body (Fig.2-1C). This indicates that the PDF+ LNvs regulate the fat body clock in the absence of external environmental cues.

Rhythmic Expression of Fat Body Clock-Independent Transcripts Requires an Intact Molecular Clock

The fat body clock regulates roughly 60% of circadian genes in the fat body; the mechanisms responsible for driving daily expression of the other 40% of circadian genes in this tissue are unknown (Xu et al., 2011). There are several potential mechanisms that could explain gene expression oscillations in the absence of the local tissue-specific clock; i.e. light, nutrients or exogenous clocks. Many tissues in *Drosophila* have photoreceptors that enable tissues to respond to light (Plautz et al., 1997). Therefore, in addition to directly entraining clocks to the external environment, LD cycles can drive rhythmic transcription via clock-independent pathways (Wijnen, Naef, Boothroyd, Claridge-Chang, & Young, 2006). Nutrients are also known to be strong entrainment signals in peripheral tissues; restricted feeding can dissociate rhythms in peripheral clocks from clocks in the brain which remain strongly synchronized with light:dark cycles even in constant conditions (Xu et al, 2011).

First we explored the possibility that exogenous clocks in other tissues drive rhythmic expression of circadian genes that don't depend on the fat body clock. To test this hypothesis, we evaluated daily expression of these genes in *Clk* mutants under LD

conditions. These mutants, called *Clk*^{jrk}*,* lack functional clocks in all tissues due to a premature stop codon that eliminates the CLK activation domain (R Allada et al., 1998). We investigated genes whose fat body expression displayed a robust rhythm, meaning a large difference between peak and trough expression levels, in the absence of the fat body clock (Xu et al., 2011). *Clk*^{jrk} mutants did not display oscillations in transcript levels for any of the genes tested, suggesting that although these genes do not require an intact fat body clock they do require an intact clock somewhere (Fig.2-2). In addition to the loss of rhythmic expression in *Clk*^{jrk} mutants, there were differences in baseline transcript levels. Rhythmic gene expression of *sex-specific enzyme 2* (*sxe2*), a lipase, and *CG17562*, an oxidoreductase, was eliminated in *Clk*^{jrk} mutants to produce an intermediate level of gene expression throughout the day (Fig.2-2A,B). In contrast, overall expression, and therefore rhythmic expression, of *sex-specific enzyme 1* (*sxe1*), a cytochrome p450 important for detoxification, and *CG14934*, a purported glucosidase involved in glycogen breakdown, was abolished in *Clk*^{jrk} mutants (Fig.2-2C,D). These data suggest there may also be a non-circadian role for CLK in the baseline expression of a subset of genes.

Clocks in NPF-Positive LN$_d$s Drive Oscillations in sxe1 Expression

Although we have established that rhythmic expression of fat body clock-independent genes requires circadian clocks we do not know from which tissue(s) the circadian control of these genes originates. Our initial discovery that central clock neurons regulate the fat body clock in constant darkness led us to hypothesize that the central clock may also regulate fat body clock-independent genes. We chose to focus on the regulation of *sxe1* because it is the most robust cycler of all of the rhythmic fat body clock-independent genes. *Sxe1* was named on the basis of its function in the sex determination pathway in fly heads, and it is enriched in the non-neuronal fat body tissue

of males (Fujii & Amrein, 2002). Early microarray studies looking for cycling transcripts in *Drosophila* heads indicated that *sxe1* is regulated by the circadian system (Ceriani et al., 2002; Claridge-Chang et al., 2001; M. J. McDonald & Rosbash, 2001). *Sxe1* is a member of the cytochrome P450 family involved in xenobiotic detoxification and has been implicated in male courtship behavior (Fujii, Toyama, & Amrein, 2008). Thus, it may confer cyclic regulation to either or both of these processes; however, the nature of the circadian control of *sxe1* is unclear.

We evaluated rhythmic gene expression of *sxe1* in the presence of light cycles rather than under constant darkness because its rhythmic expression is abolished in Clk^{jrk} mutants in LD (Figure 2-2), suggesting that light cannot drive its cycling. We found that *sxe1* continues to cycle in flies expressing CLKΔ under *Pdf*-Gal4 suggesting the central clock neurons do not regulate rhythmic *sxe1* expression (Fig.2-3A).

Next we asked whether rhythms in *sxe1* expression require clocks in the DN1 cluster. DN1 neurons integrate internal and external cues such as PDF and light to regulate circadian behavior (Lear et al., 2009; L. Zhang et al., 2010; Y. Zhang et al., 2010). Furthermore, the DN1s have recently been identified as part of an output circuit regulating rhythmic rest:activity behavior (Cavanaugh et al., 2014). Clocks in the DN1s also mediate other behaviors including aspects of the male sex drive rhythm in *Drosophila* (Fujii & Amrein, 2010). To ablate the DN1 clock we used the 911-Gal4 driver. 911-Gal4 is expressed in two populations of neurons in the fly brain; DN1s and a group of non-clock neurons located in the dorsal lateral region of the brain (Cavanaugh et al., 2014). Expressing CLKΔ in the DN1s was lethal; however expressing the equivalent dominant negative version of CYC, CYCΔ, was not. We found that expressing CYCΔ in DN1s did not alter *sxe1* rhythms in the fat body (Fig.2-3B).

Under LD conditions, the LN_ds, in conjunction with other circadian neurons, drive the evening peak of locomotor activity (Grima et al., 2004; Stoleru et al., 2004). Their

functional significance outside of behavioral rhythms has not been established. Of the 6 LN_ds located in each brain hemisphere, some express NPF, sNPF, or ITP (ion transport peptide); however the role of these peptides in mediating LN_d circadian function is still not well understood (Muraro et al., 2013). To determine whether the LN_ds mediate *sxe1* expression we used a *Npf*-Gal4 driver which is expressed in three out of six LN_ds as well as a subset of the LNvs and some non-clock neurons in the brain (Hermann et al., 2012; G. Lee et al., 2006). NPF is also expressed in endocrine cells in the midgut, although the role of NPF in these cells is not known (Brown et al., 1999; Nässel & Wegener, 2011). To determine whether LN_ds regulate *sxe1* rhythms we expressed CLKΔ using Npf-Gal4. This manipulation abolished rhythms in *sxe1* (Fig.2-3C). Rhythmic expression of *per* in the fat body of these flies was similar to that of control flies, indicating the fat body clock had not been altered (Fig.2-3D). To ensure that this effect wasn't specific to the CLKΔ transgene we disrupted the molecular clock by expressing CYCΔ in NPF-positive cells (Fig.2-3E). This manipulation also abolished *sxe1* rhythms, confirming that lack of rhythmic expression of *sxe1* is due specifically to disruption of the molecular clock in NPF-positive cells.

NPF Receptor is required for Rhythmic Expression of sxe1

Now that we had determined *sxe1* rhythms are abolished following disruption of molecular clocks under *Npf*-Gal4, we sought to identify the signal responsible for conveying this circadian information. NPF is a likely candidate to act as a circadian signal because *sxe1* oscillations are only abolished when Npf-Gal4 is used to disrupt molecular clocks. Recently, NPF protein and RNA levels were reported to cycle suggesting NPF may be released in a circadian manner (C He et al., 2013). Because there are no *npf* mutants available, we abolished NPF signaling using a mutant lacking the sole NPF receptor in *Drosophila*, *npfr* (Garczynski, Brown, Shen, Murray, & Crim,

2002). The *npfr* mutants did not rhythmically express sxe1, phenocopying the daily *sxe1* expression profiles of flies expressing either CLKΔ or CYCΔ under Npf-Gal4 (Fig.2-4A). This suggests NPF regulates *sxe1* rhythms.

NPY Regulates Circadian Gene Expression in Mammalian Liver Tissue

We wondered whether this novel role for NPF in the regulation of circadian gene expression in a peripheral metabolic tissue in *Drosophila* might also be relevant in the mammalian system. Liver-specific circadian clocks play an important role in liver physiology via contributions to glucose homeostasis and xenobiotic clearance (Gachon, Olela, Schaad, Descombes, & Schibler, 2006; Lamia, Storch, & Weitz, 2008). Liver clock ablation in mice resembles fat body clock ablation in flies; specifically ablating liver clocks eliminates rhythmic expression of most, but not all, circadian liver transcripts (Kornmann, Schaad, Bujard, Takahashi, & Schibler, 2007). Furthermore, rescuing clock function specifically in the brains of *Clock*$^{\Delta 19}$ mutant mice restores rhythmic expression of roughly 20% of circadian liver transcripts (Hughes et al., 2012). These data suggest that some circadian transcripts are driven by systemic signals emanating from the suprachiasmatic nucleus (SCN), located in the hypothalamus (Mohawk, Green, & Takahashi, 2012). The SCN harbors the master pacemaker which regulates peripheral oscillators, so-called “slave” oscillators, to orchestrate circadian rhythms (Maury et al., 2010). The SCN has been shown to relay circadian information to liver oscillators through autonomic pathways, glucocorticoids, and body temperature (Cailotto et al., 2009; Kornmann et al., 2007; Oishi et al., 2005; Reddy et al., 2007). However little else is known about the pathways and molecules facilitating system-driven transcriptional rhythms in the liver.

We took a candidate approach to see if NPY is involved in circadian gene expression in the liver. Although there is no direct mammalian homologue of *sxe1*, we

noticed that a similar p450 enzyme involved in xenobiotic detoxification, *cypb210*, also continues to oscillate in animals lacking functional liver clocks (Kornmann et al., 2007). We tested whether *cypb210* expression was altered in livers of NPY mutant mice. We extracted RNA from the livers of 6-week-old mice at the documented times of trough and peak expression, ZT10 and ZT22 respectively (Hughes et al., 2009). We found that at the time of peak expression, ZT22, NPY mutants showed a strong trend toward reduced expression of *cypb210* compared to wildtype controls (p=0.051) (Fig.2-5). These data suggest that NPY may function in the regulation of peripheral gene expression in mammals similarly to NPF in Drosophila.

Discussion

We report here that neuronal clocks play an important role in the regulation of circadian gene expression in the fly fat body. We found that the central clock regulates fat body clock function in the absence of external cues and NPF-expressing neuronal clocks drive daily expression of genes that cycle independently of the fat body clock. Prior to this report, communication between neuronal clocks and the fat body had been suggested, but there was no direct evidence for it (Xu et al., 2008). This report identifies a specific clock and signal that mediate this communication and highlights the complexity of circadian gene regulation in a peripheral metabolic tissue.

Coherent rhythms in behavior and physiology are generated through a synchronized network of clocks in tissues throughout the body. In *Drosophila*, the relationship between peripheral clocks and the central clock varies. While most peripheral clocks operate independently, the clock in the prothoracic gland, which gates eclosion, is arrhythmic in the absence of the central clock (Myers et al., 2003). Previous studies had not addressed whether the central clock regulates the function or phase of

the fat body clock. We found that the fat body clock requires the central clock in constant darkness, as demonstrated by the lack of *per* rhythms in abdominal fat bodies of flies with disrupted central clocks in DD (Fig.2-1C). It is not clear why some clocks in *Drosophila* are regulated by extrinsic factors while others are not. The fat body clock is unlike other peripheral clocks in that it modulates behavioral rhythms, specifically the phase of feeding rhythms, in addition to fat body physiology. Rhythms in feeding lead to rhythms in nutrients that can drive rhythmic gene expression in the fat body but not in the brain (Xu et al., 2011). The fat body plays an important role in sensing nutrients and integrating nutrient signals into the circadian system in coordination with light signals and rhythmic behavior. The linked nature of the fat body and brain in the regulation of behavior and physiology necessitates additional mechanisms of communication to ensure coherent organization between these two tissues.

