MOLECULAR MECHANISMS THAT GOVERN SYNAPTIC PLASTICITY AND PROTEIN TRANSLATION PROMOTE SLEEP

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ABSTRACT

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Sleep remains one of the great mysteries of evolution and biology. Despite the dangers that could accompany long periods of unresponsiveness to the external environment, every studied animal exhibits a sleep state. Much scientific deliberation has been dedicated to the search for a singular sleep function that could explain the persistence of sleep across species. However, decades of sleep research has shown that sleep is driven by a complex array of molecular signals that suggest that it likely serves multiple functions for the living organism. For my dissertation, I investigated the molecular mechanisms that underlie sleep regulation in the *Drosophila melanogaster* animal model. Also known as the common fruit fly, *Drosophila* sleep exhibits a high degree of conservation with mammalian sleep and provides many technical advantages for the sleep researcher. **Chapter 1** of this dissertation provides an overview of the neurobiology of sleep in *Drosophila* based on data from most of the published literature on *Drosophila* sleep*.* This dissertation is divided into two major lines of inquiry. The first is described in **Chapter 2** where I examine the role of Homer protein and metabotropic glutamate receptor interactions in sleep regulation. The second project examines the role of the PERK pathway — a regulator of global protein translation in the cell — in promoting sleep which is described in **Chapter 3.** In these projects, I used genetic and

pharmacological approaches to interrogate the necessity of various molecules in *Drosophila* sleep. The results from these projects demonstrate a critical role for synaptic Homer proteins and PERK signaling in the endoplasmic reticulum in sleep regulation. Together, the findings from my dissertation provide greater insight into the molecular and neurobiological underpinnings of sleep behavior.

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CHAPTER 1

The Neurobiological Basis of Sleep: Insights from Drosophila

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Abstract

Sleep is a biological enigma that has raised numerous questions about the inner workings of the brain. The fundamental question of why our nervous systems have evolved to require sleep remains a topic of ongoing scientific deliberation. This question is largely being addressed by research using animal models of sleep. *Drosophila melanogaster,* also known as the common fruit fly, exhibits a sleep state that shares common features with many other species. *Drosophila* sleep studies have unearthed an immense wealth of knowledge about the neuroscience of sleep. Given the breadth of findings published on *Drosophila* sleep, it is important to consider how all of this information might come together to generate a more holistic understanding of sleep. This review provides a comprehensive summary of the neurobiology of *Drosophila* sleep and explores the broader insights and implications of how sleep is regulated across species and why it is necessary for the brain.

1. Introduction

Despite being one of the most ubiquitous behaviors in the animal kingdom, sleep remains one of nature's greatest mysteries. From an evolutionary perspective, sleep is a very peculiar phenomenon. A process that prevents an organism from perceiving and interacting with their environment for extended periods of time is likely to increase the risk for predation, yet sleep has endured across the course of evolution and been systematically studied from the simple nervous systems of C. elegans (Raizen et al., 2008) all the way to the human brain (Reviewed in Hirshkowitz, 2004). In order to have persisted in such a wide range of species (Campbell and Tobler, 1984), sleep must provide some biological benefits that outweigh any maladaptive features. Following decades of scientific inquiry aimed at disentangling the biology of sleep, there is now virtually no denying the importance of sleep to animal life. The biological necessity of sleep is best highlighted by the negative consequences associated with its loss, which include impaired cognition with increased risk of accidents (Dinges et al., 1997; Belenky et al., 2003; Van Dongen et al., 2003), metabolic dysfunction (Spiegel et al., 2009), increased disease risk (van Leeuwen et al., 2009; Reviewed in Palma et al., 2013), and in extreme cases, death (Rechtschaffen et al., 1983; Montagna and Lugaresi, 2002; Shaw et al., 2002; Stephenson et al., 2007). The detrimental repercussions of sleep deprivation are evidence that there is a critical purpose for sleep, but there is still currently no scientific consensus on the core functions of sleep.

One of the most significant milestones in modern sleep research was the discovery that *Drosophila melanogaster*, also known as the common fruit fly, exhibits a sleep-like state (Hendricks et al., 2000; Shaw et al., 2000). Initial studies found that *Drosophila* sleep shares many of the same traits as mammalian sleep, such as stereotyped posture, increased arousal threshold, and a homeostatic response to sleep

deprivation (Hendricks et al., 2000; Shaw et al., 2000). These common features make the fruit fly a rather useful animal model to study sleep. The characterization of *Drosophila* sleep has since paved the way for the widespread use of *Drosophila* as an animal model to study sleep at the molecular and behavioral level. *Drosophila* sleep is typically observed and recorded by placing flies in transparent tubes and monitoring their movements using infrared beams or video recording. Through the use of these behavioral tracking methods, it has been shown that *Drosophila* sleep exhibits a diurnal pattern that is most consolidated in the middle of the night and that is accompanied by high levels of wake and activity during the early and later portions of the day (Hendricks et al., 2000). During the middle of the day, *Drosophila* also undergo a “siesta sleep” that is sexually dimorphic, since male sleep is longer and more consolidated than sleep in female flies in the daytime (Shaw et al., 2000; Koh et al., 2006). This difference in daytime sleep largely accounts for the longer average amount of daily sleep in male flies compared to female flies. There is also variation in sleep bout duration throughout the day and night, with bouts lasting from minutes to hours (Hendricks et al., 2000). Sleep in the fruit fly is commonly defined as 5 min or more of rest, due to the fact that the arousal threshold – which can be measured by the response to mechanical stimuli – of resting flies is significantly increased after 5 min of rest (Shaw et al., 2000). Until recently, invertebrate sleep was primarily differentiated from mammalian sleep by the absence of observable sleep stages (In mammals, we can distinguish stages such as rapid eye movement (REM) from non-REM sleep). However, recent electrophysiological experiments have demonstrated that *Drosophila*sleep does have phases of varying intensity that have been captured by local field potential recordings in the fly brain (van Alphen, et al., 2013). While we cannot directly compare *Drosophila* sleep stages to the

stages of mammalian sleep, it is now evident that changes in sleep depth are also a feature of invertebrate sleep.

It should be noted that our understanding of the circadian regulation of sleep and activity owes much to the fundamental studies of circadian clock proteins in the *Drosophila* animal model. While a comprehensive exploration of circadian rhythms is beyond the scope of this review, it is impossible to discuss sleep regulation without considering the role of the clock. The daily timing of sleep is believed to be the result of interactions between circadian rhythms and homeostatic signaling, a concept known as the two-process model of sleep (Borbély, 1982). Circadian rhythms generate daily rhythmic patterning of sleep and wake states while the sleep homeostat increases the pressure to sleep following extended periods of wake. The *Drosophila* circadian molecular clock still remains one of the most well understood and thoroughly characterized circadian systems (Reviewed in Nitabach and Taghert, 2008; Allada and Chung, 2010) and our understanding of the molecular machinery underlying circadian rhythms owes a great deal to the seminal studies that identified the core clock proteins in the fly. In 1971, Konopka and Benzer were the first to uncover a genetic basisunderlying circadian rhythms when they conducted a forward genetic screen of *Drosophila* mutants that led to the seminal discovery of the circadian gene *period*(Konopka and Benzer 1971) (Reddy et al., 1984). Later work would identify multiple key players in *Drosophila* circadian regulation: *timeless* (Sehgal et al., 1994), *dClock*(Allada et al., 1998), the *Drosophila* homolog of mammalian *Clock* (King et al., 1997), and the protein DOUBLETIME, which phosphorylates PER and targets it for degradation (Price et al., 1998; Kloss et al., 1998). These genes/proteins are all required to maintain the normal diurnal rhythms of rest and activity in the fly. Most recently, Michael Young, Michael Rosbash, and Jeffrey Hall were awarded the Nobel Prize in Medicine or

Physiology for their critical contributions to the understanding of circadian rhythms in *Drosophila.* Elucidating the biological mechanisms underlying the influence of both circadian rhythms and homeostatic signaling on sleep regulation is one of the major endeavors in sleep research today (for review, see Franken and Dijk, 2009).

This review will summarize what we have learned about sleep regulation in the fruit fly since the original description of sleep in this species and highlight the advances in our general understanding of sleep biology as a direct result of *Drosophila* sleep research. When relevant, we will discuss ways in which many neurobiological processes regulating sleep in the fly are conserved in higher order systems. Our goal is to provide a unified overview of the last two decades of fly sleep research and address the larger implications for understanding sleep function. We will also address the technical and scientific considerations that will be important to keep in mind as we move towards an increasingly holistic understanding of the biology of sleep.

2. Neurotransmitters involved in wake/sleep regulation

Seven neurotransmitters have hitherto been implicated in sleep and wake regulation in *Drosophila* (see Table 1). The wake-promoting neurotransmitters are dopamine, octopamine, and histamine, while serotonin and GABA are the major sleep promoting neurotransmitters in the fly. Acetylcholine and glutamate have been found to have both wake-promoting and sleep-promoting actions in *Drosophila* through the activity of separate and distinct brain circuits.

Table 1. Summary of the major neurotransmitters regulating wake and sleep in *Drosophila melanogaster* and identified locations of their actions.

Neurotransmitter	Sleep/Wake Effect	Presynaptic cells	Postsynaptic Receptors/ Brain Regions
WAKE PROMOTING NEUROTRANSMITTERS			
Dopamine	↑wake	DA neurons in the PPL1and PPM3 clusters in the posterior protocerebrum (Liu et al., 2012b; Ueno et al., 2012) MB dopamine neurons (Sitaraman et al., 2015)	DopR (dFSB) (Liu et al., 2012b; Ueno et al., 2012) D1 receptors (MBONs) (Sitaraman et al., 2015)
Octopamine	↑wake	ASM cells in the medial protocerebrum (Crocker et al., 2010)	OAMB (PI) (Crocker et al., 2010)
Histamine	↑wake	Unknown	HisCl1receptors (Oh et al., 2013)
SLEEP PROMOTING NEUROTRANSMITTERS			
Serotonin	↑sleep	DPM neurons (Haynes et al., 2015)	5-HT1a (MB) (Yuan et al., 2006) 5-HT2b (dFSB) (Qian et al., 2017)
GABA	↑sleep	DPM neurons (Haynes et al., 2015)	Rdl$GABA_A$ receptor (l-LNvs) (Chung et al., 2009) $GABA_{AB}$-R3 receptor (MB) (Haynes et al., 2015)
DUAL FUNCTION NEUROTRANSMITTERS			
Acetylcholine	↑wake	Subset of α/β core neurons in the MB (Yi et al., 2013)	nAChRs (l-LNvs) (McCarthy et al., 2011)
	↑sleep	Subset of α/β core neurons in the MB (Yi et al., 2013)	Unknown

Neurotransmitter	Sleep/Wake Effect	Presynaptic cells	Postsynaptic Receptors/ Brain Regions
Glutamate	↑wake	Unknown	Unknown
	↑sleep	Unknown	NMDAR (Tomita et al., 2015) (Robinson et al., 2016)

2.1 Wake-promoting neurotransmitters (see Table 1)

2.1.1. Dopamine

Numerous studies have provided evidence that dopamine promotes wake in *Drosophila.* Dopaminergic cells occur in clusters throughout the *Drosophila* protocerebrum and innervate most neuropils in the central nervous system (Friggi-Grelin et al., 2003; Mao and Davis 2009). These dopaminergic cells project to the mushroom bodies and the central complex (Friggi-Grelin et al., 2003; Mao and Davis 2009), which are important sleep-regulating brain regions in the *Drosophila* central nervous system (Pitman et al., 2006; Joiner et al., 2006; Donlea et al., 2011; Liu et al., 2016).

Genetic mutations that affect dopamine signaling in *Drosophila* have major influences on sleep and have effectively demonstrated the arousal-promoting effects of dopamine. Kume et al., 2005 identified a short-sleeping *Drosophila* mutant termed *fumin* (*fmn*), the Japanese word for “sleepless”. The mutation in the *fmn* flies was determined to be the result of a loss-of-function mutation in the *Drosophila* dopamine transporter (dDAT) gene. Since dDAT is responsible for dopamine presynaptic re-uptake (Porzgen et al., 2001), it was suggested that loss-of-function of dDAT produces prolonged dopamine signal at dopaminergic synapses in *fmn* mutants and reduced sleep

(Kume et al., 2005). Makos et al., 2009 later recorded the levels of dopamine clearance *in vivo* in *fmn* mutants using fast scan cyclic voltammetry and confirmed that the *fmn* mutation does in fact impair dopamine clearance.

Conversely, pharmacologically inhibiting dopamine synthesis via oral administration of the tyrosine hydroxylase inhibitor 3-iodo-tyrosine (3IY) increases sleep during the day, providing further evidence that dopaminergic signaling influences arousal (Andretic et al., 2005). It should be noted that the time-of-day specific effect of 3IY is likely due to a ceiling effect of *Drosophila* baseline nighttime sleep. Similarly, genetic disruption of dopamine synthesis by abolishing the expression of active tyrosine hydroxylase (TH) – an enzyme that is critical for dopamine biosynthesis – in the *Drosophila* CNS causes flies to have significantly increased total sleep time (Riemensperger et al., 2011). The loss of dopamine in the brain results in less time spent awake during the day and night and a higher arousal threshold, (Riemensperger et al., 2011).

Methamphetamine is a psychostimulant that acts through dopaminergic systems in mammals (Nishino et al., 1998). Methamphetamine also promotes arousal in *Drosophila,* as flies fed methamphetamine display longer wake bouts and extended latency to sleep after the transition from day to night (Andretic et al., 2005). The effects of methamphetamine are opposite to those caused by inhibiting the production of dopamine, and it has been suggested that the arousal-promoting effects may also act through dopaminergic signaling (Andretic et al., 2005).

Dopaminergic signaling mediates the wake-promoting effects of caffeine in *Drosophila.*In mammals, caffeine is a stimulant that likely promotes arousal through nonspecific antagonism of adenosine receptors, of which there are four that are known: A_1, A_{2A}, A_{2B}, and A_3 (Fredholm et al., 1999). *Drosophila* possesses just a

single adenosine receptor, known as AdoR (Dolezelova et al., 2007). Interestingly, while the effects of caffeine in the fly are mimicked by A1 and A2 adenosine receptor antagonists (Andretic et al., 2008), studies of AdoR null mutants in *Drosophila* indicate that AdoR is not required for the effects of caffeine since these mutants do not exhibit any altered response to caffeine (Wu et al., 2009). Instead, the arousal-inducing effects of caffeine in *Drosophila* (Hendricks et al., 2000; Shaw et al., 2000) require the dopamine D1 receptor (dDA1) in the mushroom bodies (Andretic et al., 2008). The *Drosophila* mutant *dumb1* which has deficient expression of dDA1 (Kim et al., 2007) is resistant to the arousal-promoting effects of caffeine; this resistance is rescued by the transgenic expression of wildtype dDA1 in the mushroom bodies (Andretic et al., 2008). Analysis of dDA1 mRNA from *Drosophila* heads further showed that caffeine treatment leads to a decrease in dDA1 mRNA expression, suggesting that caffeine promotes wakefulness in *Drosophila* at least in part by altering transcription of dDA1 receptors (Andretic et al., 2008).

One of the benefits of a small animal model such as *Drosophila* is that the task of mapping the neural circuits of behaviors is highly amenable in a nervous system comprising a smaller number of cells, cell types, and connections than higher order animal models. The dopaminergic regulation of sleep in the *Drosophila* brain is an excellent example of the specificity with which *Drosophila* genetic tools has allowed us to localize the actions of neurotransmitter circuits that generate complex behavior. Part of the wake-promoting dopamine signal has been isolated to single neurons in the PPL1 and PPM3 clusters in the posterior protocerebrum of the *Drosophila* brain that innervate neurons in the dorsal fan shaped body (dFSB) in each hemisphere with dDA1 receptors (Liu et al., 2012b; Ueno et al., 2012). Since the identification of these wake-promoting inputs to the dFSB, the combined approach of optogenetic stimulation of dopaminergic

cells in conjunction with electrophysiological recording in dFSB neurons has confirmed that activation of TH-expressing dopaminergic cells suppresses dFSB firing to promote wake (Pimentel et al., 2016). It should also be noted that dopamine exerts wake-promoting effects outside of the dFSB, as it was found that dopaminergic activation of D1 dopamine receptors in mushroom body neurons also induces wakefulness (Sitaraman et al., 2015).

Dopamine has a conserved wake-promoting function in mammals. Similar to *Drosophila fmn* mutants, mice with impaired DAT activity (as a result of pharmacological administration of a DAT inhibitor) exhibit elevated amounts of wakefulness, suggesting that prolonged synaptic exposure to dopamine induces wake (Qu et al., 2010). Further evidence that dopamine stimulates wake has been observed in rats, where intracerebroventricular injections of D1 and D2 agonists increase time spent awake and decrease time spent sleeping (Isaac and Berridge, 2003). Finally, PET imaging in rhesus macaques and human subjects have determined that the wake-promoting drug modafinil increases levels of extracellular dopamine in the brain (Andersen et al., 2010; Volkow et al., 2009). It should also be noted that an interesting aspect of sleep regulation that is highlighted by the fruit fly dopaminergic system is the presence of functional and anatomical connections between wake-promoting and sleep-promoting brain regions. As previously discussed, PPL1 and PPM3 wake-promoting dopaminergic cells in the posterior protocerebrum of the *Drosophila* brain synapse onto DopR expressing cells in the dFSB, which is a sleep-promoting brain center (Liu et al., 2012b; Ueno et al., 2012). In mammals, arousal pathways inhibit sleep regulating centers such as the ventrolateral preoptic nucleus (VLPO) in the hypothalamus that in turn can inhibit the same ascending arousal circuits (Saper et al., 2010). This system of alternating inhibition between wake and sleep centers is the basis for the mammalian

“flip-flop” model of sleep, which suggests that states transition or “switch” when a wake-promoting region or a sleep-promoting region no longer inhibits the other (Saper et al., 2010). In this case, dopaminergic synapses onto the sleep-promoting dFSB cells suggest that there may be some general conservation of structural logic that connects sleep- and wake-promoting circuits in the brain.

2.1.2. Octopamine

Octopamine is a wake-promoting neurotransmitter in *Drosophila* that is considered to be equivalent to the mammalian neurotransmitter norepinephrine, of which it is a structural analog. In mammals, Aston-Jones and Bloom, 1981 were the first to establish that neurons of the locus coeruleus that release norepinephrine are predominantly wake-active neurons. In the *Drosophila* central nervous system, there are approximately 100 octopaminergic cells that send projections to a many distinct regions in the brain including the calyx of the mushroom bodies, parts of the central complex, the protocerebrum, as well as the optic lobes (Sinakevitch and Strausfeld, 2006; Busch et al., 2009). Thus, similar to the norepinephrine cells in the locus coeruleus (Berridge and Waterhouse, 2003), octopamine cells have widespread projections in the *Drosophila* CNS.