The local tissue-specific clock does not regulate all circadian genes in the fat body. A significant fraction of transcripts continue to cycle in the absence of the fat body clock. These transcripts are involved in most aspects of fat body physiology including carbohydrate and lipid metabolism, immune function and detoxification (Xu et al., 2011). We considered the possibility that daily expression of these genes reflect direct regulation by environmental factors such as light or nutrient availability. Wijnen et al. showed that light can regulate diurnal gene expression independently from circadian clocks (Wijnen et al., 2006). Similarly, restricted feeding paradigms can regulate the expression and phase of circadian genes in the fat body independently of the fat body clock (Xu et al., 2011). To differentiate between light-, nutrient-, or clock-driven transcripts we evaluated rhythmic gene expression in *Clk*^{jrk} mutants, which lack functional clocks. In the presence of light *Clk*^{jrk} mutants display rhythmic feeding with a delayed phase. Thus, light-driven transcripts would oscillate with the same pattern as wildtype, nutrient-driven transcripts would display a shifted phase and clock-driven

transcripts would not oscillate. Our results indicate that rhythmic expression of genes that cycle independently of the fat body clock are clock-dependent, and not driven by light or nutrients (Fig.2-2).

Metabolic homeostasis, including appropriate glycogen levels, is achieved through interactions between clocks in the brain and fat body. Ablation of neuronal and fat body clocks have opposite effects on fat body physiology (Xu et al., 2008). We wondered whether neuronal clocks influence fat body physiology independently of the fat body clock by manipulating the expression of circadian genes in the fat body. This led us to test whether neuronal clocks drive expression of fat body clock-independent circadian genes such as *sxe1*. By manipulating specific clock neurons, we found that NPF-positive clock neurons drive the rhythmic expression of *sxe1* (Fig.2-3C). Since we specifically disrupted the molecular clock by expressing CLKΔ we are presumably only affecting cells that possess circadian clock components. Therefore, non-clock NPF-positive cells should be unaffected. Although NPF expression has also been reported in the LNvs in addition to the LN_ds, it is unlikely NPF in these cells drive *sxe1* rhythms because expressing CLKΔ in the LNvs under *Pdf*-Gal4 did not abolish *sxe1* oscillations. However, we cannot formally exclude the possibility that expression of CLKΔ and CYCΔ in non-clock cells contributes to this phenotype.

We subsequently showed that NPFR mutants do not rhythmically express *sxe1* suggesting NPF conveys circadian information, through an unknown mechanism, to the fat body (Fig.2-4). The regulation of *sxe1* by NPF, which regulates feeding behavior, is consistent with the idea that regulation of feeding behavior and removal of toxic substances should be coupled. A large delay between eating toxic substances and removing them could reduce an animal’s fitness and likely cause the animal to die. The functional importance of the circadian system in the interaction between NPF and *sxe1* is unclear. NPF regulates feeding behavior but has not been implicated in driving feeding

rhythms. Likewise, *sxe1* is involved in detoxification; however a circadian role for sxe1 in detoxification has not been established. Future studies will hopefully elucidate a functional role for NPF regulation of *sxe1* oscillations.

To map the circuit driving *sxe1* expression, the relevant localization of NPFR will need to be determined. NPFR has been mapped within the adult fly brain to clock neurons in the dorsal fly brain (i.e. DN1s), the suboesophageal ganglion and neurons innervating the mushroom body (C He et al., 2013; Krashes et al., 2009). Another possibility is that NPF signals through recently identified circadian rest:activity output neurons (Cavanaugh et al., 2014). Mapping the circuitry underlying NPF regulation of *sxe1* may garner additional insight into circadian regulation of physiology.

Interestingly, not all fat body-clock-independent circadian genes are driven via NPF-positive clock neurons. For example, *sxe2* and *CG17562*, both continue to oscillate in a circadian manner when CLKΔ is expressed in NPF+ neurons (Data not shown). These results indicate that there are additional unidentified mechanisms regulating circadian rhythms in the fat body. Metabolic cells in the brain such as the insulin-producing cells may regulate the expression of some of these genes given that the fat body is a major insulin target. Multiple mechanisms may exist to drive rhythmic expression of genes with different phases or to couple gene expression to other neuronal processes.

The mammalian circadian system is organized in a hierarchical manner in which a “master” oscillator entrains “slave” oscillators in the periphery to maintain their synchrony. The *Drosophila* circadian system is not as rigidly structured, although it still maintains many similarities to the mammalian system. The conservation between the mammalian liver and *Drosophila* fat body suggested signals driving circadian gene expression in the fat body of flies may also be conserved in mice livers (Kornmann et al., 2007; Xu et al., 2011). Remarkably, this may be true for NPF regulation of peripheral

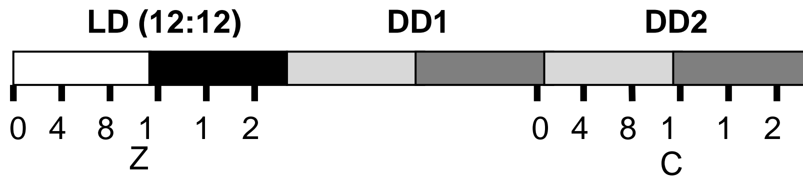
gene expression since *cyp2b10* expression is reduced in livers of NPY mutant mice (Fig.2-5). Microarray studies of NPY knockout mice livers may lead to the identification of additional NPY-regulated liver transcripts.

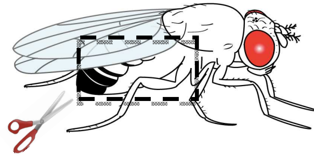
Prior to this report, NPF had largely been studied in the context of feeding behavior. Interestingly, overexpression of NPFR in NPFR neurons has been shown to increase consumption of noxious or bitter compounds in non-deprived larvae (Q. Wu, Zhao, et al., 2005). NPF mediated coordination of noxious food consumption and expression of enzymes involved in removing toxic compounds may have evolved to promote survival.

Figures

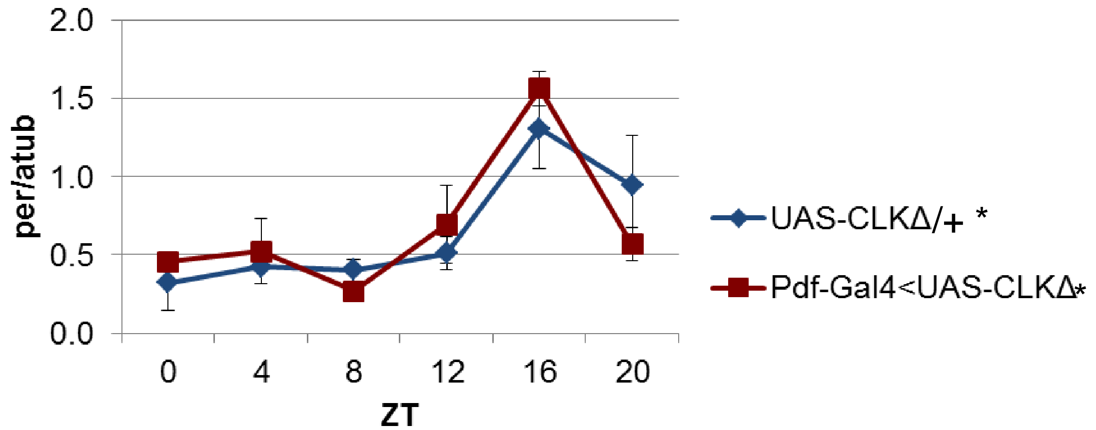
Figure 2-1

A





B



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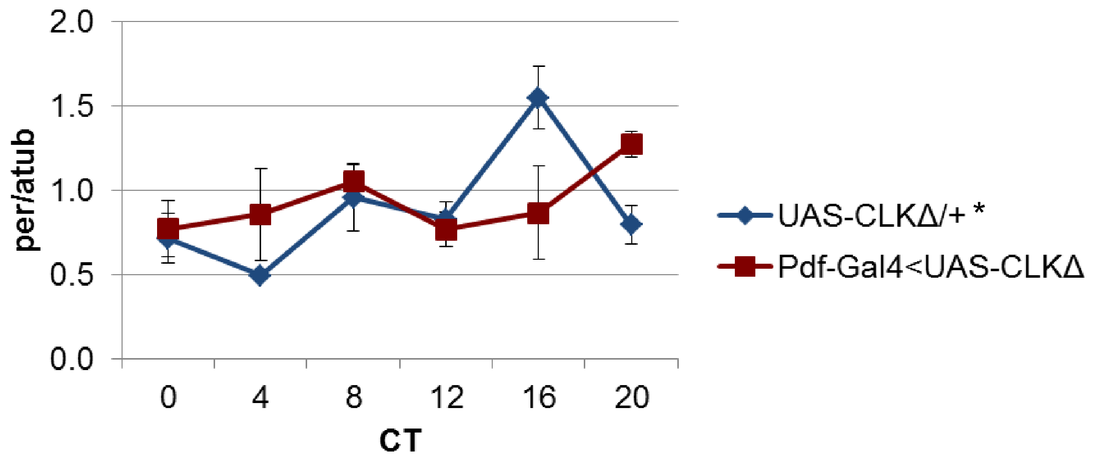


Figure 2-1. Oscillations of *per* transcript levels in the fat body over the course of the day require an intact central clock in constant darkness.

(A) Schematic of experimental design. Male flies were entrained for several days in 12 hour light: 12 hour dark cycles (LD). Flies were dissected to obtain abdominal fat bodies on either the last day in LD or on the second day of constant darkness (DD2). Graphs depict RNA levels, normalized to alpha tubulin (atub), over the course of the day in the presence of light (Zeitgeiber Time, ZT) or in constant darkness (Circadian Time, CT). Ablating the central clock (Pdf-Gal4>UAS-CLKΔ) does not affect *per* rhythms in LD (B) but abolishes *per* rhythms in DD2 compared to controls (UAS-CLKΔ/+) (C). Each experiment was repeated independently three times. Error bars denote the standard estimated mean (SEM). Significant rhythmicity was determined using JTK cycle (Hughes, Hogenesch, & Kornacker, 2010). JTK cycle p value <0.05 is indicated by asterisk (*) next to the genotype label. See table 2-1 for JTK cycle p values.