Pharmacological and genetic manipulations of octopamine signaling demonstrate the arousal-promoting effects of octopamine. *Drosophila* that are orally administered octopamine have significantly less nighttime sleep, while flies carrying mutations that disrupt key genes in the octopamine biosynthesis pathway sleep longer during the day and have a decreased latency to sleep after the transition from day to night which is indicative of an elevated pressure to sleep (Crocker and Sehgal, 2008). It should be noted that in cases of increased sleep specific to the daytime, a ceiling effect of nighttime sleep may be a contributing factor. Furthermore, increasing the excitability of

octopaminergic cells by expressing a transgene for a bacterial sodium channel in these neurons decreases total nighttime sleep, while reducing the octopaminergic cell excitability via expression of a transgenic potassium channel increases total sleep (Crocker and Sehgal, 2008).

As in the case of the dopamine arousal pathway, the neural circuitry for the wake-promoting effects of octopamine has been characterized with considerable detail. Crocker et al., 2010 utilized a mosaic analysis with a repressible cell marker (MARCM) which allows for specific temporal and spatial labeling of cells (Lee and Luo, 2001) to identify the octopaminergic cells that are responsible for the wake-promoting effect of octopamine. The wake-promoting signal of octopamine was mapped to a cluster of neurons in the medial protocerebrum; these cells activate the octopamine receptor in the mushroom body (OAMB) on insulin-like-peptide (DILP2) producing cells in the pars intercerebralis, depolarizing the DILP2 cells and increasing their cyclic-AMP (cAMP) activity (Crocker et al., 2010). The involvement of DILP2 neurons in the octopaminergic arousal pathway is supported by the reduction and increase in sleep caused by manipulating the excitability of DILP2 neurons via the transgenic expression of depolarizing or hyperpolarizing channels, respectively (Crocker et al., 2010).

In addition to being structurally similar to mammalian norepinephrine (Farooqui, 2012) octopamine also shares arousal regulating activity with norepinephrine. Norepinephrine is a critical component of the mammalian ascending arousal system and is released from the LC in the brainstem to many components of the cortex (Reviewed in Berridge et al., 2012). Early studies in rats found that noradrenergic cells in the LC produce firing bursts that precede entrance into different states of arousal (Aston-Jones and Bloom, 1981). Later work found that targeted pharmacological activation of LC cells

induced arousal, which was blocked by inhibiting norepinephrine release (Berridge and Foote, 1991).

2.1.3. Histamine

Histaminergic neurons have been shown to be important wake-promoting cells in mammals (Thakkar, 2011; Parmentier et al., 2002) and evidence suggests that histamine also promotes arousal in *Drosophila* (Oh et al., 2013). Immunohistochemicalstudies have identified 18 histaminergic cell bodies in the *Drosophila* brain (Reviewed in Nässel, 1999). These histaminergic neurons project to regions of the ventral and lateral protocerebrum of the *Drosophila* brain (Pollack and Hofbauer, 1991; Reviewed in Nässel, 1999). Pharmacological administration of the histamine receptor antagonisthydroxyzine decreases the latency to sleep (Shaw et al., 2000) and genetic hypomorphic mutations that decrease activity of histidine decarboxylase (HDC) – an enzyme critical for histamine synthesis – significantly increases daytime sleep (Oh et al., 2013). There are two histamine receptors in *Drosophila* – the histamine-gated chloride channel subunit 1 (HisCl1) and ora transientless (Ort) – but the wake-promoting effects of histamine are likely mediated only by HisCl1 since only HisCl1 null mutants display increases in sleep (Oh et al., 2013). Future work to determine which cells are required for HisC11-mediated sleep regulation will be critical for understanding the mechanism by which histamine promotes wake in the fly.

2.2. Sleep-promoting neurotransmitters (see Table 1)

2.2.1. Serotonin

Serotonin is widely expressed in the *Drosophila* central nervous system (Nässel, 1988; Vallés and White, 1988; Lundell and Hirsh, 1994; Sitaraman et al., 2008) and promotes sleep in *Drosophila* (Yuan et al., 2006). Pharmacological elevation of serotonin levels via treatment with 5-hydroxytryptophan (5-HTP), a precursor to serotonin biosynthesis, increases sleep (Yuan et al., 2006). This effect is mimicked by transgenically overexpressing tryptophan hydroxylase (TPH, known as TRH in *Drosophila*), an enzyme involved in the biosynthesis of serotonin. Conversely, blocking serotonergictransmission by expressing the transgene for the tetanus neurotoxin light chain – which disrupts exocytosis of neurotransmitter – in serotonergic cells significantly decreases sleep (Yuan et al., 2006). *Drosophila* serotonin receptors are exclusively G protein-coupled metabotropic receptors and occur in five subtypes: 5-HT1a, 5-HT1b, 5-HT2a, 5-HT2b, and 5-HT7 (Witz et al., 1990; Saudou et al., 1992; Colas et al., 1995; Qian et al., 2017). Studies in the fly demonstrate that serotonergic regulation of sleep likely acts through multiple receptors and brain regions to promote both baseline sleep (Yuan et al., 2006; Haynes et al., 2015; Qian et al., 2017) as well as homeostatic sleep rebound after sleep deprivation (Qian et al., 2017). Yuan et al., 2006 identified 5-HT1a receptors as a regulator of sleep since loss-of function mutants display significant sleep reductions (Yuan et al., 2006). Furthermore, transgenic expression of 5-HT1a in the mushroom bodies rescues the short-sleeping phenotype of the 5-HT1a *Drosophila*null mutants, implicating the mushroom bodies as the site of action for 5-HT1a sleep regulation (Yuan et al., 2006). Furthermore, Haynes et al., 2015 found that expression of *tryptophan hydroxylase* (*TRH*) RNAi in the dorsal paired medial (DPM) neurons that project to the MBs is sufficient to significantly reduce sleep. Most recently Qian et al., 2017 found that in addition to 5-HT1a and TRH, genetic knockout of the 5-HT2b receptor reduces total sleep amount and sleep bout duration

in *Drosophila*. Further genetic characterization of 5-HT2b involvement in sleep found that 5-HT2b in the dorsal fan-shaped body is required for homeostatic sleep rebound (Qian et al., 2017). Thus, serotonin mediates both sleep and sleep homeostasis via distinct sleep/wake circuits in the *Drosophila* brain.

Various studies have gathered evidence that serotonin has a role in modulating mammalian sleep, though its role is more multifaceted than in flies. Serotonergic signaling acts differentially to promote or suppress different stages of the sleep-wake cycle depending on the serotonin receptor subtype and the brain region involved (Reviewed in Ursin, 2002). Much work has yet to be done to characterize these different components of serotonergic sleep modulation in the brain. However, pharmacological manipulations of serotonergic signaling have demonstrated effects on sleep and wake in similar ways that have been seen in *Drosophila*. For example, injecting p-chlorophenylalanine – an inhibitor of serotonin synthesis – into the dorsal raphe nucleus acutely induces insomnia in rats (Gao et al., 2002) and early experiments in humans found that administration of the serotonin precursor 5-HTP increases REM sleep duration, though total sleep is not affected (Wyatt et al., 1971). Additionally, some antidepressant serotonin reuptake and serotonin receptor subtype inhibitors have been linked to improvements in subjective and objective sleep quality in depressed and non-depressed patients (Reviewed in Wilson and Argyropoulos, 2005).

2.2.2. GABA

γ-Aminobutyric acid (GABA) is considered to be the primary inhibitory neurotransmitter in vertebrates and invertebrates. In *Drosophila,* GABA-producing cells occur in small clusters that innervate large numbers of cells throughout the entire brain. (Enell et al., 2007; Okada et al., 2009). Studies in mammalian systems have found that GABAergic cells are present in the sleep-promoting region of the hypothalamus known

as the ventrolateral preoptic nucleus (VLPO) (Gong et al., 2004) as well at the median preoptic nucleus (Benedetto et al., 2012) and that GABAergic transmission promotes sleep (Gallopin et al., 2000; Benedetto et al., 2012). Likewise, the net effect of GABAergic transmission in the *Drosophila* brain promotes sleep and advances sleep onset, since reducing the excitability of GABAergic neurons in the fly by expressing the transgene for a hyperpolarizing potassium channel significantly decreases sleep time and increases the latency to sleep at night (Agosto et al., 2008). Furthermore, administration of the GABA-A agonist 4,5,6,7-tetrahydroisoxazolo-[5,4-*c*]pyridine-3-ol (THIP) significantly increases sleep in wildtype flies (Dissel et al., 2015).

Work in *Drosophila* has demonstrated that in clock cells (Parisky et al., 2008) and also in cells critical for learning and memory (Haynes et al., 2015), GABA regulates both sleep and sleep onset. *Drosophila* neurons can express ionotropic $GABA_A$ and metabotropic $GABA_B$ receptors (Mezler et al., 2001). Parisky et al., 2008 found that ionotropic $GABA_A$ signaling in clock cells regulates timing of sleep onset during the night. The Resistant to *dieldrin* (Rdl) ionotropic $GABA_A$ receptor is expressed in the large ventral lateral neurons (l-LNvs), which are a set of neurons among the approximately 150 neurons that comprised the *Drosophila* circadian network (Reviewed in Peschel and Helfrich-Förster, 2011). Of the known clock cells, the LNvs are considered to be the principal circadian pacemaker cells in the *Drosophila* brain (Renn et al., 1999; Kaneko et al., 2000; Blanchardon et al., 2001; Sheeba et al., 2008). *Rdl* is one of three $GABA_A$ subunits in *Drosophila* and is expressed in the optic lobes, antennal lobes, mushroom bodies and the central complex (Enell et al., 2007). Knockdown of the *Rdl* $GABA_A$ receptor in PDF-expressing clock cells decreases sleep (Parisky et al., 2008; Chung et al., 2009) amount while overexpression increases sleep amount (Parisky et al., 2008). Electrophysiological recordings of l-LNvs further demonstrate that GABA

inhibits the activity of l-LNvs, suggesting that inhibition of l-LNvs promotes sleep (Chung et al., 2009; McCarthy et al., 2011). Many of the molecular regulators of GABA signaling in the l-LNvs have further been identified. For example, the protein WIDE AWAKE (WAKE) increases Rdl expression at the cell membrane at the end of the day (Liu et al., 2014) while *Drosophila* Neuroligin 4 (DNlg4), a critical cell adhesion molecule, is required for proper clustering of Rdl in the l-LNvs (Li et al., 2013). Both WAKE and DNlg4 are necessary for sleep in clock cells since knockdown of *WAKE* and *DNlg4* in PDF-expressing neurons decreases sleep amount and increases the latency to sleep in *Drosophila* (Liu et al., 2014; Li et al., 2013). In contrast, Fbxl4, an E3 ubiquitin ligase modulates sleep through its promotion of Rdl degradation in clock cells (Li et al., 2017). Both genetic mutation of the *fbxl4*gene and knockdown of *fbxl4* in PDF cells increases sleep (Li et al., 2017). Thus, molecules that regulate expression and degradation of GABA consequently affect wake behavior in the fly.

Finally, GABA release from the dorsal paired medial (DPM) neurons has been shown to promote sleep by inhibiting the MBs (Haynes et al., 2015). Inhibiting GABA release from the DPM neurons decreases sleep time. Furthermore, RNAi targeted towards *Rdl*$GABA_A$ receptors and $GABA_B$-R3 receptors in the MBs recapitulates the sleep phenotype, suggesting that both ionotropic and metabotropic GABA signaling in the MBs promotes sleep. These results illustrate the role of GABA in promoting sleep and regulating sleep onset in *Drosophila.* In mammals, cells in the ventral lateral preoptic area (VLPO) of the hypothalamus – a critical sleep-promoting region in the brain – are GABAergic (Gallopin et al., 2000) and administration of GABA directly into the VLPO of rats increases sleep amount (Xiong et al., 2012). In this case, GABA-mediated inhibition of wake-promoting mushroom body neurons by the dorsal paired medial neurons shares

similarity with the inhibitory actions of the VLPO on arousal centers in the mammalian brain.

2.3. Dual function neurotransmitters (see Table 1)

2.3.1. Glutamate

Glutamate is one of the primary excitatory neurotransmitters in the mammalian central nervous system. In *Drosophila,* glutamate is the primary excitatory neurotransmitter at the neuromuscular junction (Bogdanik et al., 2004; DiAntonio, 2006; Rival et al., 2006) similar to acetylcholine in mammals. However, glutamate is also widely expressed in the *Drosophila* central brain (Sinakevitch-Pean et al., 2001). There is evidence that glutamatergic signaling has wake-promoting effects in the fly (Zimmerman et al., 2016). Transgenic activation of CNS-specific glutamatergic cells using a temperature sensitive gal80 protein to drive expression of a bacterial sodium channel in cells containing the glutamate transporter protein (VGLUT) significantly increases wake during both the day and night (Zimmerman et al., 2016). Conversely, suppressing glutamatergic signaling by expressing a heat-induced transgenic potassium channel in VGLUT cells significantly reduces wake, though the effect is only significant during the night. These results suggest that glutamate signaling has a wake-promoting effect on *Drosophila* sleep regulation.

In addition to evidence that glutamate promotes wake, there is also data that demonstrates that glutamate signaling has sleep-promoting effects in *Drosophila*. Tomita et al., 2015 found that flies with a genetic loss of the NMDA type glutamate receptor (Nmdar1) are hyperactive and demonstrate significant reductions in sleep. Additionally, flies fed the NMDAR antagonist MK-801 also displayed significant reductions in sleep (Tomita et al., 2015). Together, these results suggest

that *Drosophila* NMDA glutamate receptors likely mediate a sleep-promoting signal in the fly under normal conditions. Interestingly, pharmacological inhibition of NMDAR activity also induces wake in hibernating arctic ground squirrels (Jinka et al., 2012). Though hibernation-induced torpor is likely regulated differently than sleep, this finding does demonstrate that some wake-inhibiting action of NMDA signaling is conserved across different species. Robinson et al., 2016 also found that enhanced *Drosophila* vesicular glutamate transporter (DVGLUT) expression as a result of knocking down the RNA-editing gene Adar (Adenosine deaminase acting on RNA) in neurons significantly increases total sleep time during both the day and night. These effects involve NMDAR and AMPAR signaling, since knockdown of NMDAR subunits NR1 and NR2 as well as knockdown of the AMPA receptor GluRI rescues the sleep phenotype of Adar mutants (Robinson et al., 2016). Recently, Liu et al., 2016 found that homeostatic sleep drive after sleep deprivation is regulated by glutamatergic neurons in the ellipsoid body of the *Drosophila* brain. Extended wakefulness induces an increase in NMDA receptor expression in a subset of neurons in the ellipsoid body is required for rebound sleep after prolonged wakefulness (Liu et al., 2016). Taken together, these data demonstrate that glutamatergic signaling has sleep-promoting effects in the fly. Since it has also been demonstrated that overall increases in glutamatergic output promote wake (Zimmerman et al., 2016), it seems likely that the location of release or the postsynaptic receptors may dictate whether glutamate signaling generates a sleep or a wake-promoting signal. Furthermore, the effects of glutamate on sleep and wake may be also be differentiated by developmental and mature brain signaling effects. These effects highlight the need to understand the role of genes and transmitters in the context of defined neural circuits.

2.3.2. Acetylcholine

Acetylcholine is a major excitatory neurotransmitter in *Drosophila*. In contrast to mammals, acetylcholine is most abundant not in the peripheral nervous system, but in the central nervous system. Acetylcholine is widely expressed in regions of the fly brain such as the protocerebrum, the mushroom bodies, and the central complex (Buchner et al., 1986; Yasuyama and Salvaterra, 1999; Salvaterra and Kitamoto, 2001). In mammals, acetylcholine is a wake- and REM-active neurotransmitter (Szerb, 1967; Saper et al., 2005; Celesia and Jasper, 1966; Reinoso-Suárez et al., 2001; Vazquez and Baghdoyan, 2001). Evidence in *Drosophila* suggests that acetylcholine acts to promote both wakefulness and sleep, depending on the neuronal groups on which it acts.

Acetylcholine provides excitatory input to the wake-promoting large ventrolateral neurons (l-LNvs) – the primary circadian pacemaker cells in the *Drosophila* brain – and modulates their firing (McCarthy et al., 2011). Whole-cell recording of l-LNv activity *in vitro* demonstrates that l-LNvs fire with a synchronized rhythm and that activation of nicotinic acetylcholine receptors (nAChRs) on the l-LNvs increases their excitation and firing (McCarthy et al., 2011). Application of acetylcholine and nicotine, both nAChR agonists, depolarized the l-LNvs and caused action potential firing while treatment with the nAChR antagonists curare and α-BuTX eliminated action potential firing of the l-LNvs (McCarthy et al., 2011). Acetylcholine also induces wakefulness via G-protein coupled signaling in a subset of wake-promoting mushroom body neurons (Yi et al., 2013). Thus, acetylcholine likely promotes wake by exciting a part of the circadian circuitry in *Drosophila* known to modulate wakefulness as well as by activating receptors on wake-promoting cells in the brain.

While acetylcholine provides excitatory input to wake-promoting clock cells, there is also evidence that it promotes sleep as well. Expression of RNAi against *vesicular acetylcholine transporter (vAChT)* – which is necessary for the release and transport of acetylcholine – in a subset of MB α/β core neurons significantly decreases in sleep, suggesting that acetylcholine promotes sleep via acetylcholine transmission from these cells (Yi et al., 2013). Notably, these sleep-promoting cholinergic neurons neighbor a layer of cholinergic *wake-promoting* cells as described in the previous section. Thus, neurons within the same brain region that release the same neurotransmitter can have opposing effects on behavioral state depending on their location (and likely their downstream targets as well).