Figure 2-2

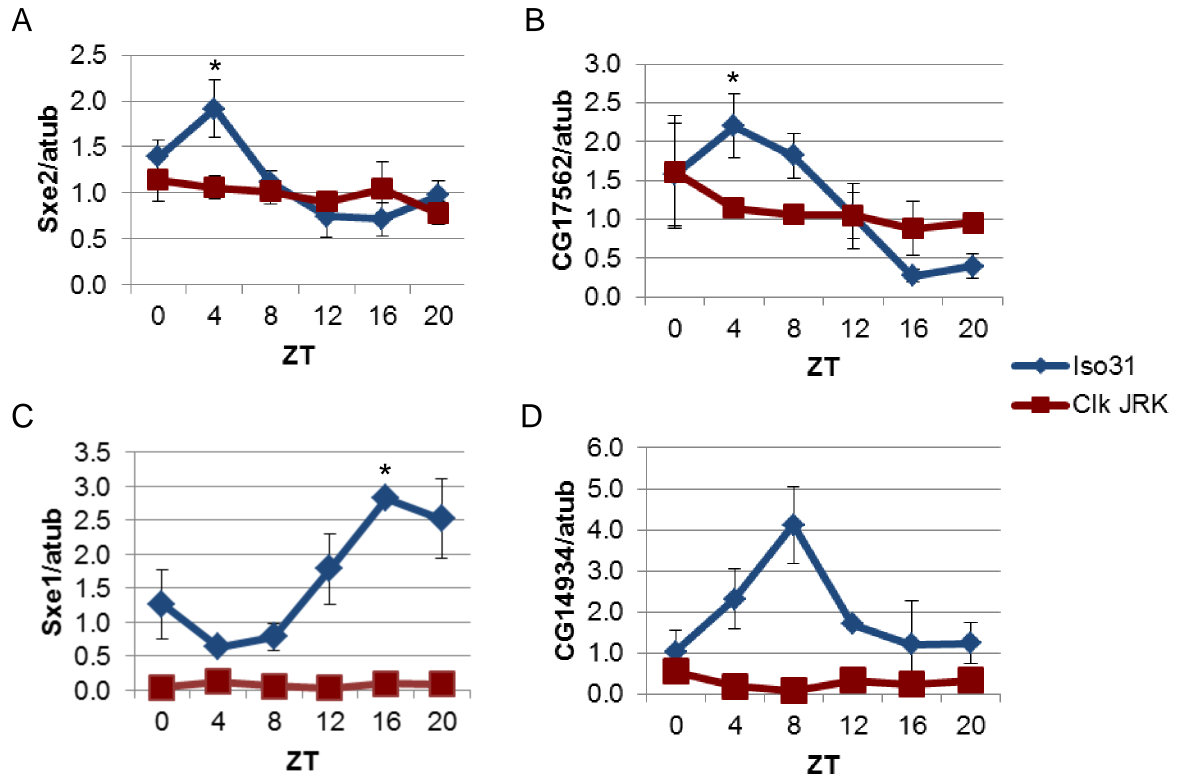
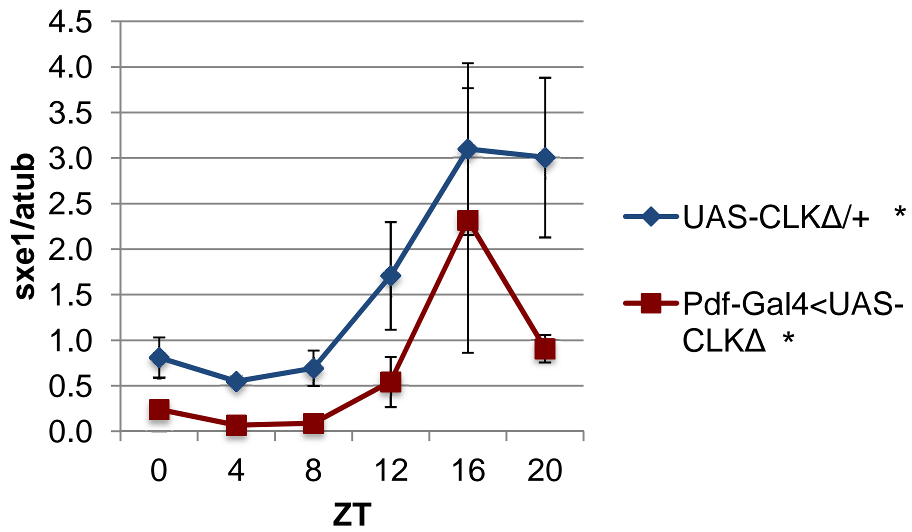


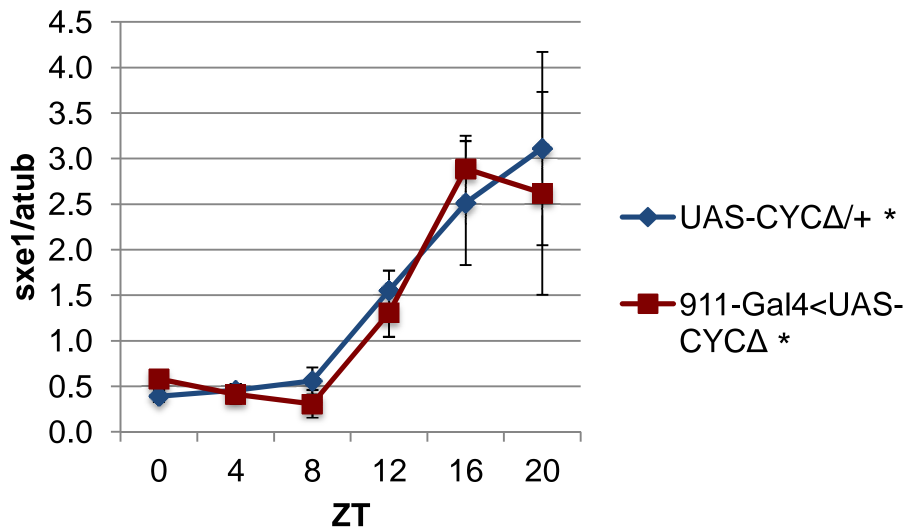
Figure 2-2. Rhythmic expression of fat body clock-independent genes requires clocks in other tissues.

Daily oscillations of several fat body clock-independent genes were tested in mutants lacking functional clocks in all tissues, Clk^{jrk} mutants, in LD. Rhythmicity of *sxe2* (A) and *CG17562* (B) was abolished in Clk^{jrk} mutants but was intact in Iso31 controls. Expression of *sxe1* (C) and *CG14934* (D) was abolished in Clk^{jrk} mutants but unaffected in Iso31 controls. All genes were normalized to alpha tubulin (atub) levels. Each experiment was repeated independently three times. Error bars denote SEM. Significant rhythmicity was determined using JTK cycle. JTK cycle p value <0.05 is indicated by asterisk at the time of peak expression. See table 2-1 for JTK cycle p values.

Figure 2-3.
A



B



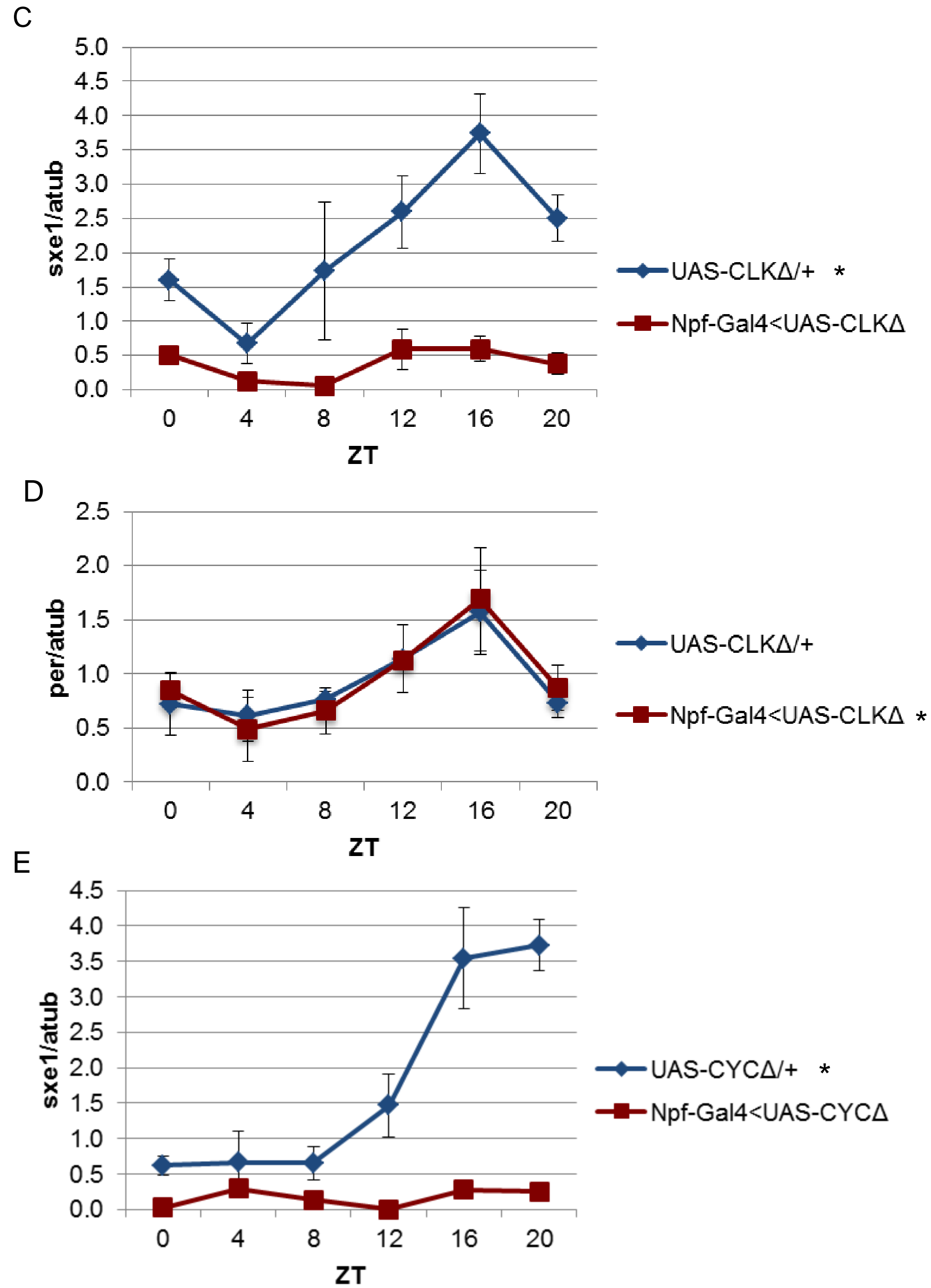


Figure 2-3. *sxe1* oscillations are regulated by NPF-expressing clock neurons.

Ablating the molecular clock by expressing CLKΔ or CYCΔ in either the LNvs (Pdf-Gal4) (A) or DN1s (911-Gal4) (B) did not eliminate rhythms in *sxe1* expression. However, expressing CLKΔ (C) or CYCΔ (E) using Npf-Gal4 abolished rhythmic *sxe1* expression. Although *sxe1* expression is independent of the fat body clock we verified that the fat body clock was still intact in flies expressing UAS- CLKΔ under Npf-Gal4 in LD (D). Each

experiment was repeated independently at least twice. Error bars denote SEM. Significant rhythmicity was determined using JTK cycle. JTK cycle p value <0.05 is indicated by asterisk (*) next to the genotype label. See table 2-1 for JTK cycle p values.

Figure 2-4

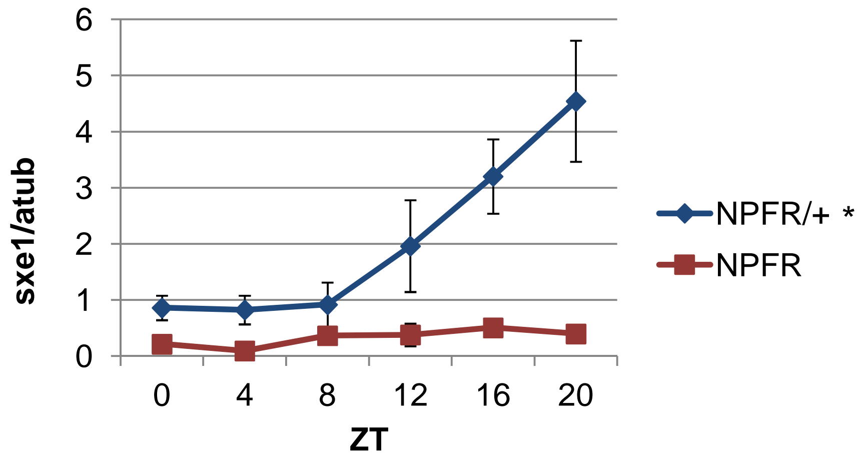


Figure 2-4. NPF is a critical circadian signal for sxe1 rhythms in the fat body.

Comparison of NPFR homozygous mutants with NPFR heterozygous mutants established that expression of *sxe1* requires the NPF receptor and suggests a role for NPF. Each experiment was repeated independently three times. Error bars denote SEM. Significant rhythmicity was determined using JTK cycle. JTK cycle p value <0.05 is indicated by asterisk (*) next to the genotype label. See table 2-1 for JTK cycle p values.

Figure 2-5

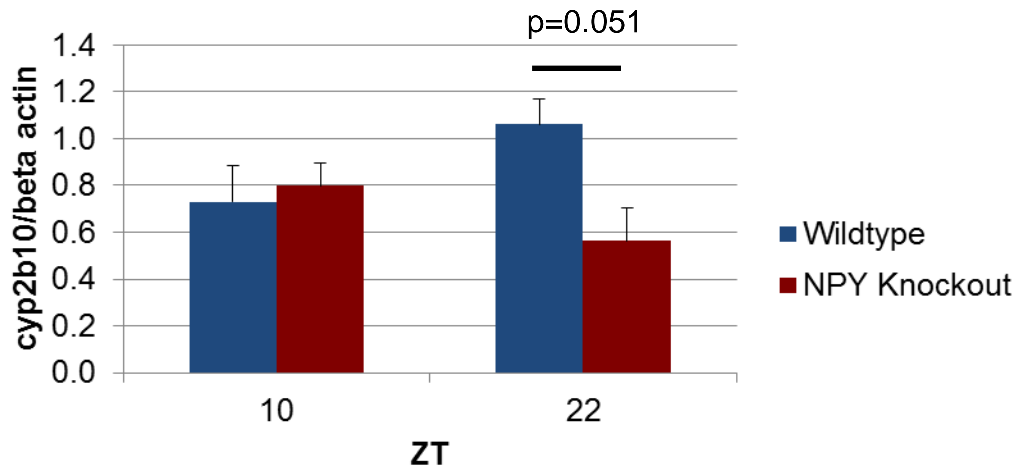


Figure 2-5. NPY regulates expression of a circadian gene in the liver.

Comparison of the times of purported peak (ZT22) and trough (ZT10) RNA levels for *cyp2b10* in NPY knockout mice and their background controls, reveals reduced *cyp2b10* expression in NPY knockouts at ZT22. N=3 mice for each genotype and timepoint. *Cyp2b10* levels were normalized to beta actin and graphed as the average ± standard error. P=0.051 by Student's t-test.

Table 2-1. JTK Cycle Output

Figure	Genotpye_Gene	P value (ADJ.P)	LAG	AMP
1B	ZT DNCLK_per	0.019365	18	0.28
1B	ZT Pdf DNCLK_per	0.009405	18	0.35
1C	CT DNCLK_per	0.004066	16	0.23
1C	CT Pdf DNCLK_per	1.000000	22	0.06
2A	Iso31_sxe2	0.001392	4	0.44
2A	Clk JRK_sxe2	1.000000	6	0.13
2B	Iso31_cg17562	0.001392	6	0.95
2B	Clk JRK_cg17562	0.694507	6	0.11
2C	Iso31_cyp4d21	0.002026	18	1.24
2C	Clk JRK_cyp4d21	1.000000	0	0.01
2D	Iso31_cg14934	0.082030	10	1.16
2D	Clk JRK_cg14934	0.542922	20	0.11
3A	ZT DNCLK_cyp4d21	0.000719	18	1.25
3A	ZT Pdf DNCLK_cyp4d21	0.000001	18	0.59
3B	DNCYC_cyp4d21	0.036315	18	1.10
3B	911 DNCYC_cyp4d21	0.004390	18	1.02
3C	DNCLK_cyp4d21	0.001392	18	1.22
3C	Npf DNCLK_cyp4d21	0.196879	20	0.23
3D	DNCLK_per	0.056785	16	0.26
3D	Npf DNCLK_per	0.044133	18	0.37
3E	DNCYC_cyp4d21	0.002907	18	1.63
3E	Npf DNCYC_cyp4d21	1.000000	0	0.05
4	NPFR het_cyp4d21	0.012763	18	1.68
4	NPFR_cyp4d21	0.196879	16	0.11

Note: *cyp4d21* is *sxe1*. LAG=phase; AMP=amplitude

Chapter 3: Independent regulation of sleep and metabolism by octopamine

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Abstract

Sleep length and metabolic dysfunction are correlated, but the causal relationship between these processes is unclear. Octopamine promotes wakefulness in the fly by acting through the insulin-producing cells (IPCs) in the fly brain. To determine if insulin signaling mediates the effects of octopamine on sleep:wake behavior, we assayed flies in which insulin signaling activity was genetically altered. We found that increasing insulin signaling does not promote wake, nor does insulin appear to mediate the wake-promoting effects of octopamine. Octopamine also affects metabolism in invertebrate species, including, as we show here, *Drosophila melanogaster*. Triglycerides are decreased in mutants with compromised octopamine signaling and elevated in flies with increased activity of octopaminergic neurons. Interestingly, this effect is mediated at least partially by insulin, suggesting that effects of octopamine on metabolism are independent of its effects on sleep. We further investigated the relative contribution of metabolic and sleep phenotypes to the starvation response of flies with altered octopamine signaling. Hyperactivity (indicative of foraging) induced by starvation was elevated in octopamine receptor mutants, despite their high propensity for sleep, indicating that their metabolic state dictates their behavioral response under these conditions. Moreover, flies with increased octopamine signaling do not suppress sleep in response to starvation, even though they are normally hyper-aroused, most likely because of their high triglyceride levels. Together these data suggest that observed correlations between sleep and metabolic phenotypes can result from shared molecular pathways rather than causality, and environmental conditions can lead to the dominance of one phenotype over the other.

Introduction

While trying to address why we sleep, we must also consider the question of what keeps us awake. Many neurotransmitters and neuropeptides are necessary for maintaining normal wakefulness in humans; these include but are not limited to dopamine, histamine, norepinephrine and orexin (Saper, Scammell, & Lu, 2005). Since maintaining a waking state involves all the major neurotransmitters and many neuropeptides, it is likely that there is overlap between sleep and other biological functions. Understanding this overlap will provide insight into the consequences of sleep deprivation, which is a common feature of modern society.

Drosophila melanogaster uses many of the same sleep-regulating neurotransmitters as mammals and has emerged as an excellent model for understanding the molecular control of sleep (Crocker & Sehgal, 2010). We recently identified octopamine, the invertebrate homolog of norepinephrine, as a wake-promoting molecule necessary to maintain normal arousal (Crocker & Sehgal, 2008; Crocker et al., 2010). Arousal in response to octopamine is mediated largely by neurons in the Pars Intercerebralis (PI), specifically by the neurosecretory cells that produce insulin-like peptides (ILPs), the fly homologs of human insulin (Rulifson et al., 2002). Since octopamine acts on the *Drosophila* insulin-producing cells to promote wake, and insulin is a major metabolic hormone in both mammals and insects (DiAngelo & Birnbaum, 2009; Saltiel & Kahn, 2001), it is possible that octopamine functions to control both sleep and metabolism by interacting with the insulin pathway. Indeed, there is considerable evidence indicating a link between sleep and metabolic activity, most notably with respect to sleep times and obesity (Saper & Scammell, 2004; Spiegel, 2004; Taheri, Lin, Austin, Young, & Mignot, 2004). In addition, octopamine signaling modulates metabolic function in other insects (Roeder, 2005). The fortuitous finding that octopamine signals

through metabolic cells in *Drosophila* provided us with a model we could use to study the relationship between these processes.

In this study, we set out to assay effects of octopamine on metabolism in *Drosophila* and to determine if metabolic signals mediate effects on sleep:wake. We show that altering the activity of the insulin signaling pathway has little effect on total fly sleep and moreover, flies lacking insulin-like peptides 2 and 3 (*ilp2-3* mutants), show normal increases in wake in response to octopamine. These data suggest that ILPs do not mediate the wake-promoting effects of octopamine. Nevertheless, octopamine does play a role in metabolism because decreasing octopamine signaling reduces triglycerides while activating octopaminergic cells increases triglycerides. An *ilp2-3* mutant background largely abrogates the metabolic effects of octopamine, which indicates that octopamine interacts with the insulin signaling pathway to alter metabolism in the fly. This metabolic phenotype is specific for octopamine; other short sleeping flies do not show consistent changes in their triglyceride levels, implying that loss of sleep per se does not lead to increased triglycerides. We also examined the activity of octopamine mutants in response to starvation, since this response typically depends upon metabolic status and involves changes in sleep (Keene et al., 2010). Surprisingly, animals with decreased octopamine signaling that sleep more under normal conditions are hyperactive while starved, suggesting that metabolic needs outweigh the need for sleep. The behavioral response of flies with increased octopamine signaling also supports the idea that the metabolic phenotype dominates under starvation conditions. These findings suggest a role for octopamine signaling in the control of sleep and metabolism and in the coordination of these processes to achieve overall homeostasis.

Methods

Fly Genetics- The following fly strains were used in this study: UAS-*dInR*^{A1325D} (Bloomington #8263), UAS-*myrAKT* (Stocker et al., 2002), UAS –*AktRNAi* (VDRC #2902), UAS-*ilp2* (Also known as UAS-*dilp2*) (Ikeya et al., 2002), *yolk*-Gal4 (Georgel et al., 2001), *oamb*^{286} (Gift from Dr. K. Han), *sss*^{p1} (Koh et al., 2008); *Fumin* (Kume, Kume, Park, Hirsh, & Jackson, 2005; M. N. Wu, Koh, Yue, Joiner, & Sehgal, 2008), *ilp2-3* (Bloomington #30888) and Iso31 (isogenic *w1118* stock; Bloomington #5905). The following lines were outcrossed into Iso31: *Tdc2*-Gal4 (Bloomington #9313), *Ilp2*-Gal4 (also known as *Dilp2*-Gal4) (Rulifson et al., 2002), UAS-*B16b* (*NaChBac* channel) (Bloomington #9466), UAS-*dTrpA1* (Hamada et al., 2008), *Elav*-Gal4, *Elav*-GeneSwitch, MB-GeneSwitch, and UAS-*mc** (constitutively active PKA) (the last four were reported previously in (Joiner, Crocker, White, & Sehgal, 2006).

Flies were grown on standard cornmeal-molasses medium as described previously, and maintained at 25℃ unless otherwise noted.

Triglyceride and protein assays- Triglyceride and protein measurements were performed as described in (DiAngelo & Birnbaum, 2009). Briefly, individual 4-7 day old mated female flies, entrained in a 12h:12h light:dark (LD) cycle at 25°C for at least 3 days, were collected on ice and homogenized in lysis buffer containing 140 mM NaCl, 50 mM Tris-HCl, pH 7.4, 0.1% Triton X and 1X protease inhibitor cocktail (Roche Diagnostics, Mannheim, Germany). For *TrpA1* triglyceride experiments, flies were raised at 18-21℃, shifted to 28℃ when they were 0-3 days old, and th en tested after 4 days at 28℃ to activate the *TrpA1* channel (Hamada et al., 2008). For experiments utilizing the GeneSwitch system, animals were placed on 500 mM RU486 in 1% EtOH in standard medium for 4 days to activate GeneSwitch activity as previously described (Joiner et al., 2006). Triglyceride and protein measurements were made using the Triglyceride

LiquiColor kit (Stanbio Laboratory, Boerne, TX) and bicinchoninic acid protein assay kit (Thermo Scientific, Waltham, MA), respectively, according to manufacturer's instructions. Thin layer chromatography (TLC) assays were performed as described in (Al-Anzi et al., 2009) with the slight modification that 5 female flies were used instead of 10 male flies.