These studies on individual neurotransmitter systems and their involvement in sleep regulation in *Drosophila* has led to an increasingly comprehensive understanding of the neuronal landscape of sleep regulation in the fly brain (See Table 1). For example, we can now identify brain regions such as the mushroom bodies or the dorsal fan-shaped bodies as sleep-regulatory centers and have also characterized different excitatory and inhibitory circuits that modulate sleep and wake in the fly (see Fig. 1). In mammalian systems, there is evidence of a mutual inhibitory flip-flop mechanism between wake-active and sleep-active cells (Saper et al., 2010) and while there is currently no direct evidence of this in *Drosophila,* the identification of different interacting sleep and wake circuits makes this seem likely. Unlike the cell autonomous regulation of circadian clock, the interaction of neuronal circuits fundamentally controls sleep and wake in mammals, and this appears to hold true in *Drosophila.*

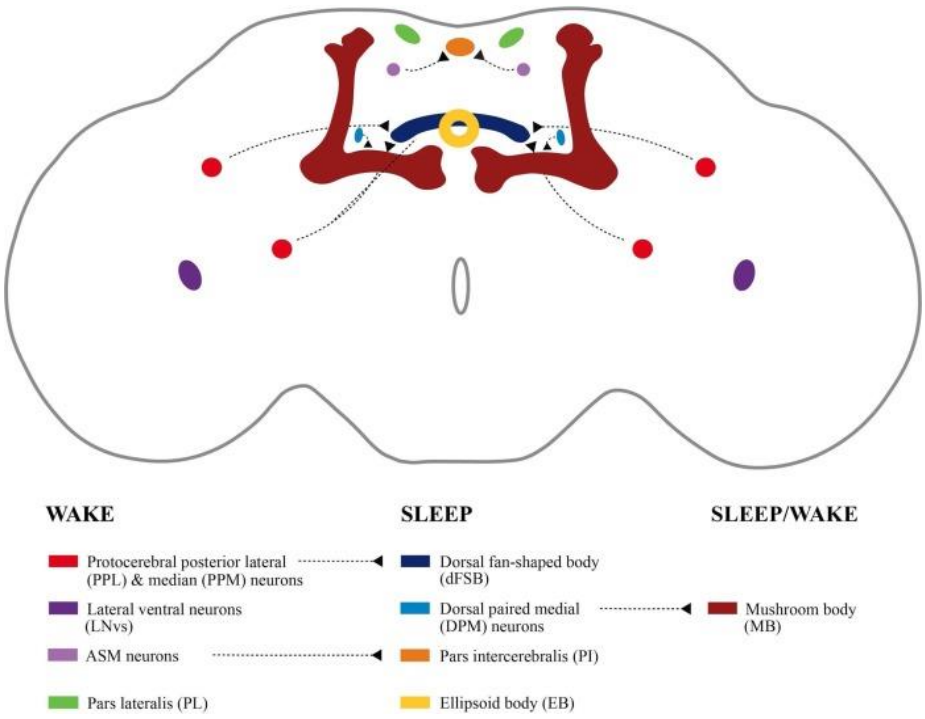

Figure 1. *Drosophila* brain regions involved in sleep and wake regulation. Dashed lines in the key indicate known functional connection between regions.

The identification of sleep- and wake-relevant neurotransmitters demonstrates that there are multiple critical brain centers that regulate *Drosophila* sleep (See Fig. 1). Some of the primary regions that regulate sleep in the *Drosophila* brain are the mushroom body, the dorsal fan-shaped body, and the pars intercerebralis and pars lateralis. The lateral ventral neurons, which are a critical part of the circadian network of the fly brain, are also required for sleep and wake regulation. Some of these regions such as the dorsal fan-shaped body primarily promote sleep while regions such as the mushroom body contain both sleep and wake promoting neurons. Additionally, there are various cells and loci throughout the brain regulate sleep and wake via synaptic connections with cells is these primary regions.

3. Signaling pathways and molecules that regulate Drosophila wake/sleep

3.1. Wake-promoting signaling pathways and molecules (see Table 2)

3.1.1. Wake-Promoting Ion Channels

Ion channels are critical components of electrical signaling among neurons in the brain. Four different ion channels have been implicated in *Drosophila* wake regulation: the Ca-α1T calcium channel (Jeong et al., 2015), the Dα3 nicotinic acetylcholine receptor(Wu et al., 2014), the potassium channel Sandman (Pimentel et al., 2016), and the TrpA1 cation channel (Lamaze et al., 2017). While mammals express three different T-type calcium channels, Ca-α1T is the single T-type calcium channel that is expressed throughout the *Drosophila* brain and it has been demonstrated to promote wake (Jeong et al., 2015). *Drosophila* mutants that do not produce Ca-α1T exhibit a significant sleep increase that suggests that Ca-α1T is wake-promoting (Jeong et al., 2015). Knockdown of Ca-α1T that is limited to neurons recapitulates the effect of the Ca-α1T genetic deficiency, demonstrating a brain-specific role for Ca-α1T in sleep/wake (Jeong et al., 2015). In mammals, different types of genetic and pharmacological examination of sleep regulation involving T-type calcium channels have yielded divergent effects on delta wave sleep (Anderson et al., 2005) (Kraus et al., 2010), so understanding how the phenotypes seen in the fly relate to more complex systems will require further investigation. The *Drosophila* nicotinic acetylcholine receptor (nAChR) Dα3 is another wake-promoting ion-channel that regulates wake (Wu et al., 2014). Wu et al., 2014tested the hypothesis that upregulation of nicotinic acetylcholine signaling was responsible for the short-sleeping phenotype of Sleepless (SSS) and Shaker mutants (both discussed in greater detail later) by administering mutants the nAChR antagonistmecamylamine (MCA) and observing the effect on sleep (Wu et al.,

2014). In support of this hypothesis, MCA rescued the short-sleep in both mutants. Furthermore, of the 10 nicotinic acetylcholine receptors in the fly, the Dα3 subunit appears to mediate changes in wakefulness since RNAi-mediated knockdown of *Dα3* rescued the short sleep in the SSS mutant background (Wu et al., 2014). In the dorsal fan-shaped body, the potassium channel Sandman acts as a wake-promoting ion channel (Pimentel et al., 2016). Translocation of Sandman to the plasma membrane is responsible for suppressing activity of dFSB neurons to increase wakefulness (Pimentel et al., 2016). Finally, the cation channel TrpA1 was recently found to be responsible for Prolonged Morning Wakefulness (PMW) – a delayed onset of daytime siesta sleep – in male flies in response to elevated temperature. Lamaze et al., 2017 found that both loss-of-function mutation of *TrpA1* as well as RNAi-mediated knockdown of *TrpA1* in neurons suppresses PMW in male flies in response to temperature increase. Thus, TrpA1 is responsible for increasing daytime wakefulness in male flies at elevated temperatures. These results demonstrate a wake-promoting role for ion signaling in *Drosophila* and reveal some of the dynamics that occur at the cellular level that directly modulate the activity of sleep/wake relevant circuits to modulate sleep behavior.

3.1.2. PKA/CREB pathway

3.1.2.1. PKA/CREB

Studies examining the role of cyclic AMP (cAMP), protein kinase A (PKA), and cAMPresponse element binding protein (CREB) have identified the cAMP-dependent pathway as one of the principal wake-promoting pathway in *Drosophila* (Hendricks et al., 2001; Joiner et al., 2006). As previously mentioned, CREB regulates circadian rhythms (Belvin et al., 1999). However, it has also been found that the activity of CREB also directly modulates sleep amount. Levels of

cAMP, PKA, and CREB are inversely correlated with the amount of sleep observed in the fruit fly (Hendricks et al., 2001; Joiner et al., 2006). Joiner et al., 2006 determined that the regulation of sleep by PKA occurs in the *Drosophila* mushroom bodies (Joiner et al., 2006). Since CREB is a transcription factor, its involvement in regulating sleep suggests that some genes whose transcription are affected by CREB will be wake-promoting genes though these genes have not been identified. It should be noted that cAMP involvement in sleep regulation is conserved in mammals as rats that are administered the drug Rolipram – which increases cAMP levels – subsequently display increased wake (Lelkes et al., 1998). Mice who have deletion of two of these isoforms of CREB, i.e, CREB hypomorph, have reduced wakefulness, particularly in the early part of the lights off period (Graves et al., 2003). CREB is also involved in regulating the daily rhythms of rest:activity in the fly. CREB is required for normal rhythms, since flies carrying a mutation in the CREB gene leading to its disrupted expression display disrupted and shortened circadian rhythms (Belvin et al., 1999). Furthermore, a loss-of-function CREB mutation results in a concurrent decrease in the expression of the gene period, a key component of the *Drosophila* clock (Belvin et al., 1999). Not surprisingly, other molecules that act on the PKA pathway have been found to play a role in sleep regulation. One of these molecules is the *amnesiac* (AMN) neuropeptide.

The *amnesiac* (*amn*) gene encodes an AMN neuropeptide that shares homology with adenylate cyclase-activating peptide (PACAP) in mammals (Liu et al., 2008). PACAPs are activators of the PKA pathway. (Figiel and Engele, 2000). The *amn* gene does not appear to regulate sleep amount. Instead, *amn* is involved in the regulation of the onset of sleep as well as the maintenance and consolidation of sleep, since flies that carry a loss-of-function amnesiac allele have fragmented sleep and a shorter latency to

sleep (Liu et al., 2008). The characterization of sleep changes in *amn* mutants suggests that distinct genes regulate sleep duration and architecture.

3.1.2.2. Calcineurin

Calcineurin is a Ca^{2+}/calmodulin-dependent serine/threonine protein phosphatase (Rusnak and Mertz, 2000) whose activity affects sleep amount in *Drosophila* (Tomita et al., 2011; Nakai et al., 2011). Calcineurin dephosphorylates target proteins to regulate their activity and studies in long-term potentiation show that calcineurin is antagonized by PKA activity (Winder et al., 1998). Tomita et al., 2011 knocked down calcineurin in neurons using RNAi and observed decreased sleep in flies. In contrast, increasing calcineurin activity by expressing a constitutively active form of calcineurin in neurons increased total sleep time. Nakai et al., 2011 similarly found that loss-of-function mutations of calcineurin subunits as well as a knockout of the calcineurin regulator *Sarah (sra)* decreased sleep. However, in their analysis of the effect of constitutively active calcineurin on sleep Nakai et al., 2011 also found that neuronal expression of active calcineurin decreased, rather than increased sleep. A possible explanation for the difference in results may be that the temporal differences in the calcineurin manipulations could have produced the different effects. Tomita et al., 2011 used a temperature-induced expression (McGuire et al., 2003) of the constitutively active calcineurin during adulthood while Nakai et al., 2011 expressed the active calcineurin throughout development. Increasing calcineurin activity during development could affect circuit formation and produce sleep effects that are independent of their effects in adulthood following normal development. Indeed, developmental effects of any manipulation are an important consideration in all animal model research. This may be particularly true for sleep which is inherently a circuit property.

3.1.3. Monoamine signaling

Vesicular transporters are responsible for packaging neurotransmitters into secretory vesicles at presynaptic sites of release (Erickson and Varoqui, 2000). The *Drosophila*vesicular monoamine transporter (VMAT) is a vesicular transporter that packages monoamine neurotransmitters into vesicles. Monoamine neurotransmitters have been shown to promote wakefulness (see above). In *Drosophila*, these monoamine neurotransmitters include octopamine, dopamine, serotonin and histamine (Busch et al., 2009; Mao and Davis 2009; Sitaraman et al., 2008; Oh et al., 2013). Nall and Sehgal, 2013 conducted a small-molecule screen by treating flies with 1280 small molecules and assaying the response to sleep. From this study, it was discovered that treatment with the VMAT inhibitor reserpine – which prevents the transport of monoamines into presynaptic vesicles – significantly increased sleep in flies, a phenotype that is recapitulated in genetic null VMAT mutants (Nall and Sehgal, 2013). The effects of reserpine were not isolated to the transmission of any single monoamine, as genetic deficiency of various monoamines did not affect the response of the flies to reserpine (Nall and Sehgal, 2013). This suggests that VMAT regulates sleep through its involvement in neurotransmission for multiple neurotransmitters. These results illustrate the wake-promoting nature of VMAT activity and highlight the utility and feasibility of drug screens in *Drosophila*.

3.1.4. Fragile-X Mental Retardation Protein

The Fragile-X Mental Retardation Protein (FMRP) modulates the activity-dependent pruning of synapses as well as synaptic plasticity in *Drosophila* (Tessier and Broadie, 2008; Mercaldo et al., 2009). FMRP is the protein product of the fragile-x

mental retardation gene (Fmr1), and levels of Fmr1 are inversely correlated to sleep time as evidenced by increased sleep being observed in flies with loss-of-function of Fmr1 and decreased sleep upon overexpression of the Fmr1gene (Bushey et al., 2009). FMRP been recently identified as a wake-promoting protein (van Alphen et al., 2013), as flies carrying a loss-of-function allele for FMRP display an increased sleep intensity during the day, as evidenced by a decreased responsiveness to mechanical stimuli (van Alphen et al., 2013). This finding illustrates a potential molecular connection linking synaptic plasticity and sleep regulation in the fly (see further below).

3.1.5. Salt-inducible kinase 3

Recently, the first forward genetic analysis of sleep in mice was conducted and identified two novel regulators of sleep, Salt-inducible kinase 2 (SIK3) and NALCN (Funato et al., 2016). Random mutagenesis of SIK3 and the sodium leak channelNALCN produced dominant mutations that produced *Sleepy (Slp)* and *Dreamless (Drl)*mice that have increased non-REM and decreased REM sleep, respectively (Funato et al., 2016). While NALCN had previously been identified as a regulator of normal circadian rhythms in *Drosophila* (Flourakis et al., 2015), Funato et al., 2016 also demonstrated that in addition to altering sleep in mice, genetic reduction of SIK3 activation in neurons increased sleep, confirming that the wake-promoting effect of SIK3 is conserved across species. This study re-emphasizes the usefulness of unbiased forward genetic screening and also reconfirmed the conservation of pathways in sleep across species. The molecules identified through these methods have further helped identify both widespread signaling pathways and distinct physical loci in the brain that regulate the sleep and wake cycle of the fly and continue to lead to new insights that are applicable across species.

3.2. Sleep-promoting signaling pathways and molecules (see Table 2)

3.2.1. Sleep-promoting ion channels

One of the important discoveries that arose from unbiased forward genetic screening in *Drosophila* was the finding that ion channels regulate sleep. In a forward genetic screen, selected phenotypes or behavioral abnormalities are identified from a population of flies that have undergone mutagenesis in order to identify genes responsible for these behaviors. Methods for generating *Drosophila* genetic mutants include ethyl methanesulfonate (EMS) mutagenesis (Konopka and Benzer, 1971), P-element insertional mutagenesis (Cooley et al., 1988), piggyBac insertional mutagenesis (Horn et al., 2003), Minos insertional mutagenesis (Uchino et al., 2007) and RNA interference (RNAi) (Rogulja and Young, 2012). Cirelli et al., 2005 identified the *Shaker* mutant from an EMS mutagenesis screen; a point mutation in the gene encoding the Shaker fast-acting voltage-gated potassium channel that results in its loss of function induces a short-sleeping phenotype (Cirelli et al., 2005). The sleep-regulatory role of Shaker appears to be conserved in mammals, as mice that are null for the Shaker-like channel Kcna2 are also short-sleepers, though notably they also exhibit frequent seizures and have significantly shortened lifespans (Douglas et al., 2007). Other mutations in genes involved in the regulation of Shaker activity were subsequently found to also affect sleep in *Drosophila*. For example, loss-of-function mutants for the gene *Hyperkinetic,* which encodes a regulatory subunit of the Shaker channel, also exhibit a short-sleeping phenotype (Bushey et al., 2007). Additionally, the protein Sleepless (SSS), identified through an independent forward genetic screen for sleep mutants, is a regulator of Shaker that is required for normal sleep regulation. Loss of SSS results in markedly reduced sleep in *Drosophila* (Koh et al., 2008). SSS is predicted

to be a member of the Ly-6/neurotoxin superfamily of proteins, which encompass proteins such as ion-channel-modulating snake neurotoxins (Tsetlin 1999; de Weille et al., 1991), cell-surface proteins and receptors (Davies et al., 1989; Wilhelm et al., 1999), and signaling molecules (Adermann et al., 1999; Tsuji et al., 2003; Huai et al., 2006). SSS was further shown to regulate Shaker expression and localization; genetic loss of SSS results in decreased Shaker expression and altered localization (Wu et al., 2010). In flies that are null for SSS, Shaker expression is greater in cell bodies than in the antennal nerve (Wu et al., 2010). Lastly, the *Drosophila* ATP-sensitive potassium channel dSur was identified as a sleep-promoting ion channel (Allebrandt et al., 2013). dSur is the fly homologue of mammalian SUR2 and knockdown of dSur in *Drosophila* neurons significantly reduces nighttime sleep, suggesting that dSur promotes sleep in the fly (Allebrandt et al., 2013).

3.2.2. EGF Signaling

The epidermal growth factor receptor (EGFR) is responsible for many different functions in the cell, including cell proliferation and differentiation. Epidermal growth factor receptor (EGFR) signaling is a sleep-promoting pathway in *Drosophila* (Foltenyi et al., 2007). This sleep-promoting role of EGFR signaling is conserved in *C. elegans* (Van Buskirk and Sternberg, 2007) and in mammals, EGFR signaling in the hypothalamus is required for circadian rhythms (Kramer et al., 2001) and promotes sleep (Kushikata et al., 1998). In *Drosophila,* there are four ligands for EGFR which must be activated by the processing proteins Star and Rho. Increasing the expression of Star and Rho increases sleep through activation of extracellular signal-regulated kinase (ERK), while decreased function of the EGFR pathway induced by RNAi of Rho decreases sleep (Foltenyi et al., 2007). Thus, activity of the EGFR pathway acts as a sleep-promoting signal in *Drosophila.* Interestingly, the role of EGF in sleep appears to be conserved in

mammalian systems, as ICV-administered EGF promotes sleep in rabbits (Kushikata et al., 1998), the EGF receptor ligand transforming growth factor-alpha (TGF-alpha) suppresses locomotion in hamsters and mice carrying a mutation resulting in hypomorphic EGF signaling are hyperactive (Kramer et al., 2001).

3.2.3. G protein signaling

G protein-coupled receptors (GPCRs) are the most abundant family of receptors in both vertebrate and invertebrate systems. GPCRs act through G proteins which are critical for the transduction of numerous intracellular signaling cascades. In *Drosophila,* G protein activity promotes both sleep and wake, depending on cell types involved. Guo et al., 2011 found the first evidence that G protein signaling in the brain promotes sleep, since pharmacogenetic enhancement of both wildtype and constitutively active Go expression in neurons led to significant increases in sleep time in the fly. This wake-promoting effect of Go on sleep was found to occur in the mushroom bodies; specific activation of Go in the mushroom bodies promotes sleep while RNAi- or pertussus toxin (PTX)-mediated inhibition of Go signaling in the mushroom bodies decreases sleep time (Guo et al., 2011). This data indicates a wake-promoting role for Go signaling, but further characterization of G protein signaling in smaller subsets of cells within the mushroom body now indicate that there are cell-type specific effects of Go and that Go signaling in some mushroom body neurons promotes sleep. Yi et al., 2013 modulated Go signaling in a small subset of α/β core cells in the mushroom body and produced the opposite effects previously seen in the previous study using a different mushroom body driver. When G protein signaling is inhibited via heat shock treatment to induce temperature-sensitive PTX in this subset of cells, the result is increased sleep (Yi et al., 2013). Thus, there is evidence that location is important for determining the role that an intracellular signaling pathway may play in regulating sleep and wake. Later work found that binding

to the G protein-coupled receptor SIFR in the pars intercerebralis (PI) by the neuropeptide SIFamide also promotes sleep (Park et al., 2014). Thus, G proteins act in different regions of the brain to modulate both sleep and wake.

3.2.4. Homer

Synapses are dynamic structures in the brain and evidence suggests that proteins within the synapse regulate behavioral state and are also in turn modulated by sleep (Gilestro et al., 2009) (Diering et al., 2016). Homer proteins are adaptor proteins within the synapse that bind to molecules such as group I metabotropic glutamate receptors and IP3 receptors (Tu et al., 1998) and are involved in processes such as calcium signaling (Shin et al., 2003) and receptor trafficking (Shiraishi et al., 2003). While mammalian systems carry three homer genes with 6 proteins due to alternate splicing, *Drosophila* contains only a single Homer gene—D-homer with one protein (Xiao et al., 1998; Kato et al., 1998). Expression of the *Drosophila* Homer gene was found to be upregulated during sleep deprivation and downregulated during sleep (Zimmerman et al., 2006). Experimental evidence that Homer proteins are involved in sleep regulation was uncovered through the observation that flies carrying a genetic deletion of D-homer have decreased and fragmented sleep, suggesting that D-homer promotes sleep and sleep consolidation (Naidoo et al., 2012). Furthermore, loss of Homer function affects the homeostatic response to sleep loss, as Homer null mutants do not display the increased bout durations of sleep that are characteristic of rebound sleep after sleep deprivation in wildtype flies. This indicates an inability for Homer null flies to maintain consolidated sleep despite increased sleep pressure (Naidoo et al., 2012). Recent work in rodents has demonstrated that the role of Homer in sleep may be related to the need for synaptic downscaling after wake (Diering et al., 2017). During sleep, synaptic downscaling has been observed in primary motor and somatosensory cortex (de Vivo et

al., 2017) and at the postsynaptic density, the Homer protein associations with molecules such as mGluR1/5 and IP3 receptors are downregulated (Diering et al., 2017). The downregulation of these physical interactions is dependent on the dominant negative immediate early gene Homer1a (Diering et al., 2017). In Homer1a null mice, the effect of sleep on these protein interactions is lost (Diering et al., 2017). Thus, Homer signaling is a molecular mechanism of synaptic plasticity that regulates sleep and wake states and also modulates excitatory signaling at the synapse. These data strongly suggest that sleep is critically important for the regulation of synaptic homeostasis and plasticity in the brain.

3.2.5. Ubiquitin-Proteasome Signaling

A role for protein degradation pathways in sleep modulation in *Drosophila* was uncovered through a forward genetics approach. An EMS mutagenesis screen identified the *Drosophila insomniac* mutant, which displays dramatically decreased and fragmented sleep (Stavropoulos and Young 2011). The *insomniac* mutation was attributed to a deletion of a region of a gene encoding a putative adaptor of the Cullin-3(Cul3) ubiquitin ligase complex (Stavropoulos and Young, 2011), which is a critical component of cellular protein degradation. Insomniac physically associates with Cul3 *in vitro* and RNAi knockdown of Cul3 and Nedd8, an ubiquitin-like protein necessary for Cul3 activity, also decreased sleep. Two additional molecules involved in ubiquitin proteasome signaling were also recently identified as regulators of sleep. Like Cul3, the E3 ubiquitin ligase gene *highwire (hiw)* also promotes sleep. Knockdown of the E3 ubiquitin ligase gene *highwire (hiw)* in the large lateral ventral neurons (l-LNvs) significantly reduces sleep in the fly (Seugnet et al., 2017). In contrast, *fat facet (faf),* a deubiquitinating enzyme, is a wake-promoting molecule whose upregulation in the large lateral ventral neurons l-LNvs reduces sleep (Seugnet et al., 2017). Interestingly, *hiw*

knockdown in the mushroom body also suppresses sleep rebound following sleep deprivation, demonstrating that *hiw* exerts its effects on sleep within different brain circuits (Seugnet et al., 2017). Thus, molecules required for ubiquitination such as Cul3 and *hiw* appear to have sleep-promoting properties. In contrast, *faf* which negatively regulates protein ubiquitination and protein degradation, promotes wake. These studies demonstrate that protein degradation pathways can modulate sleep in *Drosophila* and may also suggest that the accumulation of non-degraded proteins might somehow increase wakefulness or suppress sleep (see also further below in discussion of unfolded protein response).

3.2.6. Cellular Stress Response Signaling

3.2.6.1. Unfolded Protein Response

In the cell, the endoplasmic reticulum (ER) is responsible for the synthesis and proper processing of proteins that are either destined to be inserted in the membrane or secreted. When misfolded proteins accumulate in the cell, this leads to ER stress that triggers a cellular response known as the unfolded protein response (UPR) (Reviewed by Walter and Ron, 2011). Immunoglobulin binding protein (BiP) is a molecular chaperone protein that is upregulated during the UPR and is a key molecular component of the response to endoplasmic reticulum (ER) stress. Studies on BiP indicate that the UPR mechanism is involved in modulating the amount of recovery sleep following sleep loss in the fly. BiP mRNA and protein levels are upregulated in brain after sleep deprivation across species (Shaw et al., 2000; Naidoo et al., 2005; Naidoo et al., 2007) and BiP modulates recovery sleep in *Drosophila* as shown by the increased recovery sleep in BiP overexpressing flies and the decreased recovery sleep in BiP dominant negative mutants (Naidoo et al., 2007). Thus, BiP, as a component of the UPR, is involved in the homeostatic response to sleep loss in flies. It is not known whether BiP

alters baseline sleep however, since BiP has not been shown to change the amounts of sleep and wake in the absence of sleep deprivation. Furthermore, Brown et al., 2014 found that inducing ER stress in young flies is sufficient to significantly fragment sleep and impair the ability to recover sleep following sleep deprivation. Pharmacological administration of UPR protein inhibitors have also been shown to reduce sleep in *Drosophila* (Ly et al., unpublished observations). These studies indicate a relationship between ER stress, the UPR, and sleep.

3.2.6.2. JNK Signaling

c-Jun N-terminal Kinases (JNK) are stress-activated kinases that are involved in many diverse functions such as development, cell survival, apoptosis, cellular proliferation and differentiation (Reviewed in Davis, 2000). Basket (*bsk*), the *Drosophila* homolog of mammalian JNK, is a sleep-promoting molecule in the fruit fly. RNAi knockdown of *bsk* in neurons using the elav-GAL4 driver produces a decrease in total sleep and the same effect is mimicked by a *bsk* knockdown that is specific to the mushroom bodies (Takahama et al., 2012). The results provide evidence for the involvement of *bsk* mushroom body activity in promoting sleep and also identify other molecular mechanisms of sleep regulation originating in the sleep-regulating mushroom body region of the *Drosophila* brain.

3.2.7. Cell Cycle signaling

Proteins that regulate cell cycle signaling also play a role in *Drosophila* sleep. Rogulja and Young, 2012 identified the cell cycle protein cyclin A as a regulator of sleep in *Drosophila.* Cyclins control the progression of the cell cycle by activating cyclin-dependent kinases. After conducting a screen of RNAi lines, they found that the gene *Regulator of cyclin A1* (*Rca1*) – the *Drosophila* homolog of early mitotic inhibitor 1 (Emi1) in mammals – promotes sleep (Rogulja and Young, 2012). Loss of neuronal *Rca1* leads

to decreased sleep caused by shortened sleep bout durations (Rogulja and Young, 2012). The same effect was observed after knocking down expression of the primary target of *Rca1,* cyclin A (CycA). Loss of cyclin A in neurons via neuronal expression of CycA RNAi also led to an increased latency to sleep after lights off, suggesting that CycA may be involved in the transition from wakefulness to sleep. It was later found that CycA is regulated by TARANIS (TARA) – a transcriptional regulator – through a separate forward genetic screen (Afonso et al., 2015). TARA partially acts in the pars lateralis (PL), which is a neuroendocrine region of the brain that is considered analogous to the mammalian hypothalamus (de Velasco et al., 2007). Knockdown of *tara* in the PL significantly reduces sleep (Afonso et al., 2017). Furthermore, Cyclin-dependent kinase 1 (Cdk1), which physically interacts with CycA, acts antagonistically to CycA and TARA and promotes wake, since overexpression of Cdk1 in the PL reduces sleep (Afonso et al., 2015). Both of these studies highlight the benefits of forward genetic screening for identifying novel regulators of sleep, and are the first studies to directly demonstrate a role for cell cycle signaling pathways in sleep regulation.

3.2.8. Neuroendocrine Signaling

3.2.8.1. Ecdysone

Sleep and neuroendocrine signaling affect one another in mammalian systems (Obál et al., 2001; Meerlo et al., 2008) and flies also display evidence of neuroendocrine involvement in sleep regulation. Ecdysone is a steroid hormone that is critical for insect development and promotes sleep in *Drosophila* (Ishimoto and Kitamoto, 2010). Flies fed 20-hydroxyecdysone (20E), the active metabolite of ecdysone, slept longer and displayed longer consolidated sleep bouts. Furthermore, genetically reducing levels of 20E or levels of functional ecdysone receptors (EcRs) both cause decreases in sleep amount as well as decreased sleep rebound in response to sleep deprivation (Ishimoto

and Kitamoto, 2010). Furthermore, overexpressing EcRs in the mushroom bodies, one of the sleep-regulating brain regions, increased sleep (Ishimoto and Kitamoto, 2010). The involvement of ecdysone in sleep promotion and regulation in *Drosophila* illustrates a mechanism through which neuroendocrine signaling is involved in regulation of sleep and notably provides evidence of developmental genes regulating behavior in the adult animal.

3.2.8.2. Drosophila *insulin-like peptide*

A component of neuroendocrine signaling in *Drosophila* occurs via *Drosophila* insulin-like peptides (DILPS). There are eight DILPS in *Drosophila* (Reviewed in Kannan and Fridell, 2013) whose roles in metabolism and aging reveal that neuroendocrine signaling in *Drosophila* is highly conserved. Cong et al., 2015 recently demonstrated that DILPs and the DILP receptor DInR regulate sleep. All mutants of the DILP1, DILP2, DILP5, DILP6 and DILP7 and a *Drosophila* DInR receptor mutant exhibit total sleep reductions. Conversely, increased expression of DILP2 and DInR led to increases in sleep. Cong et al., 2015 identified a subset of clock neurons that express DILP. Furthermore, *dilp2* transcript levels were found to be downregulated by food restriction, providing a mechanism through which starvation suppresses sleep in the fly (Cong et al., 2015). Additionally, the genetic downregulation of *Dilp2* decreases sleep while *Dilp2* upregulation promotes sleep (Cong et al., 2015). This shows that starvation directly impacts a sleep-regulating pathway in the brain to inhibit sleep. Since DILPs are a critical component of *Drosophila* metabolic regulation (as insulin signaling is in mammals), their involvement in sleep points to some metabolic regulation of sleep.

4.2.8.3. Short Neuropeptide F

Another neuroendocrine signal that regulates sleep in *Drosophila* is short neuropeptide F (sNPF) (Shang et al., 2013; Chen et al., 2013; He et al., 2013). sNPF shares sequence homology to mammalian *Neuropeptide* Y (NPY) (Mertens et al., 2002), whose signaling regulates a wide variety of processes such as food intake (Gerald et al., 1996), response to stress (Wang et al., 2013), neuronal excitability (Colmers and Bleakman, 1994), and circadian rhythms (Wiater et al., 2011). The data on sNPF suggest different possibilities for its role in sleep. Shang et al., 2013 found that activating sNPF-expressing cells by heat-induction of the dTRPA1 cation channel significantly increased time spent asleep while inhibiting activity of sNPR-expressing cells by heat-induction of the Kir2.1 potassium channel decreased sleep during the day. Furthermore, expression of dominant-negative sNPF in peptidergic neurons results in sleep fragmentation which suggests that sNPF is involved in sustaining sleep (Shang et al., 2013). In contrast, Chen et al., 2013 found that flies deficient in sNPF or its receptor sNPFR have significantly *more* sleep and that flies overexpressing sNPF or sNPFR display significant reductions in sleep duration (Chen et al., 2013). sNPF increases cAMP levels *in vitro* as well as CREB activity *in vivo* (Chen et al., 2013), suggesting that the arousal promoting actions of sNPF are likely to be related to changes in the activity of the cAMP-dependent pathway. While there are varying phenostypes observed with these different manipulations of sNPF signaling, it is possible that there are developmental effects of disrupted sNPF signaling that explain the effects of constitutive sNPF or sNPFR genetic manipulation compared to those observed upon heat-induced knockdown. This adds further support for the need to differentiate effects in adult animals from those that change development. It is also possible that the activation of sNPF-expressing cells leads to the release of some other factors that may alter the observed sleep response in a way that is different to the genetic loss of the ligand or

receptor. Together, the results from these two studies suggest that sNPF signaling is critical for sleep regulation both during development as well as in the mature brain.

3.2.9. Non-Neuronal Signaling

3.2.9.1. Glial Cells

Work utilizing the *Drosophila* model has provided interesting insights into the involvement of non-neuronal cells in sleep modulation. Recently, studies in rodent models have led to the emergence of evidence that glial cells are involved in sleep regulation (Halassa et al., 2009; Schmitt et al., 2012; Xie et al., 2013). In *Drosophila,* glial cell signaling also regulates sleep, with data pointing to the Notch signaling pathway as one of the molecular mechanisms through which this occurs. Notch is a transmembrane receptor (Wharton et al., 1985) that regulates a signaling pathway that is critically involved in nervous system development and cell fate (Reviewed in Louvi and Artavanis-Tsakonas, 2012). In *Drosophila,* Notch is expressed in glial cells (Seugnet et al., 2011b) and modulates the homeostatic response to sleep loss as well as the effects of sleep loss on learning in *Drosophila* (Seugnet et al., 2011b). Increasing Notch activity by p-element disruption of the transcription factor *bunched* in the mushroom bodies – which negatively regulates Notch activity – abolishes the homeostatic response to sleep loss. Similarly, increasing Notch signaling by overexpressing its receptor ligand *Delta* in the mushroom bodies reduces the compensatory increase that occurs in sleep in response to sleep loss as well as rescuing learning impairments that occur after sleep deprivation in flies (Seugnet et al., 2011b). Since the *Delta* ligand is expressed in neurons, binding of *Delta* to glial-expressed Notch receptors are potential mechanisms through which glial cells interact with neurons to regulate sleep in the fly. Furthermore, since glial cells are important for synaptic plasticity (Reviewed in Ben Achour and Pascual, 2010), understanding the molecular involvement of glial cells in sleep may

uncover novel mechanisms that underlie the relationship between sleep, learning and memory. In addition to Notch signaling, glial Amyloid Precursor Protein Like (APPL), the *Drosophila* homologue of amyloid precursor protein (APP), is necessary to promote wake (Farca Luna et al., 2017). Genetic knockdown of APPL in cortex glia and astrocyte-like glia – two of four different glial subtypes in the *Drosophila* brain – increases sleep at night while overexpression of APPL decreases sleep (Farca Luna et al., 2017). Knockdown of glial APPL leads to reduced expression of glutamine synthetase (GS) and the gap junction innexin 2 (Inx2), which are both involved in glutamate recycling at the synapse. Glial-specific double knockdown of both GS and Inx2 mimics the increased sleep phenotype seen following APPL knockdown. Furthermore, increasing the glutamate reuptake capabilities of glial cells through overexpression of the glutamate transport dEAAT1 rescues the sleep increases following APPL knockdown (Farca Luna et al., 2017). These results demonstrate a role for APP signaling in sleep regulation that occurs within glial cells and involves regulation of glutamate recycling pathways. Given that sleep dysfunction is associated with Alzheimer's disease (Ju et al., 2013; Lim et al., 2013), the following findings have important implications for understanding the molecular mechanisms that may regulate both sleep as well as neurodegenerative disease pathology.

3.2.9.2. Fat Bodies

Sleep and immunity are processes that exert reciprocal influence on one another. Pro-inflammatory molecules are upregulated following insufficient sleep and sleep alterations are often associated with infection and disease (Reviewed in Imeri and Opp, 2009; Gamaldo et al., 2012). Williams et al., 2007 found that the transcription factor Relish, a critical component of the *Drosophila* immune response system, is upregulated during sleep deprivation and promotes sleep in the fly (Williams et al., 2007). Relish is

the *Drosophila* homolog of NF-kappaB (NFκB) and flies heterozygous for a null mutation in the *Relish* gene display decreased and fragmented sleep patterns (Williams et al., 2007). Notably, altering Relish expression in the fat bodies, and not neurons, regulates sleep as shown by the sleep response to rescue or knockdown of Relish specifically in the fat bodies (Williams et al., 2007). Relish RNAi expression in the fat bodies significantly decreased sleep while expressing Relish in the fat bodies of Relish null mutants rescues their disrupted sleep. Changing expression of Relish in neurons had no effect (Williams et al., 2007). Furthermore, inducing an immune response by exposing flies to bacteria or injury increases sleep and requires Relish activity, as mutants null for Relish, do not display increases in sleep induced by immune response (Kuo et al., 2010). This is rescued by producing expression of Relish only in the fat bodies (Kuo et al., 2010). Thus, molecular signaling in the fat bodies is capable of altering sleep. Since fat bodies are equivalent to the liver/visceral fat in mammalian systems, this observation opens up a new avenue of investigation—how does fat influence sleep? Conversely, how does sleep affect metabolic function and energy balance? Given the increasing prevalence of obesity (James, 2004; Ogden 2012) this is an important question to investigate this mind-body interaction and will likely be a productive area of inquiry.