RNA isolation, cDNA synthesis and quantitative PCR – Total RNA was isolated from abdomens of 3-8d old female flies using Trizol Reagent (Life Technologies, Grand Island, NY) according to the manufacturer's protocol. RNA was reverse transcribed using the High Capacity cDNA Reverse Transcription kit (Life Technologies, Grand Island, NY) and random hexamers. Quantitative PCR was performed on an ABI Prism 7000 using Sybr Green (ABI, Life Technologies, Grand Island, NY). Primer sequences used for QPCR were: *dFAS* (sense 5' CTGGCTGAGCAAGATTGTGTG 3' and antisense 5' TCGCACAACCAGAGCGTAGTA 3'), *dACC* (sense 5' AGATGCAGAACGATGTCCGC 3' and antisense 5' CTCTTTGTGAAGCAGCTCCG 3'), *dATPCL* (sense 5' CACGACAGATTGGTCCAAGCTC 3' and antisense 5' CTTGCTCTTCACGTCGGCTAAC 3'), *bmm* (sense 5' ACGTGATCATCTCGGAGTTTG 3' and antisense 5' ATGGTGTTCTCGTCCAGAATG 3'), *whd/dCPTI* (sense 5' GCCAATGTGATTTCCCTGCTTC 3' and antisense 5' CTTTGCCCTTCAGGATTTCCTC 3') and *rp49* (sense 5' GACGCTTCAAGGGACAGTATCTG 3'and antisense 5' AAACGCGGTTCTGCATGAG 3'). Genes were normalized to rp49 for analysis.

Behavior and starvation experiments- All flies were kept on a 12:12 LD cycle at 25°C except for flies associated with *TrpA1* sleep experiments, which were measured for baseline sleep at 21℃ and shifted to 28℃ at light s on to activate the *TrpA1* channel. For these experiments nighttime sleep was plotted instead of total sleep because the increase in temperature affected daytime sleep in control and experimental animals.

The wildtype flies used in this experiment (Fig.3-1B) contained an *ilp2*-mcherry construct in their background (to be discussed elsewhere). For starvation experiments, 1-7 day old female flies were placed in 65 X 5 mm tubes containing 5% sucrose and 2% agar and maintained in LD cycles for ~3 days prior to the initiation of starvation and/or high temperature treatment. Locomotor activity was monitored using the Trikinetics DAM system (Trikinetics, Waltham, MA) for one baseline day followed by the entire duration of the starvation protocol. Flies were starved by placing them in tubes containing 2% agar only. Activity levels under starvation were defined as the total number of beam crossings over a specified period of time (1, 3, 6, 12, 24 hrs) following the start of food deprivation (2% agar tubes). Baseline activity was defined as the total number of beam crossings over a period of time the day prior to starvation and corresponds to the same time of day as assessed under starvation. Statistical tests were done as described in figure legends.

Results

Wake promoting effects of octopamine are not mediated by insulin.

Since octopamine acts through the insulin-producing neurons of the fly brain to regulate sleep, we asked whether alterations in insulin signaling lead to the changes in sleep seen with octopamine modulation. In *Drosophila*, there are 7 insulin-like peptides (*ilps*) but only 3 (*ilps* 2, 3 and 5) are expressed in the octopamine-sensitive PI neurons in the adult fly brain (Brogiolo et al., 2001; S. J. Broughton et al., 2010; Ikeya et al., 2002). However, all ILPs signal through a single insulin receptor (*dInR*) (Robert S Garofalo, n.d.; Oldham & Hafen, 2003). Whether this receptor is expressed in the adult brain is controversial, but it is known to be highly expressed in the fat body, the fly equivalent of liver and adipose tissue (R S Garofalo & Rosen, 1988; Hwangbo et al., 2004). The main action of ILPs in flies is to regulate growth throughout development, although the pathway has also been shown to regulate aging, reproduction and metabolism in adults

(DiAngelo & Birnbaum, 2009; Partridge, Alic, Bjedov, & Piper, 2011; Marc Tatar, Bartke, & Antebi, 2003). However, the contribution of this pathway to the control of sleep is unclear.

To determine whether the wake-promoting effects of increased octopamine are due to increased insulin signaling, we first overexpressed *ilp2* in the PI neurons. *ilp2* was chosen since it has the highest homology to human insulin and its expression can rescue the growth phenotype observed by partially ablating PI neurons (Rulifson et al., 2002). Overexpression of *ilp2* in the PI neurons had very little effect on total sleep (Fig.3-1A). In addition, flies with increased octopamine signaling in either a heterozygous or homozygous *ilp2-3* mutant background showed decreases in nighttime sleep comparable to those seen in a wildtype background (Fig.3-1B). Consistent with these findings, activating the insulin signaling pathway by expressing a constitutively active form of *dInR* ($dInR^{A1325D}$) or a myristylated form of the kinase *dAkt* (myrAKT) in either the adult fly fat body or in the brain led to only minor effects on total sleep (Fig.3-1C and D). Moreover, any effects observed were in the opposite direction from those seen with increased octopamine signaling; in other words, there was an increase in sleep rather than wake. However, the small increase in sleep may not be physiological as it occurred even when insulin signaling was decreased through expression of Akt RNAi in the fat body (Fig.3-1C). Together, these data suggest that the insulin signaling pathway is not directly responsible for the sleep effects observed when octopamine signaling is altered.

Octopamine signaling regulates fat levels independent of its effects on sleep.

Since octopamine has actions on metabolism in other invertebrates and its wake-promoting actions are mediated through the insulin-producing neurons of the brain

(Crocker et al., 2010; Roeder, 2005), we hypothesized that octopamine regulates metabolic function in Drosophila. To test this hypothesis, we first measured ilp2 levels in animals with increased or decreased octopamine signaling. Unfortunately, the ilp2 mRNA and protein measurements, by qPCR and immunohistochemistry respectively, were very variable (data not shown). Similarly, the phosphorylation status of dAKT, a downstream mediator of insulin signaling, varied in the fat bodies of animals with altered octopamine signaling (data not shown). While phosphorylated dAKT levels were consistently low in flies carrying a null mutation in the oamb receptor, they were highly variable when octopamine signaling was increased. Since insulin signaling regulates triglyceride levels (DiAngelo & Birnbaum, 2009), we measured triglycerides in the fat bodies of flies with increased or decreased octopamine signaling. We found that activation of octopamine-producing neurons, through the use of a bacterial Na+ channel (NaChBac) driven by Tdc2-Gal4 (Gal4 expressed in octopamine and tyramine-producing neurons), increased triglycerides (Fig.3-2). To address whether the effect on triglycerides was developmental, we also activated these neurons in adult flies by inducing expression of a heat-sensitive TrpA1 channel. As with NaChBac, triglyceride levels increased with TrpA1 activation (Fig.3-2B). Most of our triglyceride measurements used a colorimetric assay (DiAngelo & Birnbaum, 2009; Hildebrandt, Bickmeyer, & Kühnlein, 2011), but we also verified effects of altered octopamine levels on lipids through thin layer chromatography assays (Fig.3-2C-E) (Al-Anzi et al., 2009). While the oamb mutants had robust effects on triglycerides regardless of nutrient conditions, we found that the metabolic phenotype of flies with increased octopamine, particularly the ones that had increased octopamine throughout development (NaChBac), was more evident under low nutrient conditions (Fig.3-2E).

Effects of increased octopamine on triglycerides were not due to increased feeding as feeding assays indicated that these animals actually eat less (data not shown). As octopaminergic neurons also produce tyramine, we considered the possibility that the effect on triglycerides was mediated by tyramine. However, decreased triglycerides in flies mutant for the octopamine receptor, *oamb* (Fig.3-2B), support a role for octopamine in increasing triglyceride levels.

To determine if insulin signaling mediates the metabolic effects of octopamine, we increased octopamine signaling in an *ilp2-3* mutant background and found that the effect on triglycerides was reduced (Fig.3-2B). These data suggest that octopamine acts, at least in part, through the insulin signaling pathway to alter triglyceride levels.

Changes in overall triglyceride storage can occur by modifications in fatty acid and triglyceride synthesis, breakdown or a combination of both. To further understand the mechanism whereby octopamine promotes fat storage, we measured the expression of the *Drosophila* homologs of enzymes important for the synthesis of fatty acids (fatty acid synthase (dFAS), acetyl-CoA carboxylase (dACC), and ATP citrate lyase (dATPCL), as well as the lipase brummer (bmm), and the enzyme responsible for mitochondrial transport and subsequent oxidation of fatty acids, carnitine palmitoyltransferase (dCPTI) (Baker & Thummel, 2007; Zinke, Schütz, Katzenberger, Bauer, & Pankratz, 2002). *oamb* mutants displayed decreased *dFAS* mRNA levels, suggesting a decrease in the synthesis of fatty acids in these mutants (Fig.3-3). Conversely, *bmm* and *dCPTI* mRNA levels were augmented in the *oamb* mutants, indicating an increase in fat breakdown also (Fig.3-3). Surprisingly, *dATPCL* expression was also increased in *oamb* mutants (Fig.3-3), which is perhaps a compensatory response to the decreased *dFAS* levels. Together, these data suggest that octopamine controls triglyceride homeostasis by promoting fatty acid synthesis and inhibiting lipid

breakdown; alterations in the expression of enzymes important for these processes may account for the triglyceride phenotypes observed in flies with altered octopamine signaling.

Given that sleep deprivation is implicated in obesity (Spiegel, 2004) and flies with increased neuronal octopamine signaling sleep significantly less, it is possible that the sleep loss itself contributes to the increased triglycerides. In order to address this issue, we measured triglycerides in other short-sleeping flies. We examined three different genotypes previously shown to have decreased sleep: *sleepless* (*sss*) and *fumin (fmn)* mutant flies, and flies expressing a constitutively active protein kinase A (PKA) transgene in the mushroom body (Joiner et al., 2006; Koh et al., 2008; Kume et al., 2005). In contrast to flies with increased octopamine signaling, the *fumin* mutants show no differences in triglyceride levels compared to controls (Fig.3-4). Moreover, the *sss* mutants and the flies that express constitutively active PKA in the mushroom body have decreased triglycerides (Fig.3-3). Together, these data suggest that the triglyceride phenotypes observed in animals with altered octopamine signaling are due to specific effects of the octopamine pathway and are not secondary to the changes in sleep observed in these animals.

Coordination of sleep and metabolic needs during starvation.

States of long-term sleep deprivation stimulate hunger and promote feeding while starvation suppresses sleep in mammals as well as flies (Keene et al., 2010; MacFadyen, Oswald, & Lewis, 1973; Rechtschaffen & Bergmann, 2002). Since the response to starvation also depends upon metabolic status, it provides a good example of a situation where metabolic and sleep needs must be balanced (Horne, 2009; Keene et al., 2010). Because octopamine affects both these physiological processes, we asked how flies with altered octopamine respond to starvation. Thus, we subjected flies with

increased (*Tdc2*-Gal4<UAS-*NaChBac*) and decreased (*oamb* mutants) octopamine activity to starvation medium (2% agar) and assayed their survival on this medium as well as their activity during this time. Consistent with their higher triglyceride levels, flies with increased octopamine signaling live longer on starvation medium than controls (Fig. 3-5A). Conversely, flies with decreased octopamine signaling are more sensitive to starvation, which is consistent with their lower triglyceride levels (Fig.3-5A).