Table 2

Summary of Signaling Mechanisms Regulating sleep in *Drosophila Melanogaster*

WAKE-PROMOTING SIGNALS	
Pathway / Signaling Mechanism	**Molecules Involved**
Ion Channel Signaling	Ca-α1T (Jeong et al., 2015) Dα3 (Wu et al., 2014) Sandman (Pimentel et al., 2016) TrpA1 (Lamaze et al., 2017)
PKA/CREB Pathway	PKA/CREB (Joiner et al., 2007) Amnesiac (Liu et al., 2008) Calcineurin (Tomita et al., 2011) (Nakai et al., 2011)
Monoamine Signaling	VMAT (Nall and Sehgal, 2013)
FMRP	FMRP (van Alphen et al., 2013)
Salt-inducible Kinase 3	Salt-inducible kinase 3 (Funato et al., 2016)
SLEEP PROMOTING SIGNALS	
Pathway / Signaling Mechanism	**Molecules Involved**
Ion Channel Signaling	Shaker (Cirelli et al., 2005) Hyperkinetic (Bushey et al., 2007) Sleepless (Koh et al., 2008) SUR2 (Allebrandt et al., 2013)
EGF Pathway	Star (Foltenyi et al., 2007) Rho (Foltenyi et al., 2007) ERK (Foltenyi et al., 2007)
G-protein Signaling	Go protein (Guo et al., 2011) (Yi et al., 2013) SIFamide (Park et al., 2014)
Homer	Homer (Naidoo et al., 2012)
Ubiquitin-Proteosome Signaling	Cullin-3(Cul3) (Stavropoulos and Young, 2011) Fat facet (faf) (Seugnet et al., 2017) Highwire (hiw) (Seugnet et al., 2017)

Cellular-Stress Signaling	UPR factors (Brown et al., 2014) JNK (Takahama et al., 2012)
Cell Cycle Signaling	Cyclin A (Rogulja and Young, 2012) TARANIS (Afonso et al., 2015)
Neuroendocrine Signaling	Ecdysone (Ishimoto and Kitamoto, 2010) Dilp2 (Cong et al., 2015) sNPF (Shang et al., 2013) (Chen et al., 2013)
Non-Neuronal Signaling	Notch (Seugnet et al., 2011b) Relish (Williams et al., 2007) (Kuo et al., 2010) Amyloid precursor protein Like (Farca Luna et al., 2017)

4. Conservation of mechanisms and insights into sleep function

The question of why sleep is a necessary behavior applies to nearly every animal in the animal kingdom. Sleep is observed in a broad and diverse range of organisms; elephants (Tobler, 1992), seals (Lyamin et al., 2017), giraffes (Tobler and Schwierin, 1996) cockroaches (Tobler and Neuner-Jehle, 1992), birds (Roth et al., 2006), zebrafish (Yokogawa et al., 2007), jellyfish (Nath et al., 2017) and the *C. elegans*roundworm (Raizen et al., 2008) are just some examples of animals that exhibit sleep states. Clearly, sleep must serve some fundamental function that makes it necessary for survival in order to have persisted in so many different animals and environments over the course of evolution. Underlying sleep and wake behavior are also many regulatory mechanisms that are conserved across multiple species, including *Drosophila* (Allada and Siegel, 2008; Zimmerman et al., 2008a). The cross-species examination of sleep is important to understanding this behavioral phenomenon because it highlights the core principles of sleep neurobiology. The precise function of sleep is an ongoing debate and it is still not entirely clear why such a great portion of every day is spent in an unconscious state. By understanding how the brains of different species have evolved to accomplish a similar behavioral endpoint, we can pinpoint the core biological needs that drive sleep and ultimately come to a conclusion about the biological functions of sleep.

As described in the previous sections, many of the neurotransmitters and molecules in *Drosophila* that regulate sleep have parallel roles in mammals. Of the neurotransmitters that are known to affect sleep and wake in *Drosophila,* dopamine, octopamine/norepinephrine, GABA, serotonin and glutamate are all conserved in mammalian sleep regulation (Isaac and Berridge, 2003; Aston-Jones and Bloom, 1981; Gallopin et al., 2000;Xiong et al., 2012; Ursin, 2002; Fuller et al., 2011). In addition to neurotransmitters, other molecules that share sleep-regulating function

across *Drosophila* and other mammals include cAMP (Lelkes et al., 1998), EGFR (Kushikata et al., 1998; Kramer et al., 2001), and voltage-gated potassium channels (Douglas et al., 2007). The shared molecular components of sleep regulation in the fruit fly have further shed light on some key principles that underlie important theories about sleep function. There are currently many theories regarding the function of sleep (Krueger et al., 2016) and emerging evidence supporting various theories has arisen from work in the *Drosophila* animal model. Here, we explore some of these theories about sleep function by examining evidence that is shared between *Drosophila* and other studied species (see Table 3).

4.1. Synaptic homeostasis and plasticity

Plasticity is an inherent feature of all biological systems; organisms must constantly respond and adapt to changes in the environment in order to survive. Within the brain, shifting demands on different neuronal connections lead to adjustments in both synaptic transmission strength and structure (Reviewed in Holtmaat and Svoboda, 2009). There is a large amount of evidence that suggests that sleep is modulates synaptic plasticity and maintains synaptic homeostasis in the brain. Early studies found that molecular markers of synapse formation are upregulated during wakefulness and downregulated during sleep in both rats (Cirelli and Tononi, 2000a) and *Drosophila*(Gilestro et al., 2009). Furthermore, increased wakefulness is correlated with increases in the number of synapses and synapse size in *Drosophila* (Bushey et al., 2011). Using electron microscopy, de Vivo et al., 2017 recently compiled structural evidence that synaptic spine downscaling occurs during sleep in mice. Together, these data suggest that sleep is critical for modulating synaptic plasticity in the brain and that across species, sleep likely contributes to

synaptic downscaling. This notion is known as the synaptic homeostasis hypothesis, which posits that sleep is required to prune and downscale synapses synaptic connections that are strengthened during wake (Reviewed in Tononi and Cirelli, 2006). This may be important for minimizing cellular stress, increasing the signal-to-noise ratio of circuits, and recalibrating the energy demands in the brain. Electrophysiological studies in mice and rats have also found evidence supporting the theory of downscaling, since spontaneous excitatory output of cortical neurons decreases in frequency and amplitude after sleep (Liu et al., 2010). During sleep, spine formation also occurs throughout the brain (Yang et al., 2014; Diering et al., 2017), though the recent evidence strongly suggests that the net effect across all synapses is a downregulation of synapse number and size following sleep (Diering et al., 2017; de Vivo et al., 2017).

Another predominating theory about sleep function that relates to its role in plasticity is that sleep is critical for the consolidation of memories and learned information and tasks (Reviewed in Walker and Stickgold, 2006; Diekelmann and Born, 2010). Numerous studies in humans, rodents, and *Drosophila* have shown that sleep loss is consistently followed by impairments in cognitive functioning and memory (Graves et al., 2003; Killgore et al., 2006; Palchykova et al., 2006; Li et al., 2009; Tucker et al., 2010). In *Drosophila,* cells that regulate sleep have been shown to mediate learning and memory (Donlea et al., 2011; Haynes et al., 2015). For example, inducing sleep through transgenic activation of sleep-promoting dFSB-projection neurons after courtship training is sufficient to generate long-term memories that last for multiple days and affect courtship behavior (Donlea et al., 2011). Additionally, sleep induced by dFSB activation suppresses the activity of MP1 and MV1 neurons that mediate memory elimination (Berry et al., 2012; Berry et al., 2015), identifying another unique learning circuit whose activity is modulated by sleep. In a different study, both transgenetic and

pharmacological induction of sleep rescues learning and memory deficits in *Drosophila* learning mutants as well as in flies carrying a mutation in *Presenilin* that is relevant to Alzheimer's disease (Dissel et al., 2015). Thus, it is evident that in *Drosophila,* sleep and memory are functionally and anatomically linked.

4.2. Metabolism

Insufficient or disrupted sleep is associated with metabolic dysfunction in many species (Gangwisch et al., 2005; Spiegel et al., 2009; Barf et al., 2010; Wang et al., 2014), which suggests that sleep may be required for the maintenance of metabolic homeostasis. In humans, wake is associated with an upregulation of energy expenditure (Jung et al., 2011; Markwald et al., 2013) and in rodents sleep is associated with an enrichment of transcripts related to metabolic function in the periphery (Anafi et al., 2013). It has been postulated that sleep serves to regenerate energy stores that are exhausted while the brain is awake (Benington and Heller, 1995) (Scharf et al., 2008). One of the ways in which sleep might mediate metabolic homeostasis is by modulating feeding behavior. In response to starvation or hunger, sleep is suppressed in rats (Borbély, 1977) and *Drosophila* (Keene et al., 2010); this may be a conserved adaptive response to promote foraging behaviors when energy stores are low. Indeed, sleep deprivation in humans promotes food-seeking (Chapman et al., 2013) and increases the activation of regional brain activity in response to food (St-Onge et al., 2012). Interestingly, the suppression of sleep following starvation in *Drosophila* requires functional taste perception (Linford et al., 2015) which validates the postulation that sleep and feeding are functionally linked. Multiple lines of evidence in *Drosophila* also experimentally link lipid metabolism to changes in sleep and survival. For example, Thimgan et al., 2015 found that *Drosophila cycle* clock mutants that are

extremely sensitive to sleep deprivation (10 h of sleep loss is enough to induce death), starvation mitigates the effects of sleep deprivation and alterations in putative lipid metabolism gene expression underlie the protective effects of starvation against sleep deprivation in these flies. Slocumb et al., 2015 also observed that flies selected for starvation-resistance express higher levels of triglyceride stores and display increased sleep compared to controls, demonstrating that individual responses to nutrient availability are correlated with changes in sleep and energy storage efficiency. Interestingly, both starvation-resistance and longer sleep can be inherited across many generations suggesting that there is genetic component to the relationship between sleep and metabolic fitness in the fruit fly (Masek et al., 2014). Recently, the RNA/DNA binding protein *translin (tsrn)* was identified as a critical molecular component of starvation-induced sleep. *Tsrn* signals in neurons expressing Leucokinin (LK) to suppress sleep (Murakami et al., 2016). RNAi-mediated knockdown of *tsrn* in LK cells abolishes starvation-induced sleep (Murakami et al., 2016). Genetic *tsrn* flies do not have altered feeding behavior after starvation, which demonstrates that *tsrn* regulates sleep after starvation independent of feeding (Murakami et al., 2016). To date, numerous studies have identified signaling pathways that underlie a functional relationship between sleep, feeding and metabolism (Reviewed in Shukla and Basheer, 2016). In mammals, there is a growing amount of evidence of a reciprocal interaction between sleep and energy homeostasis. Recently, work in mice has demonstrated that food consumption can directly influence sleep architecture (Perron et al., 2015). Based on work in *Drosophila* and other species, it seems apparent that a conserved role of sleep may be to regulate metabolic function. Given the high prevalence of metabolic disorders, there is great public health incentive to understand how sleep and metabolism are functionally linked.

4.3. Brain development

Sleep duration is the longest during early development in humans (Iglowstein et al., 2003) and many other species (Jouvet-Mounier et al., 1970), which suggests that sleep is also vital for normal brain development. During development the growth and pruning of synapses is critical for the establishment of mature connections that will later serve the mature adult organism. Multiple lines of evidence indicate that quality of sleepduring development impacts health and cognitive function later in life. Shorter sleep durations during early life correlate with decrements in cognitive performance (Touchette et al., 2007) and irregular sleep schedules during adolescence are correlated with decreased white matter integrity as measured by fractional anisotropy(Telzer et al., 2015). Similar to mammals, *Drosophila melanogaster* sleep for longer periods of time during early life (Shaw et al., 2000; Kayser et al., 2014) and sleep deprivation during early adult development is sufficient to induce lasting learning and memory impairments (Seugnet et al., 2011a). The longer sleep times and higher arousal thresholds observed in *Drosophila* during developmental sleep are mediated by decreased inhibition of dopamine onto sleep-promoting dFSB cells (Kayser et al., 2014). Artificially overexciting these cells during development disrupts the formation of a subset of olfactory glomeruli cells and impairs courtship behavior in adulthood (Kayser et al., 2014). By identifying specific loci in the brain that regulate and are affected by developmental sleep in the fly, this study also proves that sleep is necessary for proper brain development. It also shows conclusively that altering sleep during development affects behavior in adults. In rodents, one of the developmental correlates of sleep is increased synapse elimination (Yang and Gan, 2012). In the developing mouse somatosensory cortex, synaptogenesis occurs during both sleep and wake, but synapses are eliminated at higher rates during sleep (Yang and Gan, 2012). Sleep also enhances ocular

dominance plasticity following monocular deprivation during the critical period of visual development (Frank et al., 2001). These data suggest that during development, sleep may be particularly important for synaptic remodeling.

4.4. Protein homeostasis

Protein homeostasis, also known as proteostasis, is a key feature of healthy cellular function that involves tight regulation of transcription and translation, protein folding, chaperone protein activity, and protein degradation. Wakefulness likely places many cellular demands on the brain, as neuronal connections are active and proteins are turned over or generated. As previously discussed, extended wakefulness upregulates the transcription and translation of the unfolded protein response (UPR) chaperone protein BiP in *Drosophila* (Naidoo et al., 2007), mice (Terao et al., 2003; Naidoo et al., 2005) and rats (Cirelli and Tononi 2000b; Cirelli et al., 2004). This conserved response to extended wake suggests that sleep may be critically important for modulating cellular proteostasis. Furthermore, the study of age-related changes in sleep and overall brain health points to growing evidence that intracellular proteostatic regulation and sleep are linked. One of the behavioral consequences of aging across both flies and humans is reduced sleep time and fragmented sleep architecture, while a molecular correlate of aging is an impaired unfolded protein response to sleep deprivation (Koh et al., 2006) (Naidoo et al., 2008). The presence of both dysfunctional sleep patterns and disrupted cellular stress response during aging suggests that healthy sleep may mediate healthy protein balance in the cell. Furthermore, sleep has been implicated in the disease pathogenesis in Alzheimer's disease, in which cellular protein aggregation is a key feature. Sleep dysfunction is both a common feature and risk factor of Alzheimer's disease (Ju et al., 2013; Lim et al., 2013), which is characterized by the

accumulation of intracellular tau and amyloid protein aggregation in the brain (Reviewed in Peter-Derex et al., 2015). There is mammalian evidence that sleep might promote the expulsion of unwanted proteins from the brain since injections of radiolabeled amyloid beta (Aβ) are cleared twice as fast in sleeping mice compared to those that are awake (Xie et al., 2013). Recently, it has also been demonstrated that the transgenic expression of human amyloid beta 42 (Aβ42) induces sleep fragmentation in *Drosophila* (Gerstner et al., 2017). Thus, there is evidence in different species that amyloidogenic alterations in proteostasis may be mediated by sleep and may conversely alter sleep patterns as well. A requirement for sleep to maintain protein homeostasis may underlie some of the correlation between poor sleep and disease risk and the fact that sleep disturbance is often a comorbidity of neurodegenerative disease pathogenesis as well as a feature of aging. As previously discussed, disrupting cellular proteostasis by inducing endoplasmic stress in *Drosophila* leads to impairments in normal sleep architecture (Brown et al., 2014), suggesting that the relationship between sleep and protein homeostasis is bidirectional. Similarly, in rodents, modulation of UPR pathways is sufficient to alter sleep patterns (Methippara et al., 2009). Pharmacological enhancement of the UPR PERK pathway signal via central infusion of the drug salubrinal, which prevents dephosphorylation of eukaryotic initiation factor 2α (eIF2α), increases total time spent in slow wave sleep in rats (Methippara et al., 2009). Evidence across species thus suggests that sleep regulates protein homeostasis in the brain and can be modulated by signals involved in maintaining protein homeostasis.

4.5. Oxidative stress

Oxidative stress is another known cellular consequence of sleep deprivation (Reviewed in Villafuerte et al., 2015). Sleep loss leads to lower levels

of antioxidantenzyme superoxide dismutase (SOD) in the brain of rats (Ramanathan et al., 2002), and oxidative stress markers are upregulated in the spleen and bone marrow lymphocytes of sleep-deprived mice (Lungato et al., 2016). Mackiewicz et al., 2007conducted a microarray study of cortical and hypothalamic brain tissue in mice and found transcriptional upregulation of genes encoding antioxidant proteins during sleep. Koh et al., 2006 found that flies fed paraquat – an oxidative stress-inducing compound – on a regular basis significantly shortened their lifespans and led to reduced and fragmented sleep as well as reductions in circadian rhythmicity. These studies have not yet illustrated a causative link between sleep and oxidative stress repair but certainly emphasize a relationship between oxidative stress and disrupted sleep and point to the possibility that one of the restorative purposes of sleep is to counteract oxidative stress in the brain.

4.6. Immune function

Finally, there is a great deal of evidence that suggests that sleep plays a critical role in modulating immune function (Reviewed in Imeri and Opp, 2009). A study in humans found that short sleep durations are associated with an increased susceptibility to illness and when human subjects are exposed to rhinovirus, an increased likelihood of developing a cold is associated with short sleep durations (Prather et al., 2015). Interestingly, genetic manipulations in *Drosophila* that increase sleep by inhibiting the cellular activity of wake-promoting cells also increase the chance for survival after bacterial infection (Kuo and Williams, 2014b). Furthermore, acute sleep deprivation has been shown to alter immune factor levels in multiple species (Williams et al., 2007; Wilder-Smith et al., 2013). These studies highlight the importance of sleep for

maintaining resilience against illness and also demonstrate that the relationship between immune function and sleep is conserved.

Table 3

Some putative functions of sleep based on conserved mechanisms of sleep regulation

PROPOSED FUNCTION	EVIDENCE ACROSS SPECIES*
Synaptic Plasticity	*Drosophila* (Gilestro et al., 2009; Bushey et al., 2011) Rodents (Cirelli and Tononi, 2000a; Liu et al., 2010) (Diering et al., 2017)
Learning and Memory	*Drosophila* (Donlea et al., 2011; Haynes et al., 2015; Berry et al., 2015) Rodents (Graves et al., 2003; Palchykova et al., 2006; França et al., 2015) Humans (Killgore et al., 2006; Touchette et al., 2007; Tucker et al., 2010)
Energy and Metabolism	*Drosophila* (Masek et al., 2014; Slocumb et al., 2015; Thimgan et al., 2015; Cong et al., 2015; Linford et al., 2015) Rodents (Gangwisch et al., 2005; Nikonova et al., 2010; Wang et al., 2014; Naidoo et al., 2014; Perron et al., 2015; Hakim et al, 2015) Humans (Gangwisch et al., 2005 ; Spiegel et al., 2009; Barf et al., 2010; St-Onge et al., 2012)
Development	*Drosophila* (Kayser et al., 2014) Rodents (Yang and Gan, 2012) Humans (Touchette et al., 2007; Telzer et al., 2015)
Proteostasis	*Drosophila* (Naidoo et al., 2007; Brown et al, 2014) Rodents (Terao et al., 2003; Cirelli and Tononi 2000b; Cirelli et al., 2004; Naidoo et al., 2005; Terao et al., 2006; Naidoo et al., 2008; Kang et al., 2009 ; Hakim et al, 2015)
Oxidative Stress Repair	*Drosophila* (Koh et al., 2006) Rodents (Ramanathan et al., 2002; Mackiewicz et al., 2007)

Immune Function	*Drosophila* (Williams et al., 2007; Kuo et al., 2010; Kuo and Williams, 2014a; Kuo and Williams, 2014b) Rodents (Everson, 1993; Zager et al., 2007) Humans (Drake et al., 2000; Wilder-Smith et al., 2013; Prather et al., 2015)

**Some of these references cite correlational studies*

5. Technical Considerations and Future Directions

Here, we have provided an overview of the findings from many studies that have spanned almost twenty years of scientific research. Across the board, there are some technical considerations that are important to recognize and address, since they have broad implications for how we interpret previously published data as well as how the field of *Drosophila* sleep research can continue to improve as we move forward. Some of the primary issues that should be considered when looking at all of the studies in fly sleep thus far are: limitations in single-fly analysis of sleep, sex differences in sleep, and the possible role of brain development-specific effects on sleep relating to the known sleep regulators.

At present, the most notable limitation of most systems that monitor and record *Drosophila* sleep is that they require individual flies to be separated into tubes for data collection. This limits the analysis of sleep to flies that are socially isolated throughout the experiment and introduces the confound of social isolation effects on sleep patterns. Like rodents (Kaushal et al., 2012) and humans (Kurina et al., 2011), *Drosophila* sleep is affected by social experience (Lone et al., 2016; Brown et al., 2017). Social isolation reduces *Drosophila* sleep amount (Brown et al., 2017) while social experience increases sleep (Lone et al., 2016). The changes in sleep following social isolation also leads to

cellular stress in the brain (Brown et al., 2017). For this reason, understanding the implications of findings from these traditional sleep experiments requires some consideration for the effects of social experience and studying sleep in groups (Liu et al., 2015) will provide even more information about how sleep is regulated in the brain. Additionally, there are technical differences in the way sleep is recorded from isolated flies in locomotor tubes. In the traditional set-up, fly sleep is recorded using single infrared beam breaks, where flies are placed in locomotor tubes in which a single infrared beam separates the two ends of a transparent tube. When a fly crosses this beam, activity is recorded. The benefit of this system is that it has allowed for very high throughput analysis of sleep in flies over many different genotypes and under various conditions. However, in this system, flies moving on one of side a tube may not cross the beam in the center and thus “appear” to be sleeping. Recently, work examining changes in sleep in female flies after mating found that the post-mating increases in sleep were not detectable using a single-beam system (Garbe et al., 2016). This demonstrates the necessity for higher-resolution sleep recording techniques such as multibeam systems (Garbe et al., 2016) or video systems (Zimmerman et al., 2008b) in order to capture certain sleep changes and also emphasizes the point that the absence of measurable sleep changes in a single beam system does not exclude the possibility that a sleep phenotype still exists.

Another important point to consider as we look at all of the data that has been collected on fly sleep is the sexual dimorphism of sleep in *Drosophila* (Shaw et al., 2000; Koh et al., 2006). New data is demonstrating that some modulation of sleep behavior is indeed specific to either females (Garbe et al., 2016) or males (Beckwith et al., 2017). Many of the seminal studies on *Drosophila* sleep report findings within a specific sex. It is possible that some of what we know about sleep in the fly may not apply broadly to

both sexes, depending on the type of regulation that we are studying. These current studies examining sex-specific sleep behavior will provide insight into whether this might be the case and if so, what types of sleep regulators should be focused on in the context of sex-specific sleep regulation.

Finally, we must also bear in mind the possible role that development plays in producing phenotypes from specific genetic mutations in the fly. Any mutation that is present during development may exert their effect on sleep by altering the development of interacting circuits in the brain. This is especially important for understanding sleep and wake regulation. Unlike cell-autonomous clock mechanisms, sleep and wake regulation is dependent on multiple interacting circuits. Furthermore, individual proteins or molecules may have differential expression or function during development that could affect its role on sleep in the brain at various stages of life. Many more recent studies have employed conditional induction or repression of genes using conditional expression systems (Nicholson et al., 2008) in adult flies to circumvent these caveats. When considering developmental changes in brain development, we should also think about the time-frame during which behavioral data is collected. Some brain circuits do not fully mature until about a week after eclosion (Sinakevitch et al., 2010; Kayser et al., 2014). Despite this, many studies are conducted in flies that are few days post-eclosion. This raises the possibility that the same flies studied at older ages might reveal slightly different phenotypes that could differentiate the developmental role of various genes from the role of the gene in the adult brain. Overall, keeping all of these technical considerations in mind will allow researchers to better discern the implications of their own results as well as design stronger experiments to minimize technical confounds in their data.

Moving forward, we believe that it will be important to directly investigate the

functional relationships between the already identified players in *Drosophila* sleep. Thus far, most of the results from different published studies provide support for the findings of one another. For example, the study of sleep regulation by dopamine has uncovered a functional relationship between the wake-promoting PPL and PPM neurons (Liu et al., 2012b; Ueno et al., 2012) and the dorsal fan-shaped body (Donlea et al., 2011; Pimentel et al., 2016). Even this immensely well-characterized circuit leaves room for further investigation, as it has not, for example, been demonstrated that activation of only the PPL and PPM neurons directly suppressed dFSB activity. The knowledge gleaned thus far on sleep in the fly initiates new and more focused lines of inquiry. Furthering our understanding of the known sleep and wake regulatory pathways will allow us to determine what pathways are acting in parallel to regulate sleep and which instead converge to promote either sleep or wakefulness in the fly. This will ultimately provide greater insight into how the brain operates on a large scale to orchestrate complex outputs like sleep. While this is no simple task and will require careful dissection and methodical examination, this endeavor remains well-suited to be answered in the fly in ways that are similar to the methods used by the studies that have been described here.

6. Conclusion

More than a century since Thomas Hunt Morgan became one of the first scientists to use the fruit fly model to study genetic inheritance (Morgan, 1910), *Drosophila melanogaster* remains at the forefront of scientific inquiry. In the early days of modern *Drosophila* research, the fruit fly was the ideal eukaryotic model for forward genetic screening which led to a deeper understanding of the genetic basis of complex behavior. Today, modern advances in genetic techniques have broadened the scope of

Drosophila sleep studies. In the time since the complete sequencing of the *Drosophila* genome (Adams et al., 2000), a wide array of tools has been developed that present novel and elegant ways to study and manipulate neural function in the fly. For example, transgenically expressed visual markers label cells and circuits in the brain (Clyne et al., 2003; Lee and Luo, 2001; Nicolaï et al., 2010), real-time neural activity can be captured *in vivo* with genetically encoded calcium indicators and two-photon microscopy (Bushey et al., 2015), both light and heat can be used to activate or suppress neuronal activity (Reviewed in Owald et al., 2015), and electrode recordings of neurons can be conducted in waking flies (Maimon et al., 2010). The level of genetic tractability of the fruit fly is presently the forte of the *Drosophila* model. These tools allow for dynamic spatiotemporal control of the fly genome that has unprecedented precision and specificity; in some cases, these tools have mapped out the neuroanatomy of complex neural processes and behaviors with single-cell resolution (Liu et al., 2012a; FreIFeld et al., 2013; Shuai et al., 2015; Yapici et al., 2016).

The study of sleep in small model organisms has important implications for our understanding of sleep in higher order systems as well. It is now understood that many of the major neurochemical components and signaling pathways that regulate sleep in *Drosophila* are also conserved in mammalian sleep. New research discoveries continue to confirm that many of the general biological principles dictating how sleep and wake are controlled in the fly are also present in more complex species. For example, the identification of an interconnection between sleep and circadian rhythms has been recapitulated in many mammalian models since the early *Drosophila* research identifying the circadian genes and proteins that affect patterns of sleep and wake. Even at the circuit level, fruit fly sleep bears resemblance to sleep in higher order organisms. For example, sleep and wake states are partially regulated and determined by the activation

or inhibition of specific brain circuits. Within these sleep circuits, there are data which suggests that mutually inhibitory connections may be fundamental for state switching; this is true in both the fly and mammals. *Drosophila* studies have also demonstrated that there are multiple neuronal groups involved in sleep regulation that are analogous to mammalian nuclei, and a complete understanding of the *Drosophila* sleep circuit may further elucidate mammalian sleep circuits. Interestingly, there is also an increasing focus on sleep regulation by extraneuronal cell types that has also been moved forward by some work in the fly. For example, in *Drosophila,* sleep signaling from the fat bodies provided experimental evidence that peripheral signals can change sleep states. There is also evidence in mammals that peripheral tissues are affected by sleep and also that peripheral signaling contributes to the regulation of behavioral state. Thus, studies in the fruit fly continue to align with the growing body of work that is demonstrating that sleep is not only controlled by the brain but also by the interaction between the brain and the rest of the body. In all species studied to date including *Drosophila,* it is evident that sleep affects a very wide range of biological processes. This suggests that sleep likely serves multiple functions that are not only specific to the brain but have importance for peripheral organs as well. This notion that is not particularly surprising given the pervasiveness of sleep across the animal kingdom and throughout evolution.

Though sleep shares an observable similarity to death, it is critical for most life on this planet. We are hard pressed to find animals that can survive without sleep and even acute perturbations in sleep carry measurable consequences across many different types of species. Our growing understanding of sleep has provided vital insight into both the biological vulnerabilities of the brain as well as its remarkable capabilities for plasticity and repair. Perhaps the most exciting aspect of our ever expanding knowledge of sleep is the deepening complexity with which we can posit new scientific questions.

For example, *Drosophila* research has taught us that *how* certain intracellular signals affect behavior is just as important as *where* in the brain they do so. Beyond identifying new regulators of sleep, understanding what cell types or brain regions are involved in regulation by factors that have already been discovered will be just as critical to constructing a complete understanding of sleep's neurobiological mechanisms. The impressive resolution with which we currently understand of the *Drosophila* nervous system certainly suggests that we are well on our way not only to fully understanding how sleep affects the brain, but also to identifying the core functions of this mysterious behavior. Though tiny, the fruit fly has revealed much about the neurobiology of sleep and the brain. Based on the current state of *Drosophila* sleep research, it appears likely that the *Drosophila* animal model will continue to expand the boundaries of our understanding of sleep and neuroscience in the years to come.

CHAPTER 2

Homer proteins promote sleep through their interactions with metabotropic glutamate receptors

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Abstract

Homer proteins mediate plasticity and signaling at the postsynaptic density of neurons and are necessary for sleep and synaptic remodeling during sleep. The goal of this study was to investigate the mechanisms of sleep regulation by Homer signaling. Using the *Drosophila* animal model, we demonstrate that knockdown of Homer specifically in the brain reduces sleep and that *Drosophila* Homer proteins bind to the sole *Drosophila* mGluR, known as DmGluRA. This is the first evidence that DmGluRA, which bears greatest homology to group II mammalian mGluRs, also has functional homology with group I mGluRs, which couple to Homer proteins in mammals. Analysis of sleep in a *DmGluRA* genetic null suggests that DmGluRA signaling alone does not affect sleep amount since null mutants do not exhibit alterations in daily sleep time relative to wildtype. However, Homer and mGluR proteins have been shown to dissociate at the synapse during sleep, we sought to determine the functional necessity of this interaction in sleep regulation. Using the CRISPR/Cas9 gene editing system, we generated a targeted amino acid replacement of the putative binding site for Homer on DmGluRA in order to prevent Homer and DmGluRA protein binding. We found that loss of the conserved proline-rich PPXXXF sequence on DmGluRA prevents Homer/DmGluRA associations and significantly reduces sleep amount. Thus, we identify a conserved mechanism of synaptic plasticity in *Drosophila* and demonstrate that the interaction of Homer with DmGluRA is necessary to promote sleep.

Introduction

Sleep is a complex behavioral process that remains puzzling to all who seek to understand its evolutionary origins and biological machinations. Identifying the molecular mechanisms is an important endeavor towards understanding the biological function of sleep. A broad range of published evidence suggests that sleep modulates synaptic plasticity in the brain (Abel et al., 2013) (Kuhn et al., 2016). While it has been shown that the expression and localization of synaptic proteins change during sleep (Bushey et al., 2011), less is known about how plasticity-relevant proteins at the synapse might conversely act to regulate sleep and wakefulness.

At the neuronal postsynaptic density, Homer proteins regulate calcium signaling and synaptic signaling and plasticity (Kammermeier et al., 2000) (Thomas, 2002). Homer proteins maintain synaptic structure and function through their interactions with receptors and scaffolding proteins at or near the synapse (Soloviev et al., 2000) (Tu et al., 1998) (Hayashi et al., 2009). Previously, work in our lab has demonstrated that Homer proteins promote sleep and sleep consolidation; genetic loss of *Homer* in *Drosophila melanogaster* results in a short and fragmented sleep phenotype (Naidoo et al., 2012). Recently, it has also been shown that Homer signaling is required for synaptic downscaling during sleep in rodents (Diering et al., 2017). The mechanism through which Homer signaling affects sleep remains an open question. The roles of single genes and proteins in sleep regulation are commonly explored but less is known about how interactions among multiple proteins affect sleep and wake. Thus, given the nature of Homer coupling to other partners at the synapse, we sought to determine whether binding of Homer to one of its partners may mediate its effects of sleep.

In mammalian systems, Homer proteins act as adaptor molecules to group I metabotropic glutamate receptors (mGluRs) at the synapse (Brakeman et al., 1997) and

couple them to various calcium and potassium channels (Tu et al., 1998) (Kammermeier et al., 2000). mGluRs are transmembrane g-protein coupled receptors that activate intracellular signaling cascades in order to modulate synaptic plasticity and neurotransmission (Conn and Pin, 1997). While it has been shown that mGluR signaling is necessary for sleep-associated memory consolidation (Diering et al., 2017), little is known about whether mGluR signaling itself regulates sleep and sleep amount. Some rodent studies have characterized the effect of knockdown of different individual mGluR subtypes in animal models and found that loss of different mGluR subtypes produce various changes in sleep and wake (Pritchett et al., 2015) (Ahnaou et al., 2015). Genetic loss of the group II mGluRs increases wakefulness and light-mediated shifts in circadian rhythms (Pritchett et al., 2015) while null mGluR5 mice exhibit altered sleep responses after sleep deprivation (Ahnaou et al., 2015). These results suggest that different mGluRs may regulate sleep, though further analysis is required to exclude redundancy or compensatory effects from other mGluR subtypes that might follow the loss of any single mGluR.

In the following study, we investigated the role of Homer, mGluR, and Homer/mGluR interactions in regulating sleep in the *Drosophila melanogaster,* also known as the common fruit fly. In mammals, mGluRs exist as 8 different subtypes that can be classified into 3 groups (type I, II, or III) based on their amino acid sequence homology and signal transduction mechanisms (Niswender and Conn, 2010). In contrast to mammals, *Drosophila* carries just one functional gene for mGluR, known as *DmGluRA* (Parmentier et al., 1996). This confers an experimental advantage for the questions we want to answer about sleep regulation since the absence of multiple gene isoforms allows us to directly probe gene function in the absence of redundancy or compensatory actions from alternative subtypes of the same molecules. We studied

sleep/wake behavior in flies that have various deficits in Homer and mGluR signaling in order to further understand how these proteins regulate sleep/wake behavior.

Here, we demonstrate for the first time that Homer and mGluR interactions are conserved in *Drosophila*. Until now, it was unknown whether Homer couples to mGluR in the fly. We find that DmGluRA, a homolog of the mammalian group II mGluRs, physically associates with Homer proteins in *Drosophila melanogaster*. Thus, DmGluRA exhibits some conservation of mammalian group I mGluR function. Since previous studies examining the effects of genetic Homer signaling disruption have not been cell-specific, we conducted an RNAi knockdown of Homer in neurons and show that brain-specific Homer signaling is required to promote sleep. Furthermore, no study has examined the requirement of total mGluR signaling on sleep. Since *Drosophila* have only one mGluR, we examined sleep in a *DmGluRA* null mutant to address this question. Unlike Homer, total loss of DmGluRA signaling does reduce sleep, but rather, changes the distribution of sleep across the 24 hour day so that null mutants sleep more during the day and less during the night compared to wildtype animals. Finally, we show that the physical interaction of Homer with DmGluRA is necessary for Homer to promote sleep. Following a CRISPR Cas9-mediated mutation of the putative Homer binding site on DmGluRA, we find that flies become significantly shorter sleepers. These results suggest that unlike Homer, mGluR signaling in *Drosophila* is not sleep-promoting on its own but that its coupling to Homer is critical for Homer to promote sleep. Thus, we have uncovered a key feature of synaptic regulation that is conserved across species and opens the door to its continued study in the *Drosophila* model.

Results

Homer signaling in neurons is required to promote sleep

Drosophila sleep has a diurnal rhythm consisting of multiple sleep bouts that occur across both the day and night. Sleep bouts range in length from a few minutes to approximately a couple of hours and are the longest and occur primarily during the night (Hendricks et al., 2000). Sleep during the day – which occurs primarily in the middle of the day – is often referred to as a midday "siesta" (Wijnen and Young 2008). Data in both *Drosophila* and mice has demonstrated that genetic loss of either *Drosophila* Homer or the mammalian dominant negative Homer 1a disrupts normal sleep patterns and neuronal plasticity during sleep (Naidoo et al., 2012) (Diering et al., 2017). Since Homer signaling regulates synaptic transmission in both the central and peripheral nervous system, we wanted to determine whether brain-specific Homer signaling is required for sleep. We knocked down *Homer* specifically in neurons using the GAL4/UAS system (Brand and Perrimon, 1993) to express transgenic RNAi against the *Homer* gene using a neuron-specific $elav^{C155}$ GAL4 driver. We found that *Homer* knockdown in neurons significantly reduced the amount of sleep in the fly (**Figure 1A and 1B**). Analysis of wake activity shows that Homer knockdown does not lead to any changes in hyperactivity (**Figure 1E**). This recapitulates the effect of global loss of the *Homer* gene in the fly, demonstrating that Homer signaling in the brain is required to promote sleep. Analysis of sleep architecture shows that the reduction is sleep is due to fewer average sleep bouts per day but not a change in the average sleep about length (**Figure 1C and 1D**). This is different from the phenotype of a complete Homer knockdown, where sleep bout length decreases and sleep bout number increases in the *Drosophila* null Homer mutant (indicating a sleep fragmentation phenotype). From this data, it appears that loss of Homer reduces sleep similarly to complete genetic knockout

but that the sleep fragmentation phenotype is specific to the null mutant. We can conclude here that Homer signaling in the brain is required to promote sleep.