Starvation was previously shown to induce hyperactivity and also suppress sleep in *Drosophila* (Keene et al., 2010; G. Lee & Park, 2004). Both of these effects probably reflect the activation of a foraging or food-seeking response. We hypothesized that under starvation conditions flies with increased sleep pressure due to decreased octopamine signaling (*oamb* mutants) would be less active relative to controls. We found, however, that although both *oamb* mutants and background controls suppress sleep by a similar amount relative to their baseline levels, *oamb* mutants become hyperactive prior to controls (Fig.3-5B). This response probably results from the decreased triglycerides in these flies. Investigation of flies with increased octopamine signaling (*Tdc2*-Gal4<UAS-*NaChBac*) revealed a similar dominance of the metabolic phenotype. We found that while these flies increase their activity in response to starvation, the increase reflects enhanced activity specifically during wake. In fact, these flies fail to suppress sleep under starvation conditions (Fig.3-5C). These data indicate that sleep and activity during starvation are based on an animal's metabolic demands, rather than its sleep drive, supporting the idea that the need for nutrients is dominant over an animal's need for sleep.

Discussion

In this study, we addressed the role of the biogenic amine octopamine in controlling both sleep and metabolism and the relationship between these processes in

the context of octopamine signaling. While the PI neurons are activated by octopamine to promote wakefulness (Crocker et al., 2010), the effect is not mediated by the insulin-like peptide, ILP2, secreted by these neurons, as overexpressing ILP2 in the PI neurons and manipulating downstream insulin signaling in the brain or the fat body does not increase wake (Fig.3-1). Furthermore, increasing octopamine signaling in an *ilp2-3* mutant background does not prevent a reduction in nighttime sleep (Fig.3-1B). This leaves open the question of what in these insulin-producing neurons is the wake-promoting signal. Although we show here that it is not *ilp2* or *ilp3*, we cannot rule out *ilp5*, which is also expressed in these neurons. We believe, however, that this peptide is also an unlikely signal as it would act through the same downstream insulin signaling pathway, which we show is dispensable for proper sleep regulation with respect to octopamine (Fig.3-1C and D). It is possible that other unidentified peptides are produced in the PI neurons and are released in response to octopamine stimulation to control sleep. Alternatively, octopamine may stimulate synaptic transmission from the PI neurons to increase wakefulness. Regardless, a more in depth characterization of these neurons will be needed to determine the mechanism whereby octopamine promotes wake.

We also present data that octopamine increases fat levels and it does so by regulating the expression of enzymes important for fat synthesis and breakdown (Fig. 3-2,3). Effects on lipids appear to be independent of changes in sleep as other short sleeping flies do not show the same increases in triglycerides (Fig.3-4). In addition, unlike the sleep phenotype, effects of octopamine on metabolism are mediated by ILPs, which is consistent with known effects of insulin signaling on lipid accumulation in flies and mammals (DiAngelo & Birnbaum, 2009; Saltiel & Kahn, 2001). Although analysis of ILP2 expression in PI neurons and phospho-dAKT levels in the fat bodies of animals

with altered octopamine signaling yielded variable results, we found that increases in triglycerides in flies with increased octopamine signaling were partially suppressed in an *ilp2-3* mutant background (Fig.3-2B). The lack of complete suppression may be due to compensation by *ilp5*, which is known to be increased in *ilp2-3* mutants and may be further increased upon octopamine stimulation (Grönke et al., 2010). Changes in the transcript levels of metabolic enzymes involved in lipid synthesis and breakdown correspond with decreased triglyceride storage in *oamb* mutants (Fig.3-3). In mammals, some of these metabolic enzymes, particularly fatty acid synthase, have been shown to be transcriptionally regulated by insulin (Kershaw et al., 2006; Paulauskis & Sul, 1989; Sul, Latasa, Moon, & Kim, 2000). These data suggest that the insulin signaling pathway contributes to the metabolic effects of octopamine.

An interesting condition where sleep and metabolism interact is during times of starvation. Periods of starvation suppress sleep in flies and humans most likely due to the need for the animal to search for food (Keene et al., 2010; MacFadyen et al., 1973). We show here that despite their large drive to sleep, *oamb* mutants increase their activity in response to starvation even before controls do (Fig.3-5B). Thus, these animals rapidly initiate their foraging response, which is consistent with their decreased triglycerides sensitizing them to metabolic stress, and indicates that metabolic need is dominant over the drive to sleep. This is supported by the response of flies that have increased octopamine signaling through activation of octopaminergic neurons. These flies fail to suppress sleep when starved. Thus, while they show an increase in activity, the activity is restricted to times when they are normally awake. Given their heightened arousal, the inability of these animals to curtail sleep again indicates that the metabolic status, which in this case is that of high triglycerides, dictates the response. One question that still needs to be addressed is how the sleep system of the animal senses

changes in nutrient conditions and alters its activity accordingly. Keene *et al*. (2010) showed that the suppression of sleep during starvation is indeed due to a caloric deficit, raising the possibility that the activity of neurons that control sleep depends upon nutrient flux through metabolic pathways.

It was shown previously that two circadian mutants with short-sleeping phenotypes, Clk^{Jrk} and cyc^{01}, have enhanced sleep suppression under starvation conditions. Nevertheless, this is not a general characteristic of short-sleeping flies. The short-sleeping mutants *fmn* and $shaker^{minisleep}$ reduce sleep similar to controls (Keene et al., 2010), and we report here that the short sleepers produced by increased octopamine signaling do not suppress sleep at all. The relevant *Clk*-expressing neurons were localized by Keene *et al* to circadian clock neurons, specifically to a subpopulation of the dorsal neurons (DN1s) and the dorsally located lateral neurons (LNds). The authors showed that driving *TrpA1* in these neurons abolished sleep suppression during starvation. It was previously suggested that oscillatory signals received by the DNs from central clock neurons are subsequently propagated to the PI (Jaramillo et al., 2004; M. Kaneko, Park, Cheng, Hardin, & Hall, 2000). We found that stimulation of octopaminergic neurons, whose projections include the ILP-producing PI neurons, results in an abnormal response to starvation in that while they increase their activity they do not suppress their sleep (Fig.3-5C). We propose that sleep suppression during starvation ultimately depends on the activity of cells within the PI. Thus, stimulating these neurons by activating inputs to them from either clock neurons or octopaminergic neurons prevents sleep suppression. Further studies are needed to address this hypothesis.

Recently much attention has been paid to interactions between sleep and metabolism in organisms from the fly to humans (Keene et al., 2010; Thimgan, Suzuki,

Seugnet, Gottschalk, & Shaw, 2010). While the mechanisms underlying these interactions are still being elucidated, the data presented in this study favor a model where the octopamine signaling pathway controls both sleep and metabolism independently and so one is not affected as a consequence of the other. This is similar to the effects of norepinephrine, the mammalian homolog of octopamine, on biological processes that use overlapping circuitry. In mammals, the norepinephrine-producing neurons in the LC increase their firing in response to heightened arousal (Sara, 2009). The LC is also known to activate the stress pathway and coordinate the brain's response to stress (Goddard et al., 2010). Thus, it is likely that in human sleep deprivation studies norepinephrine affects not only arousal, but also the stress pathway, which in turn can affect metabolism (Dallman et al., 2003). The parallels between these regulatory processes in mammals and flies indicate that *Drosophila* is a powerful model to understand the mechanisms underlying the coordination between sleep and metabolism.

Figures

Figure 3-1

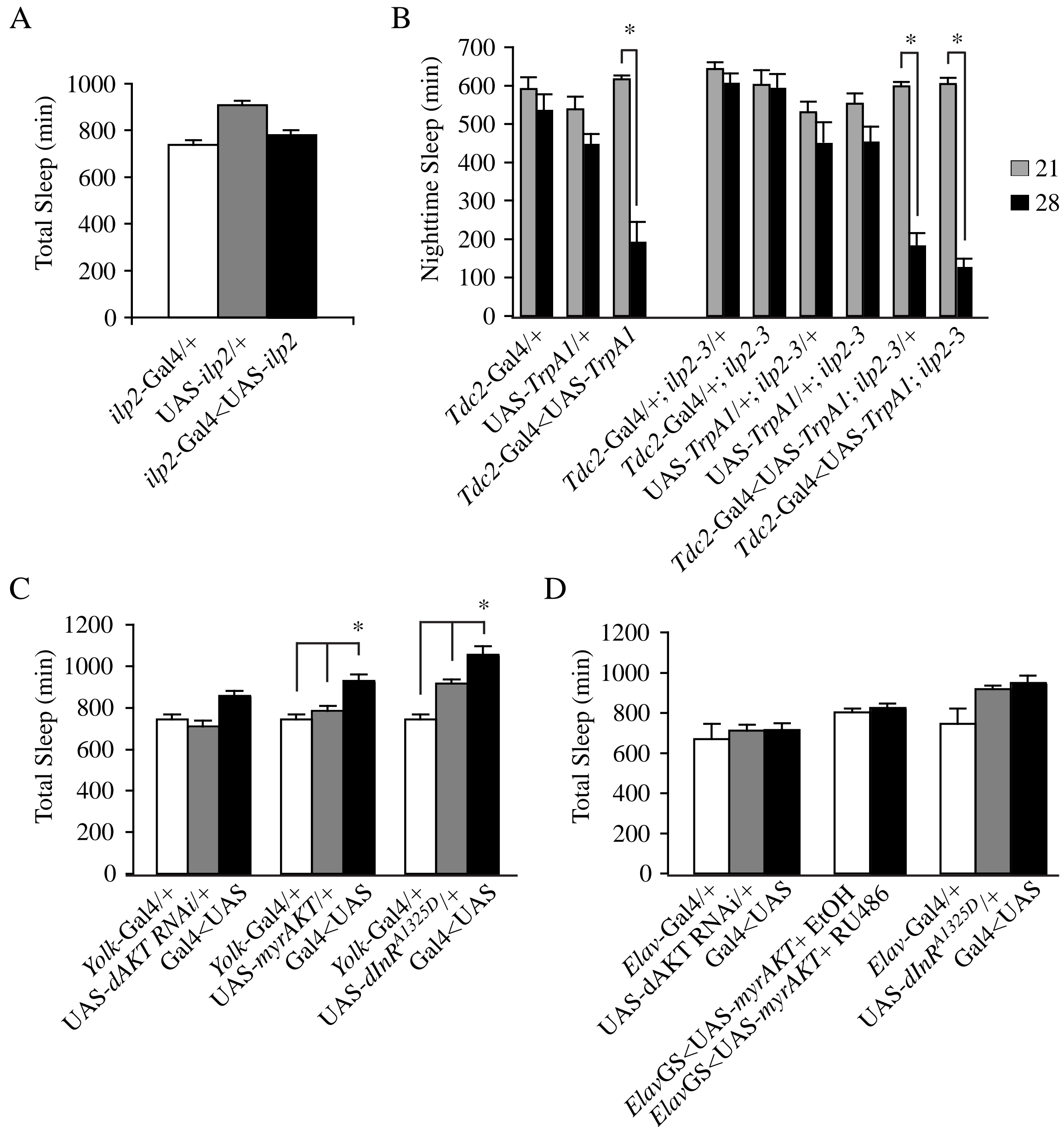

FIGURE 3-1. The insulin pathway does not mediate wake-promoting effects of octopamine.