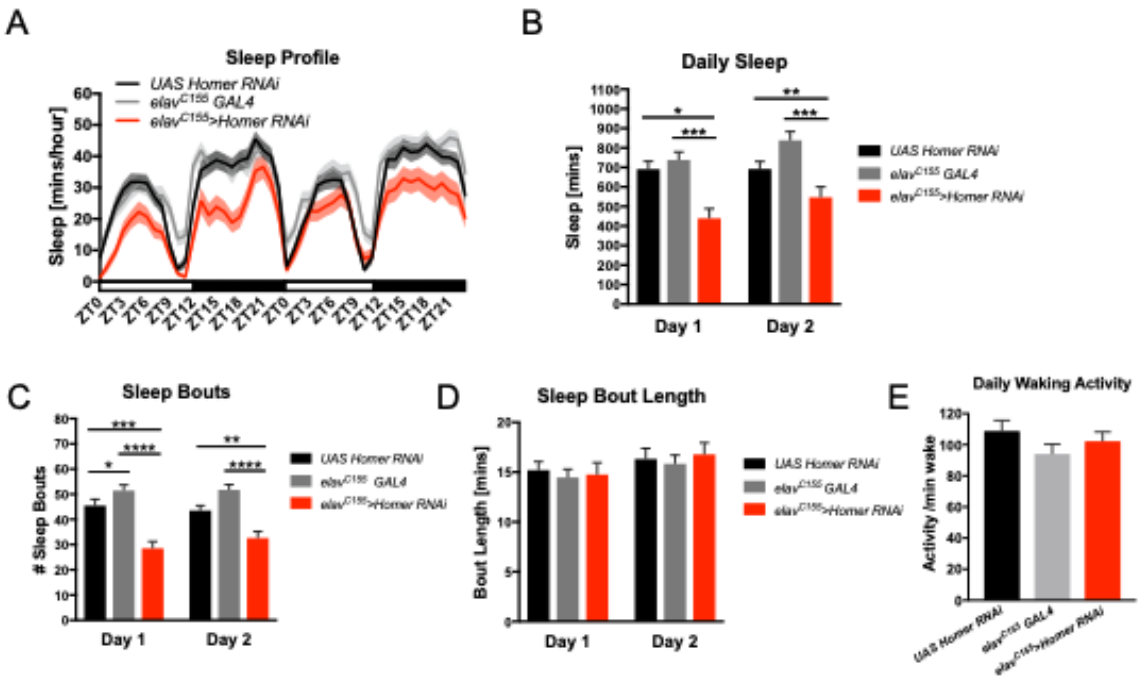


Figure 1. Neuron-specific knockdown of Homer reduces sleep in *Drosophila*
(A) Sleep profile of flies expressing Homer RNAi in neurons compared to uncrossed parental controls. Expression of Homer RNAi in neurons (expressing *elav*) reduces sleep *(N≥ 35, shaded area represents SEM)* (B) Quantification of daily sleep amounts. Total sleep is significantly reduced following Homer RNAi expression in neurons *(N≥ 35, *P<.05, **P<.01,***P<.001, ****P<.0001)* (C) The average number of sleep bouts is reduced in flies expressing Homer RNAi in neurons *(N≥ 35, *P<.05, **P<.01,***P<.001, ****P<.0001)* (D) Homer RNAi expression in neurons does not significantly change average daily sleep bout length compared to parental controls (*N≥ 35)* (E) Waking activity levels are not significantly different between flies expressing Homer RNAi in neurons compared to parental controls *(N≥ 35)*

DmGluRA is required for normal sleep patterns across the day and night

In mammalian systems, Homer proteins couple mGluR proteins to other proteins at the synapse to regulate mGluR signaling. To assess the role of mGluR signaling in regulating sleep behavior, we measured sleep in *Drosophila* mutants lacking the *Drosophila* mGluR gene, *DmGluRA.* These mutants carry a null allele of *DmGluRA* and

do not express DmGluRA protein (Bogdanik et al., 2004). After outcrossing the null *DmGluRA* mutant strain into the wildtype white Canton-Special (wCS10) genetic background, we reconfirmed that DmGluRA protein is not expressed in the null *DmGluRA* mutants (**Figure 2A**). Null *DmGluRA* mutants exhibit an altered daily sleep/wake profile relative to wildtype flies that is characterized by an increase in sleep during the day and a decrease in sleep during the night (**Figure 2B and 2C**). Genetic loss of *DmGluRA* did not change daily sleep amounts (**Figure 2D**). To confirm that null *DmGluRA* mutants are not simply hypoactive or hyperactive, we measured the rate of activity of the null *DmGluRA* mutants and confirmed that *DmGluRA* mutants did not exhibit any significant changes in activity while awake compared to wildtype flies during the sleep experiments (**Figure 2E**). We also measured climbing ability of null *DmGluRA* mutants to confirm that the null mutants do not demonstrate any basal locomotor deficits compared to wildtype flies (**Figure 2F**).

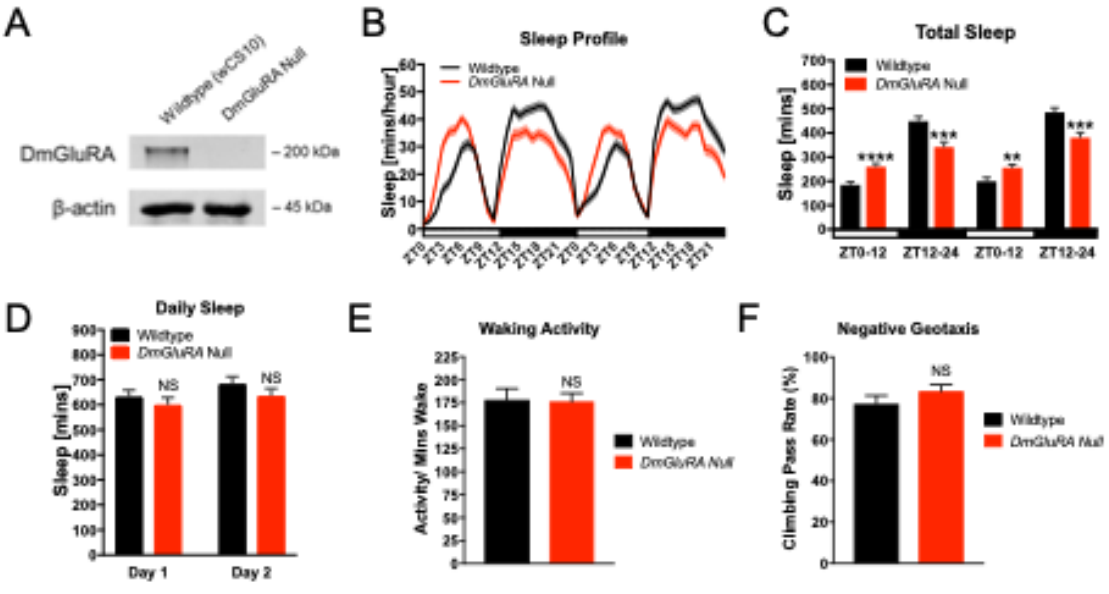


Figure 2. Genetic loss of *DmGluRA* alters the distribution of sleep across the day and night

(A) Null *DmGluRA* mutants do not express DmGluRA protein, as seen from the expression of the ~200kDa DmGluRA dimer that is expressed in wildtype flies and not in the null mutant. β-actin is shown and was used as a loading control (B) Daily sleep profile of wildtype wCS10 flies and null DmGluRA mutants averaged over the course of the entire 72 hour experiment. Null *DmGluRA* mutants exhibit an altered distribution of sleep across the day and night compared to wildtype flies *(N=70, shaded area represents SEM)* (C) Quantification of total sleep amount during the day and night. Sleep amount is higher during the day and lower during the night in null *DmGluRA* mutants relative to wildtype flies *(N=70, *P<.05, ***P<.001)* (D) Quantification of total sleep per day in wildtype flies compared to *DmGluRA* null mutants. Loss of *DmGluRA* has no significant effect on the total amount of sleep per day (E) Rate of activity is unchanged in null *DmGluRA* mutants relative to wildtype flies *(N=70)* (F) Null *DmGluRA* mutants do not have impaired locomotor ability as measured by the climbing pass rate in a negative geotaxis assay *(N=110)*

Given the daytime- and nighttime-specific effect of changes in sleep following *DmGluRA* knockdown, we sought to determine how light entrainment might contribute to the *DmGluRA* mutant sleep phenotype. We therefore measured sleep in null *DmGluRA* mutants in the absence of external light cues. While null *DmGluRA* mutants initially exhibit more sleep during active periods and less sleep during inactive periods in the first few days in the dark, after multiple days without any light cues the sleep and wake rhythms in null *DmGluRA* mutants become more similar to those observed in wildtype flies (**Figure 3A**). After multiple days without light, the sleep amount is similar during the subjective day and night between wildtype flies and null *DmGluRA* mutants (**Figure 3B**). This suggests that *DmGluRA* regulates behavioral state according to light onset and offset, maintaining wakefulness during light periods while promoting sleep during dark periods. Additionally, sleep architecture is similar between wildtype flies and null *DmGluRA* mutants in constant darkness (**Figure 3C and 3D**), further demonstrating that the effects of *DmGluRA* knockdown on sleep and wake is dependent on light entrainment.

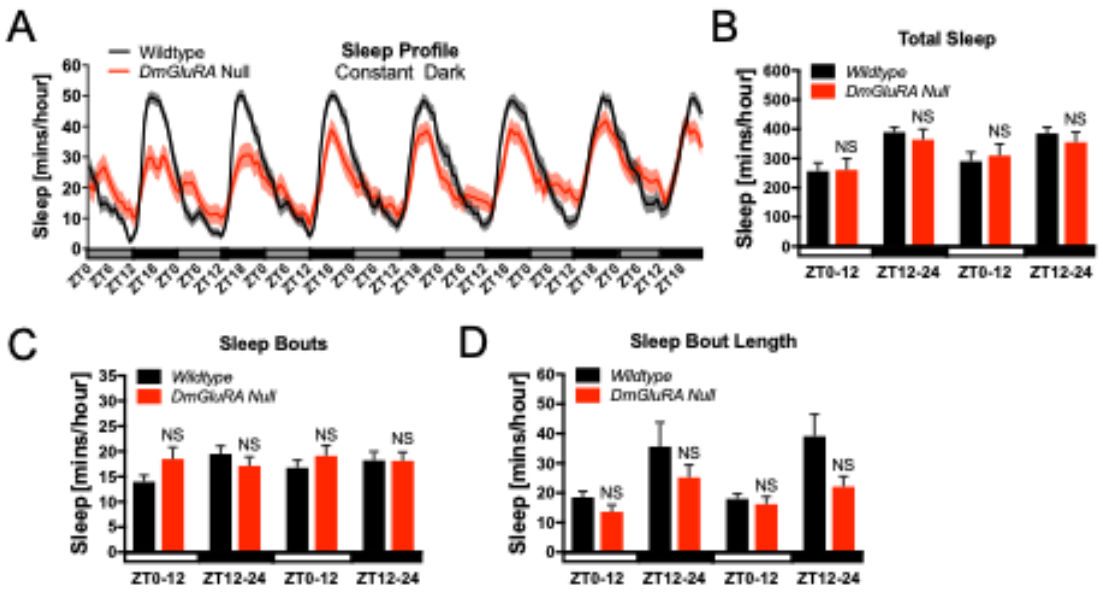


Figure 3. Null *DmGluRA* mutants have a similar sleep profile to wildtype flies in constant dark conditions
(A) 7 day sleep profile of wildtype wCS10 flies and null DmGluRA mutants in constant darkness *(N≥ 34, error bars represent SEM)* (B) Total sleep (on the last two days and nights of the sleep recording) during the subjective day and night are not significantly different between null *DmGluRA* mutants and wildtype flies *(N≥ 34)* (C) Null *DmGluRA* mutants do not exhibit any changes in the average number of sleep bouts or in the average sleep bout length (on the last two days and nights of the sleep recording) compared to wildtype flies in constant dark conditions.

Genetically altering the Homer binding site on DmGluRA reduces sleep in *Drosophila*

Though loss of DmGluRA signaling itself does not appear to change sleep amount, we wanted to address the possibility that Homer binding to DmGluRA is required for its sleep-promoting effects in *Drosophila*. To address this question, we employed the CRISPR/Cas9 system (Qi et al., 2014) (Bassett et al., 2014) for targeted gene editing to alter the putative binding site for Homer on DmGluRA in order to produce a *Drosophila* mutant in which Homer binding to DmGluRA in inhibited. In mammals, Homer binds to a proline rich PPXXF motif (Tu et al., 1999) on the C-terminus mGluR. The *Drosophila DmGluRA* gene encodes a PPGTRF amino acid sequence in the c-

terminal region of the DmGluRA gene that we identified as the putative binding site for *Drosophila* Homer proteins. Thus, we designed a guide RNA to specifically alter this site using CRISPR/Cas9 homology directed repair (**Figure 4A**). Mutant flies express alanines in place of the endogenous PPGTRF sequence (**Figure 4A**). Using co-immunoprecipitation, we demonstrate that DmGluRA co-immunoprecipitates with Homer proteins in *Drosophila* confirmed that amino acid replacement of the proline-rich region of the DmGluRA protein prevents Homer/DmGluRA association (**Figure 4B**). We examined sleep in these mutant flies and found that the binding site mutation results in significantly reduced sleep compared to genetic background controls (**Figure 4C and 5D**). Both average sleep bout number and sleep bout length are reduced in the mutant fly (**Figure 4E and 4F**). Analysis of waking activity in these mutants suggests that these flies to not exhibit any hyperactivity that might contribute to changes in recorded sleep measurements (**Figure 4G**). We analyzed Homer expression in the brains of the mutants compared to wildtype and confirmed that Homer expression is unchanged by the *DmGluRA* mutation (**Supplemental Figure 1**). We also sleep deprived flies to determine whether the PPGTRF mutation affects sleep rebound and found that sleep rebound after 6 hours of sleep deprivation at the end of the night is unchanged in the mutant flies (**Supplemental Figure 2**). These results suggest that the interaction between Homer and DmGluRA is necessary to promote sleep.

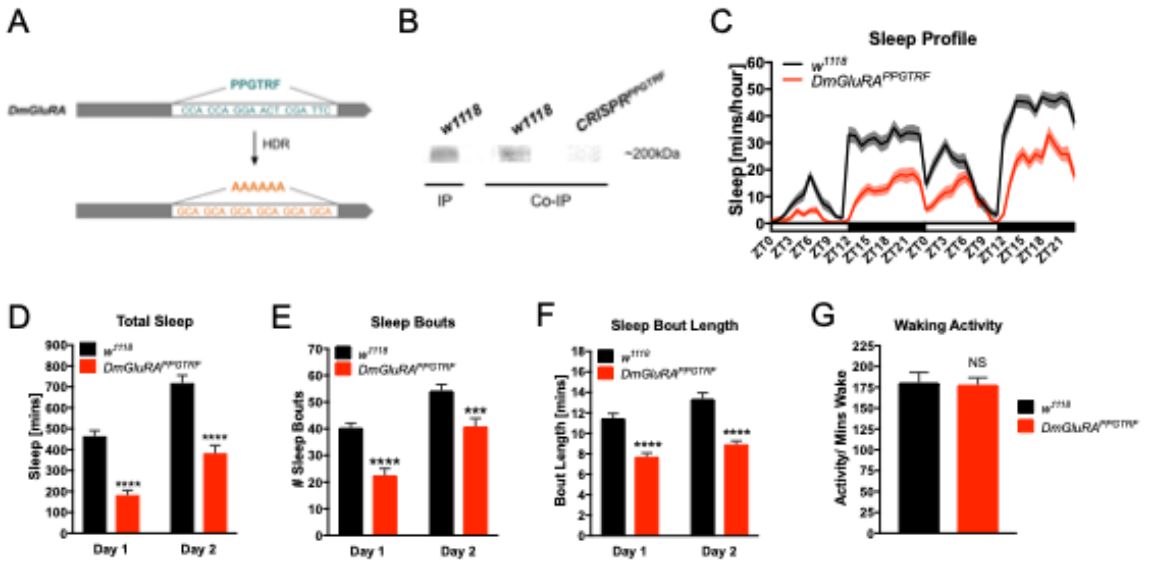


Figure 4. Homer and DmGluRA associations are required to promote sleep
(A) Schematic of the CRISPR-mediated homology directed repair of the putative binding site for Homer on DmGluRA. The PPGTRF amino acid sequence of DmGluRA has been replaced with alanines (AAAAAA) (B) Co-immunoprecipitation of mutant flies confirmed that replacement of the PPGTRF motif disrupts binding of Homer and DmGluRA protein (C) Sleep profile comparing wildtype (w1118) sleep to flies carrying the *DmGluRA*^{PPGTRF} allele over two days and nights *(N=27).* Mutant flies lacking the PPGTRF Homer binding motif have significantly reduced sleep compared to wildtype controls.(D) Quantification of sleep from Figure 5b. Mutant flies have significantly reduced sleep compared to wildtype controls (*N=27, **** P <.0001)* (E) Mutant flies have significantly fewer sleep bouts per day compared to wildtype controls (*N=27, *** P <.001,**** P <.0001)* (F) Mutant flies have significantly shorter average sleep bout lengths compared to wildtype controls (*N=27, **** P <.0001)* (G) Mutant flies lacking the PPGTRF Homer binding motif do not exhibit significant changes in waking activity compare to wildtype controls.

Discussion

In this study, we demonstrate that Homer promotes sleep through its interaction with DmGluRA in *Drosophila melanogaster*. Until now, Homer and mGluR interactions were only shown to exist between mammalian Homer proteins and group I mGluR subtypes. It was not known that the sole *Drosophila* DmGluRA – which shares the greatest homology with mammalian group II mGluRs – could interact with Homer proteins in the *Drosophila* nervous system. Conservation of this binding interaction in *Drosophila* provides evidence of functional pleiotropy in mGluR proteins in lower-level

organisms. One might speculate that over the course of evolution, the emergence of multiple gene subtypes with specified functions provided greater modulation of cellular function in more complex and higher-order animals. This finding has important implications for our understanding of metabotropic glutamate receptor function and plasticity in across species.

Previously, published work from our lab demonstrated that the Homer gene is required for normal sleep behavior, since genetic Homer null flies exhibit short and fragmented sleep (Naidoo et al., 2012). In this study, we conducted a brain-specific knockdown of Homer to show that brain-specific Homer signaling is required to promote sleep. We also demonstrate that complete genetic loss of *DmGluRA* does not impact total sleep amount but instead leads to a redistribution of daily sleep where null *DmGluRA* mutants have higher amounts of daytime sleep and lower amounts of nighttime sleep compared to wildtype controls. Notably, the changes in daytime and nighttime sleep amount did not persist in constant dark conditions, suggesting that *DmGluRA* regulates day/night somnolence in a light-dependent manner. Previous work has demonstrated that cell-specific genetic knockdown of *DmGluRA* in light-sensitive PDF clock cells in the *Drosophila* brain increases locomotor activity after light offset and before light onset (Hamasaka et al., 2007). Since we observe more wakefulness during these times in our experiments, it suggests that *DmGluRA* signaling from these clock cells are likely contributing to the observed sleep phenotype in the null mutant. Because DmGluRA protein is also broadly expressed throughout the *Drosophila* brain (Devaud et al., 2008), it is likely that mGluR signaling in other brain regions may also play a role in regulating sleep/wake, and future investigation will be necessary to address this. This study is the first to examine the sleep effect of complete genetic loss of mGluR signaling and has important implications for our understanding of processes regulated by mGluRs.