(A): Total sleep over a 24-hour period in female flies overexpressing ILP2 in *ilp2*-producing neurons (*ilp2*-Gal4<UAS-*ilp2*), compared to control flies that contain the *ilp2*-Gal4 or UAS-*ilp2* transgene only. Total sleep is shown as mean ± SEM. n=18 for each genotype. **(B):** Nighttime sleep in flies with increased octopamine signaling in the

presence or absence of *ilps* 2 and 3 is shown as mean ± SEM. n≥12 for each genotype. *=p<0.01 as compared to 21℃ baseline using student s t-test. **(C,D):** Total sleep in flies with altered insulin signaling. Either a fat body driver (*yolk*-Gal4) **(C)**, or a pan-neuronal driver (*Elav*-Gal4 or an RU486 inducible *Elav*GS-Gal4) **(D)**, was used to express UAS-*dAkt RNAi*, UAS-*myrAKT* or UAS-$dInR^{A1325D}$ (constitutively active). *Elav*GS-Gal4 was activated by RU486 in 1% ethanol (EtOH) for 4 days and compared to flies given 1% EtOH without RU486. Flies containing both the Gal4 and UAS transgenes (Gal4<UAS) are compared to their Gal4 and UAS transgene alone controls. Total sleep is shown as mean ± SEM. n=32 for each genotype. *=p<0.01 as compared to each control line (Gal4 and UAS alone) using one-way ANOVA followed by Tukey-HSD post hoc test. *Elav*GS= *Elav* Gene Switch; *myrAKT*= myristylated AKT; *dInR*= *Drosophila* Insulin Receptor.

Figure 3-2

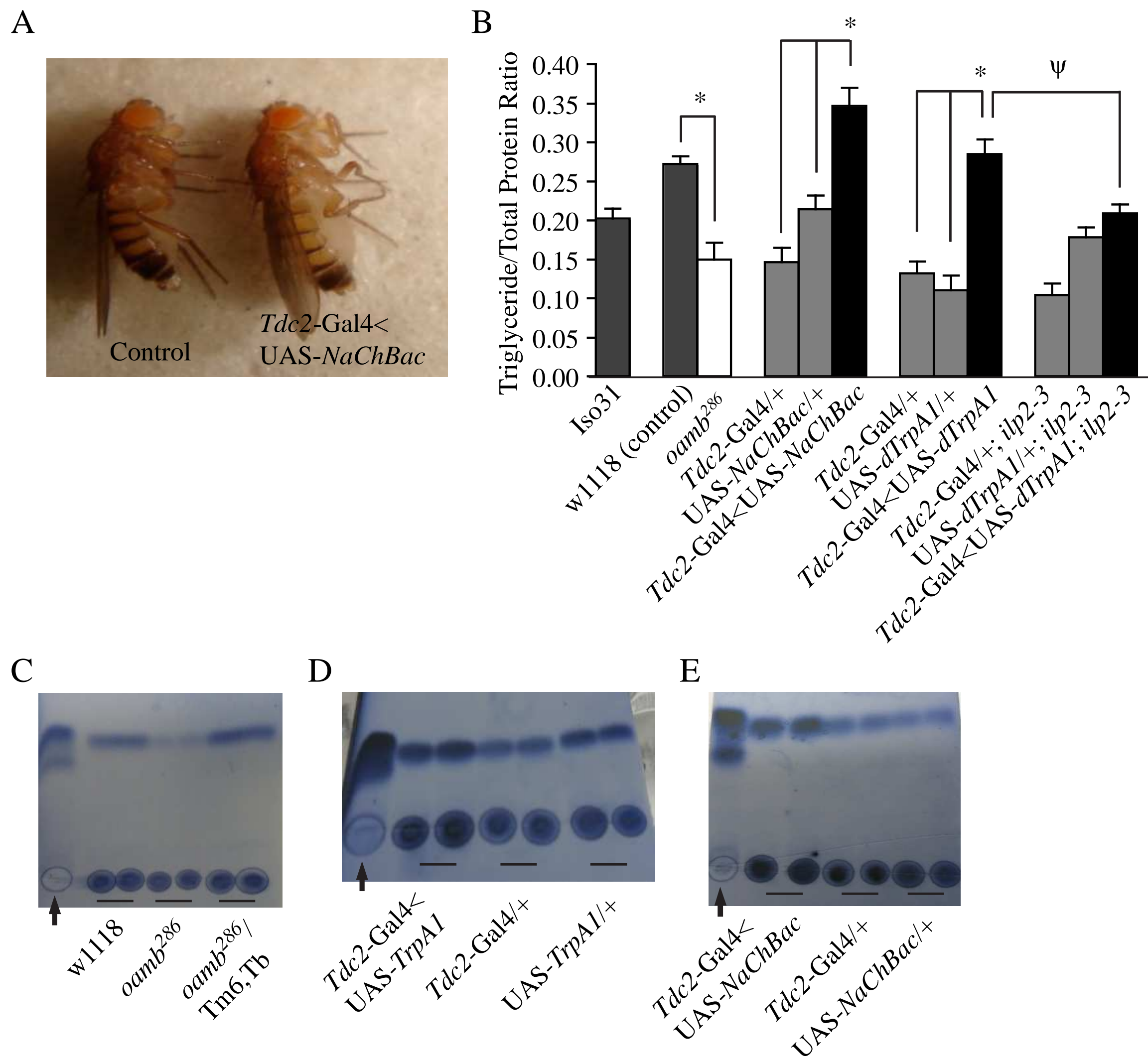

FIGURE 3-2. Triglyceride levels are changed in flies with altered octopamine signaling.

(A) Image shows a control fly (UAS-*NaChBac*/+) on the left and a fly with increased octopamine signaling, through activation of octopaminergic neurons (*Tdc2*-Gal4<UAS-*NaChBac*), on the right. Note the enlarged abdomen of the *Tdc2*-Gal4<UAS-*NaChBac* flies. **(B)** Octopamine signaling was increased either throughout development (*Tdc2*-Gal4<UAS-*NaChBac*) or conditionally during adulthood (*Tdc2*-Gal4<UAS-*TrpA1*) and triglyceride/total protein ratios were compared to control flies possessing only the Gal4 or UAS transgene. The effect of octopamine on triglycerides was reduced in an *ilp2-3* mutant background. Octopamine signaling was decreased using an octopamine receptor mutant, *oamb*^{286}, and triglyceride levels were compared to those of their background control (w1118). Each experiment was performed 3 times and values represent mean ± SEM of the pooled data. n≥12 for each genotype. *= p<0.05 relative to both control lines carrying Gal4 and UAS transgenes alone as determined by one-way ANOVA followed by Tukey-HSD post hoc test. Ψ= p<0.01 by Student's t-test. Triglycerides were also analyzed using Thin Layer Chromatography: **(C)** *oamb*^{286}

mutants and **(D)** flies with increased octopaminergic signaling (*Tdc2*-Gal4<UAS-*TrpA1*) on standard food. **(E)** Flies with increased octopaminergic signaling (*Tdc2*-Gal4<UAS-*NaChBac*) on low nutrient food (2% agar, 5% sucrose). Arrows point to the butter standard. Each duplicate spot (indicated by a line below the two spots) on the plate represents a technical replicate for that genotype. Each experiment was replicated two or more times.

Figure 3-3

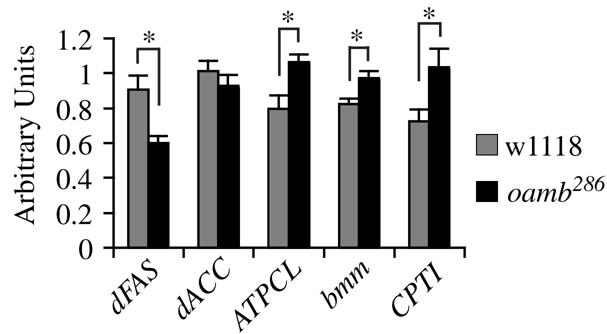

FIGURE 3-3. Synthesis and breakdown of lipids altered in *oamb* mutants.

Analysis of transcript levels of enzymes involved in either lipid synthesis (*dFAS*, *dACC*, *ATPCL*) or lipid breakdown (*bmm*, *CPTI*) in the fat bodies of *oamb* mutants and their background control (w1118). The experiment was performed twice with the data pooled to include 6 biological replicates for each genotype, and values represent mean ± SEM. *=<0.05 by Student's t-test.

Figure 3-4

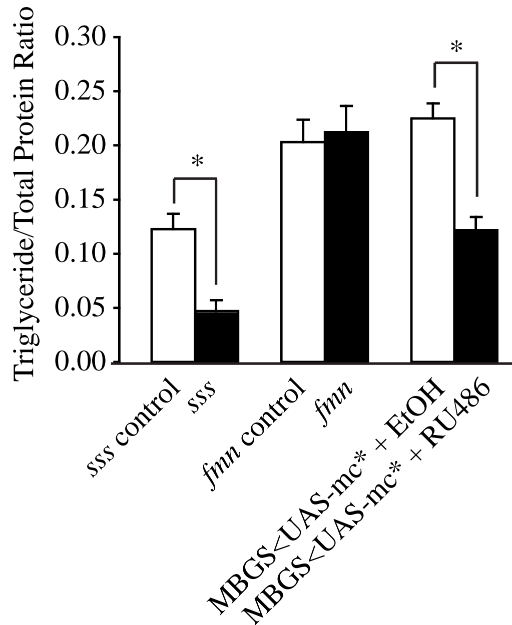

FIGURE 3-4. Reduced sleep does not cause increased triglycerides.

Triglyceride/total protein ratios of short sleeping mutants *sleepless* (*sss*) and *fumin* (*fmn*) were compared to their respective background controls. Triglycerides were also measured in flies where sleep was reduced in a wildtype genetic background by expressing a constitutively active form of PKA (mc*) in the mushroom body using an RU486 inducible driver (MBGS). Controls were given 1% ethanol (EtOH) without RU486. Plotted values equal the average of data pooled from at least three experiments $\pm$ SEM. $n \geq 13$ for each genotype *= $p<0.01$ by Student's t-test compared to each appropriate control.

Figure 3-5

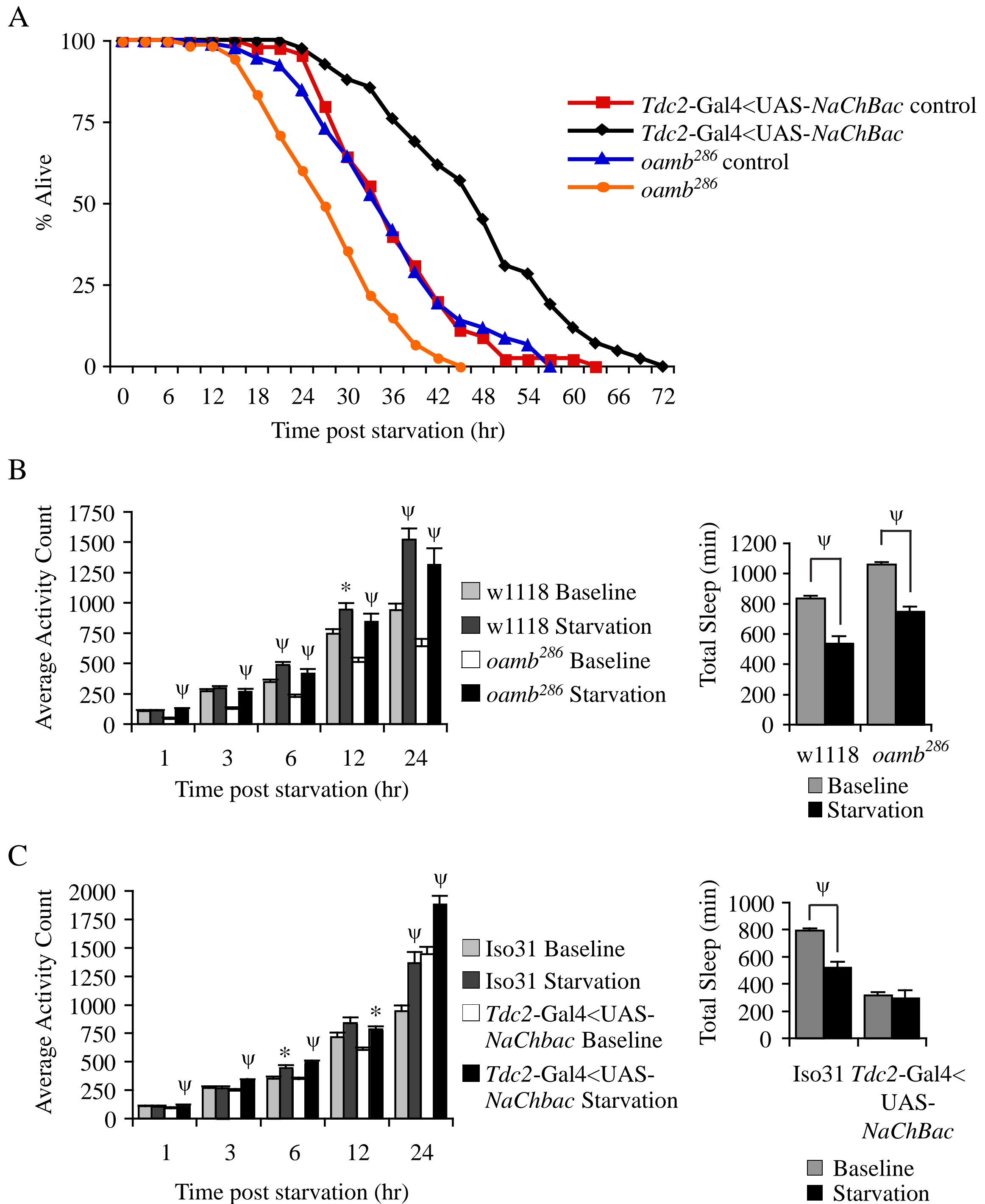

FIGURE 3-5. The response to starvation is dictated by metabolic need, rather than sleep pressure.

(a) Lifespan of flies with increased (*Tdc2*-Gal4<UAS-*NaChBac*) or decreased (*oamb*^{286}) octopamine signaling on starvation medium. Survival time reflects lifespan following the

start of food deprivation. The number of dead flies was determined every 3 hours. The graph summarizes the results of at least two experiments. **(b)** *oamb*^{286} mutants have exaggerated activity in response to starvation although they reduce sleep similar to controls. Total sleep over a 24-hour period was determined for the first day flies were starved on 2% agar (starvation) and the day before starvation in which flies were given sucrose food (baseline). **(c)** Flies with increased octopamine signaling do not suppress sleep in response to starvation. Flies expressing *NaChBac* in octopaminergic cells also display increased activity in response to starvation; however, they do not display similar reductions in sleep. Activity is plotted as the average of data pooled from three independent experiments. The activity value equals the average number of beam crossings over a period of time (1, 3, 6, 12, 24 hours) following starvation. Error bars reflect ± SEM. Statistical significance was determined using pooled data. Ψ=$p<0.01$ and *=$p<0.05$ correspond to comparisons of activity and sleep following starvation to the baseline values for each genotype by one-way ANOVA followed by Tukey-HSD post hoc test and Student's t-test respectively.

CHAPTER 4: Summary and Perspectives

For my dissertation, I sought to better understand the influence of sleep and circadian rhythms on metabolism. The study of interactions between these processes has gained significant traction due to the alarming growth of metabolic dysfunction in modern society. The relationship between circadian clocks or sleep and metabolic homeostasis has mostly been studied in mammals rather than flies. However, this is beginning to change due in part to the wider adoption of flies as a model of energy metabolism (Padmanabha & Baker, 2014). The relative simplicity of fly neuroanatomy and physiology, vast array of available genetic tools, and conservation across many organisms, makes *Drosophila* an ideal model to dissect complex interactions between physiological systems. I used *Drosophila* to investigate circadian regulation of gene expression in a peripheral metabolic tissue as well as the effects of intersecting sleep and metabolic neural circuitry.

Clocks and Metabolism

Since the initial discovery of the first core clock gene *period* by Konopka and Benzer in 1971, significant progress has been made regarding clock function at the level of molecules, cells and tissues. Indeed, researchers have identified many mechanisms involved in setting clock periodicity and phase, we have a basic appreciation for integration of temporal signals within the cell and the functional significance of tissue-specific clocks is becoming increasingly apparent. *Drosophila* has primarily been used to dissect the genes and neural circuits underlying regulation of overt behavioral rhythms of rest:activity. However, flies display a vast repertoire of other circadian rhythms for which the genetic and cellular basis have not been thoroughly investigated or are not well

understood (Fujii, Krishnan, Hardin, & Amrein, 2007; Krishnan, Dryer, & Hardin, 1999; J.-E. Lee & Edery, 2008; Sakai & Ishida, 2001). Little is known about the mechanisms downstream of the molecular clock responsible for communicating and translating circadian information into circadian rhythms. Understanding circadian rhythms at a systems level, particularly broader interactions between different tissues and other physiological systems, is the next frontier for which *Drosophila* will be an essential tool.

In flies, regulation of peripheral clocks by the central clock had been examined with respect to some peripheral clocks, however prior to our work, this did not include the fat body clock. We found that the fat body clock depends on the central clock under conditions of constant darkness, similar to the clock in the prothoracic gland (PG), which is the only other known peripheral clock regulated by the central clock in *Drosophila* (Myers et al., 2003). The phase of the clock in oenocytes, which regulate sex pheromone production and mating behavior, is controlled by PDF release from neurons in the abdominal ganglion (Krupp et al., 2008, 2013a). The complexity of peripheral clock regulation in flies has not been thoroughly investigated. Currently it is unclear why some clocks in *Drosophila* are autonomous while others are not. Future work will hopefully elucidate mechanisms responsible for differentiating regulation of peripheral oscillators.

The circadian system exerts much of its control through regulation of circadian gene expression. Tissue-specific gene expression patterns are generated in part by local clocks. Microarray studies comprehensively evaluating rhythmic gene expression in the presence and absence of local clocks has not been carried out for most tissues but was completed for the fat body. By profiling gene expression in the presence and absence of the fat body clock, the authors were able to establish the extent to which the fat body clock contributes to local tissue rhythms (Xu et al., 2011). Although this provided some insight into the function of the fat body clock, few robust physiological or behavioral

circadian outputs controlled by the fat body clock have been investigated. The fat body clock has been shown to regulate the phase of rhythmic feeding, and synchrony between the fat body and brain clocks is important for overall reproductive fitness; however metabolic rhythms have not yet been identified (Xu et al., 2011, 2008). An interesting area of future study will be to determine whether metabolites circulating in fly hemolymph display circadian rhythms and whether the fat body clock, as well as potentially other clocks, drive them.

The fat body clock regulates rhythmic transcription of genes involved in lipid and carbohydrate metabolism, immunity, and detoxification which in turn influence fat body physiology (Xu et al., 2011). Nonetheless, the fat body clock does not regulate rhythmic expression of all cycling genes in the fat body. For example, the cytochrome P450 enzyme, *sxe1*, continues to cycle in the absence of the fat body clock (Xu et al., 2011). We found that even though these genes, including *sxe1*, cycle independently of the fat body clock, they are still regulated by circadian clocks, as demonstrated by the absence of oscillations in Clk^{jrk} mutants. Furthermore, we went on to identify the neurons responsible for driving the rhythmic expression of *sxe1* as NPF-expressing clock cells (most likely LN_ds). In addition, we found that the NPF receptor is required for daily *sxe1* oscillations suggesting NPF facilitates *sxe1* rhythms. Lastly, we showed that this mechanism might also be relevant in mammals as peak expression of *cyp2b10,* an analogous cytochrome P450 enzyme that cycles independently of the liver clock, is reduced in livers of NPY mutants. Systemic regulation of peripheral oscillations in gene expression has not previously been studied in *Drosophila*, and only a limited number of systemic signals have been identified in mammals.

My dissertation suggests a novel role for the metabolic neuropeptide NPF/NPY as a circadian signal conserved between flies and mammals. Previously, NPF had only

been implicated in circadian rhythms and was known primarily as a regulator of feeding behavior. Thus, it is not surprising that the role of NPF in circadian rhythms may be to coordinate the expression of xenobiotic detoxification with consumption of toxic food. Although molecular links between circadian rhythms and feeding, like the *Drosophila takeout* gene, are known to exist, NPF's role in regulating feeding rhythms has not yet been investigated (Sarov-Blat, So, Liu, & Rosbash, 2000). NPY-lesioned animals exhibit disrupted circadian feeding patterns and abnormal sleep-wake patterns (Wiater et al., 2011). Therefore, NPY may coordinate sleep:wake, fasting:feeding and metabolic cycles to ensure circadian coherence and optimal function. In order to fully understand the role of NPY in coordinating these cycles, the role of NPY in each of these individual processes needs to be further investigated. Although NPY is implicated in sleep homeostasis, its role is unclear and varies based on the site of NPY injection (Dyzma, Boudjeltia, Faraut, & Kerkhofs, 2010). Whether NPY knockout mutants have altered sleep patterns is not known and would be an interesting area of future study.

Sleep and Metabolism

Metabolic homeostasis is not only influenced by the circadian system, but can also be modulated by sleep. Short sleep duration and poor sleep quality are associated with metabolic dysfunction (Spiegel, Tasali, Leproult, & Van Cauter, 2009). Therefore, the interaction between sleep homeostasis and metabolism is a very attractive and important area to study. To gain insight into the genetic and neural basis of interactions between sleep and metabolism we investigated implications of intersecting sleep and metabolic neural circuitry.

In *Drosophila*, octopamine, the insect equivalent of norepinephrine, promotes wakefulness by binding to octopamine receptors on the cell membranes of Insulin-

Producing Cells (IPCs) (Crocker & Sehgal, 2008; Crocker et al., 2010). We found that the major output of the IPCs, *Drosophila* insulin-like peptides (DILPs), do not act downstream of octopamine to promote wake (Erion, Diangelo, Crocker, & Sehgal, 2012). Increasing octopaminergic signaling in a *dilp2-3* mutant background results in decreased nighttime sleep comparable to increasing octopaminergic signaling in a wildtype background. In addition, manipulations that either decreased or increased insulin signaling in the brain or fat body did not decrease sleep (Erion et al., 2012). These data do not rule out the possibility that sleep homeostasis is modulated by insulin signaling but does strongly suggest that it is not responsible for relaying the wake-promoting effects of octopamine. The wake-promoting molecule expressed by the IPCs remains unknown. The identification of this molecule will aid in comprehensively mapping the entire octopamine sleep circuit.

Although insulin does not mediate the wake-promoting effects of octopamine, we showed that it does mediate metabolic phenotypes caused my increased octopamine signaling. Thus, octopamine affects sleep and metabolism through independent pathways. Sleep and metabolism appear to influence each other; sleep deprivation leads to metabolic disease and metabolic disease such as obesity leads to sleep disorders such as sleep apnea. Identifying the molecules and genes at the intersection of sleep and metabolism will improve the treatment of both sleep and metabolic disorders.

Finally, in this dissertation we provide insight into the hierarchal structure of basic physiological needs. Since feeding and sleeping are mutually exclusive behaviors, the fly must determine which is more important based on internal and external signals. Previously, experiments had not been done to determine whether eating or sleeping supersedes the other in the case where the need to sleep and the need to eat are both

high. We found that a fly that has both a high drive to sleep due to a mutation in an octopamine receptor and a high drive to eat due to starvation, obtaining food was more important than sleeping (Erion et al., 2012).

Ultimately, the complexities inherent to physiology can be better understood by studying the molecules and genes that lie at the intersection of behavioral and metabolic processes. My dissertation highlights the great power of *Drosophila* as a model organism and exemplifies its use in investigating interactions between physiological systems.

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