For example, mGluRs – including *DmGluRA* – are critical for learning and memory (Schoenfeld et al., 2013) (Diering et al., 2017). In the periphery, *Drosophila DmGluRA* signaling is required for development of the neuromuscular junction (NMJ). Null *DmGluRA* mutants have been previously shown to exhibit altered NMJ morphology and changes in cellular excitability at the NMJ (Bogdanik et al., 2004). A limitation of analysis of sleep in the *DmGluRA* mutant is that examination of sleep in a genetic null does allow us to rule out changes in development or in peripheral signaling as contributors to the observed sleep phenotype. While we did not identify any changes in baseline locomotor ability or activity that would indicate a behavioral contribution of peripheral mGluR signaling in null flies, we cannot definitively attribute the sleep effects to brain-specific mGluR signaling. Additionally, we must also consider whether changes in development of glutamatergic synapses as a result of *DmGluRA* knockdown might mediate sleep behavior. Future directions will be to examine the effects of conditional genetic knockdown of mGluR signaling in the brain. Furthermore, as previously discussed, mammalian systems express multiple mGluR subtypes, and different mGluR subtypes may have distinct roles in regulating sleep and aging. Future investigation will be necessary to address this.

Finally, we provide new evidence that mGluR coupling to Homer is a requirement for sleep. Using targeted gene editing, we generated a highly specific mutation on the *DmGluRA* gene that prevents it from coupling with Homer proteins in the cell. We observed that this dramatically reduces sleep in the fly and suggests that the interaction between Homer and DmGluRA is necessary to promote sleep. The benefit of this targeted gene editing approach is that we did not knockout either DmGluRA or Homer and we do disrupt Homer interactions with other proteins. This provides more mechanistic specificity to our observed phenotype than can be achieved with whole

gene knockout. While the role of individual molecules in sleep is commonly investigated, the requirement for protein-protein interactions in sleep regulation is much less explored. Thus, this represents a highly novel discovery about the molecular regulation of sleep.

Our sleep findings in the Homer/DmGluRA binding mutant raise many important questions about how Homer and mGluR might modulate both neuronal and behavioral output in the fly. An outstanding question that remains is whether Homer and mGluR associations potentiate or inhibit neuronal activation. Since Homer is critical for mediating calcium dynamics in the cell (Worley et al., 2007), it will be useful to characterize changes in intracellular calcium as a result of our mutation in the future as well as record from cells in organisms during different behavioral states to get a better understanding of the electrophysiological consequences that Homer and mGluR binding dynamics exert on the neuron. In addition to measuring the electrophysiological output of the neurons, it will also be important to understand how Homer binding changes mGluR activation and downstream signaling cascades. mGluR signaling effects not only the second messenger cascades downstream of g-protein activation but also impacts the activity of other glutamate receptors in the cell membrane, so this will be another area of investigation in the future. Analysis of mGluRs in cultured suprachiasmatic nucleus (SCN) neurons has demonstrated that mGluR activation inhibits ionotropic glutamate receptor-mediated calcium increases in the cell (Haak, 1999). Furthermore, mGluR activation leads to a stable reduction in the expression of AMPA receptors at the cell surface (Sanderson et al., 2011). Interestingly, a reduction in AMPA receptors at the membrane has been found to be a correlate of sleep (Lanté et al., 2011) and during sleep, mGluR signaling is necessary for memory consolidation (Diering et al., 2017). Future directions will be to further characterize the molecular and electrophysiological changes that result from uncoupling DmGluRA from Homer proteins in the cell.

Identifying molecular regulators of sleep regulation may have important clinical implications for the treatment of diseases in which sleep disturbances are comorbid or a risk factor for poor outcomes. There is great deal of evidence linking poor sleep to negative health outcomes and better sleep quality to increased longevity and improved health in the elderly (Reviewed in Grandner et al., 2010). If sleep has functional consequences for health and disease, it is possible that therapies that improve sleep might concurrently improve other outcomes for diseases such as Alzheimer's or Parkinson's disease, where sleep disturbances are common symptoms (Musiek et al., 2015) (Knie et al., 2011). Thus, studies such as this one are not only critical for our understanding about the basic biology of sleep, but may bear important implications for human health and disease moving forward.

Experimental Procedures

Drosophila *stocks and husbandry*

Wildtype, mutant, and transgenic flies in the following study are in the white Canton-Special (wCS10) genetic background strain (unless otherwise noted). The wCS10 strain was originally a gift from Ronald Davis (Scripps Research Institute, Jupiter, FL). UAS Homer RNAi was obtained from Bloomington Stock Center. The elavC155 GAL4 strain was a gift from Amita Sehgal (University of Pennsylvania, Philadelphia, PA). The *DmGluRA* null mutant carries the $DmGluRA^{112}$ null allele (Bogdanik et al., 2004) and was a gift from Tom Jongens (University of Pennsylvania, Philadelphia, PA). UAS Homer RNAi, elavC155 GAL4, and the null *DmGluRA* mutant were outcrossed into the wCS10 laboratory background strain for 10 generations prior to all molecular and behavioral experimentation. The $DmGluRA^{PPGTRF}$ mutation was

generated on a w1118 background using homology directed repair in the CRISPR/Cas9 System. All mutants described here are homozygous for the *DmGluRA*^{PPGTRF} allele. Targeting of Cas9 to the putative Homer binding site of *DmGluRA* was conducted with the following 20-bp gRNA sequence: 5′-TAATAGAATCGAGTTCCTGG-3′ and single stranded DNA to replace the sequence with CGACGACGACGACGA. Sequencing of the *DmGluRA* gene to confirm the change in the PPGTRF motif was conducted using the following primers: DmGluRAf2 5'-CAGCGCCTTAAGTATATTAGTCC-3' and DmGluRAf2 5'-CCTCATAGGAATTTCCAGTTCC-3'. gRNA injection and homozygosing of the mutant allele was conducted by Rainbow Transgenic Flies, Inc. (Camarillo, CA). All flies were raised on standard dextrose media (University of Pennsylvania Cell Center, Philadelphia, PA) and maintained in a 12 hour light:dark cycle at 25°C prior to and during all sleep recordings, except in those sleep recordings conducted in constant dark conditions (see *Drosophila* Sleep Assays*)*. Female flies were utilized for all experiments.

Drosophila *Sleep Assays*

Flies were collected under CO_2 anesthesia after eclosion and allowed to grow to one week of age before recording. For all sleep assays, female flies were placed in glass locomotor tubes containing standard dextrose media and allowed to acclimate for one full day in the recording chamber before the start of data collection. Sleep was recorded by video and analyzed as previously described (Zimmerman et al., 2008). Sleep is defined as 5 or more minutes of continuous inactivity (Shaw et al., 2000). For sleep deprivation experiments, flies were monitored using the *Drosophila* activity monitoring system (DAMS) (TriKinetics, Waltham, MA, USA) to allow for continuous data collection throughout the deprivation period. DAMS monitors were placed on an automatic sleep deprivation platform that delivered a mechanical pulse every 20 seconds at random intervals. Mechanical sleep deprivation was scheduled from ZT18 to ZT24 (the last 6

hours of the night) and sleep data was collected until the end of the following day. For constant dark condition recordings, flies were placed in a recording chamber in which all light had been removed and sleep was recorded for 7 days and nights starting on the following day.

Negative Geotaxis Assay

Negative geotaxis was measured as previously described (Ali et al., 2011). Briefly, negative geotaxis was observed in groups of 10 flies at a time during which flies were placed inside two conjoined plastic vials (Genesee Scientific, San Diego, CA, USA) and gently tapped to the bottom of the lower vial. An 8cm demarcation was placed above the bottom of the plastic vial. For each trial, the number of flies passing the 8cm mark after being tapped to the bottom of the vial was recorded. Climbing rate was recorded in each group for a total of 10 trials with 1 minute of rest provided between trials.

Western Blotting and Co-Immunoprecipitation

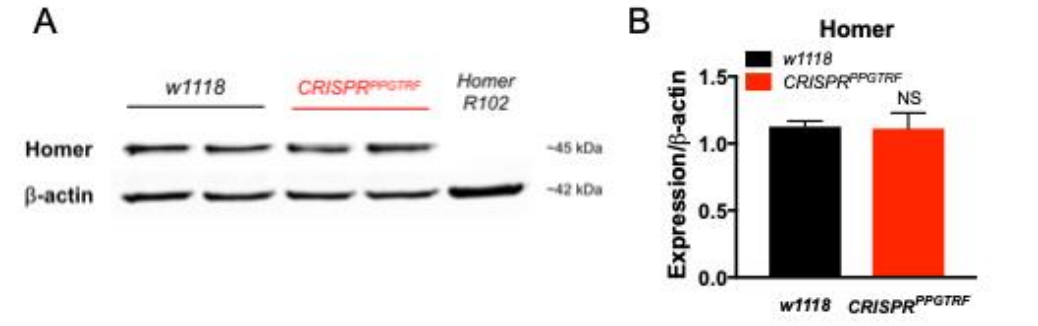
For Western blot analysis, flies were sacrificed over dry ice and single heads were homogenized in chilled standard lysis buffer (10mM Tris-HCl, 1mM EDTA, 10% Glycerol, 1% Triton-X, 150mM NaCl) containing Halt™ Protease Inhibitor Cocktail (ThermoFisher Scientific, Waltham, MA, USA). Head lysates were centrifuged to remove cellular debris, and protein was run on sodium dodecyl sulfate (SDS) polyacrylamide gels (10% Tris-HCl), transferred to nitrocellulose membranes (Bio-Rad), blocked in Odyssey® TBS Blocking Buffer (LI-COR, Lincoln, Nebraska, USA) and incubated with mouse DmGluRA 7G11 primary antibody (1:10, European Molecular Biology Laboratory, Heidelberg, Germany). 7G11 antibody was co-incubated with rabbit β-actin primary

antibody (1:1000, Cell Signaling, Danvers, MA USA) as a loading control. Membranes were subsequently incubated with goat anti-mouse IRDye®800RD (1:1000, LI-COR, Lincoln, Nebraska, USA) and donkey anti-rabbit IRDye®680RD secondary antibodies (1:10000, LI-COR, Lincoln, Nebraska, USA). Western blot analysis had been previously conducted in our lab to confirm the specificity of the DmGluRA antibody. Protein expression was detected and analyzed using the Odyssey® Infrared Scanner (LI-COR, Lincoln, Nebraska, USA). Co-immunoprecipitation was conducted using the Dynabeads Protein A Kit (ThermoFisher Scientific, Waltham, MA, USA). Dynabeads were bound to guinea pig Homer antibody, which was a gift from Uli Thomas. Eluted antigen was run on sodium dodecyl sulfate (SDS) polyacrylamide gels (10% Tris-HCl) and processed as described above. After the blocking step, membranes were incubated with the mouse DmGluRA 7G11 primary antibody (1:10) and visualized using goat anti-mouse IRDye®800RD (1:1000) and visualized on the Odyssey® Infrared Scanner.

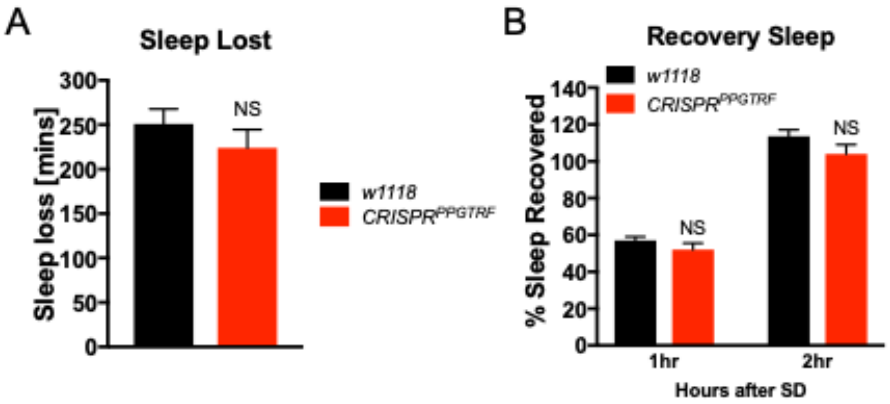
Statistical Analysis

Student's t tests were used to compare sleep between genotypes. When relevant, analyses of daytime and nighttime sleep were performed separately. Statistical analysis was conducted using GraphPad Prism software (La Jolla, CA, USA).

Supplemental Figures



Supplemental Figure 1. Mutation of the PPGTRF site on *DmGluRA* does not alter Homer expression.
(A) Western blot analysis of Homer expression in *Drosophila* heads. Homer is expressed in both w1118 wildtype flies as well as CRISPR mutants and not in the Homer102 null *(N≤7)* (B) Quantification of Homer expression relative to beta-actin. Levels of normalized Homer expression is not altered by mutation of the *DmGluRA* Homer binding site *(N≤7).*



Supplemental Figure 2. Mutation of the PPGTRF site on *DmGluRA* does not alter homeostatic sleep rebound
(A) Quantification of minutes of sleep lost (relative to a baseline recording the night before) between wiltdtype and CRISPR mutant flies. Mutant and wildtype flies were sleep deprived during the last 6 hours of the night *(N≤24)* (B) Amount of recovery sleep following sleep deprivation was not significantly changed in CRISPR mutants relative to wildtype controls *(N≤24).*

CHAPTER 3

The protein translational regulator PERK is required for sleep

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Abstract

Sleep disturbances are a major risk factor for neurodegenerative disease, suggesting that sleep may have an important role in regulating protein homeostasis – or proteostasis – in the brain. Indeed, acute sleep deprivation has been shown to activate intracellular signaling pathways that modulate proteostatic balance in the cell. One of these pathways, known as the protein kinase R (PKR)-like endoplasmic reticulum kinase (PERK) pathway, transiently inhibits protein translation in response to endoplasmic reticulum (ER) stress and misfolded protein accumulation. Here, we examined the role of the PERK pathway in sleep regulation. We provide the first evidence that PERK signaling is not only activated in response to sleep deprivation, but that PERK signaling is functionally required for sleep. We show that pharmacological inhibition of the PERK pathway has cross-species effefts on sleep, significantly reducing sleep in both *Drosophila* and zebrafish. We also find that both constitutive and acute transgenic knockdown of the PERK pathway in neurons suppress sleep in *Drosophila,* while transgenic overexpressionof PERK enhances sleep*.* This suggests that PERK is a cross-species regulator of sleep and wake behavior. Furthermore, we show that changes in PERK signaling modulate the release of the wake-promoting neuropeptide pigment dispersing factor (PDF) in the *Drosophila* brain, uncovering a mechanism through which proteostatic pathways affect sleep and wake behavior. Together, these results demonstrate that the PERK pathway is a cross-species regulator of sleep and wake.

Introduction

During sleep, the brain exhibits significant plasticity that has been measured as changes in the size and number of synapses (Bushey et al., 2011) (de Vivo et al., 2017) (Yang et al., 2014) as well as alterations levels of protein synthesis (Seibt et al., 2012). At present, much remains to be understood about how and whether sleep might be functionally responsible for regulating the translation and expression of proteins in the brain. Experiments in mice have demonstrated during sleep, proteins and toxins are actively cleared from the brain (Xie et al., 2013), lending strength to the notion started by early observations in synaptic changes that sleep modulates protein balance that wakefulness might inherently perturb. This idea is supported by the fact that sleep dysfunction is associated with increased risk for neurodegenerative disease (Schenck et al., 2013) (Zhou et al., 2017) (Ju et al., 2013) (Lim et al., 2013) (Hahn et al., 2014) and may even contribute directly to disease pathogenesis (Kang et al., 2009) (Rothman et al., 2013) (Di Meco et al., 2014). A primary pathological feature of neurodegenerative disease is the aggregation of misfolded proteins in the brain that occur along with neuronal loss (Ross and Poirier, 2004), highlighting the possibility that sleep is necessary to maintain protein homeostasis — or proteostasis — in the brain.

Cellular proteostasis involves the proper maintenance of protein synthesis, folding, and trafficking inside the cell (Vendruscolo et al., 2011). When proteostatic balance is disrupted and misfolded proteins accumulate in the cell, a set of intracellular signaling pathways known collectively as the Unfolded Protein Response (UPR) become activated (Ron and Walter, 2007). The UPR originates in the subcellular organelle known as the endoplasmic reticulum, or the ER. Here, secretory and membrane proteins are produced, folded, packaged, and post-translationally modified for transport out into the cell (Ron and Walter, 2007). UPR activation is mediated three signaling transducers:

PKR-like ER kinase (PERK), inositol-requiring protein-1 (IRE1), and activating transcription factor-6 (ATF6) (Ron and Walter, 2007). Together, these transduction molecules activate signaling cascades that inhibit protein synthesis, upregulate protein chaperone activity, and degrade transcript and protein products in the cell (Ron and Walter, 2007). Thus, the primary purpose of the UPR is to reinstate cellular proteostasis in response to ER stress and to prevent cell death.

Wakefulness and sleep deprivation are associated with UPR activation across multiple species including rats (Cirelli and Tononi, 2000) (Cirelli et al., 2004), mice (Terao et al., 2003) (Naidoo et al., 2005), *Drosophila* (Naidoo et al., 2007) and white-crowned sparrows (Jones et al., 2008). In these studies, transcript or protein levels of the UPR chaperone molecule binding immunoglobulin protein (BiP) are significantly elevated in the brain. Previously, our lab has shown that BiP promotes recovery sleep after sleep deprivation in *Drosophila melanogaster* (Naidoo et al., 2007), identifying some of the first evidence that UPR signaling is functionally relevant to sleep regulation. However, much remains unknown about UPR signaling and sleep and in particular, the involvement of the individual UPR signaling transduction pathways in sleep regulation remains unexplored.

In this study, we investigated the role of the PERK pathway in regulating sleep and wake behavior. Because levels of protein biosynthesis are a key determinant of cellular proteostasis (Vendruscolo et al., 2011), understanding how PERK signaling affects sleep is a critical early step towards understanding the molecular mechanisms that tie sleep to cellular health. PERK suppresses protein translation in response to ER stress (Harding et al., 2000). In the critical rate-limiting step of protein synthesis, GTP-bound eIF2 recruits the initiator Met-tRNAi to the 40S ribosomal subunit to form the 43S preinitiation complex necessary for the translation initiation (Pestova et al., 2008).

Activated PERK phosphorylates the alpha subunit of eukaryotic initiation factor 2 (eIF2α) which halts translation of new proteins in the cell (Harding et al., 2000). Previously, it has been shown that pharmacological inhibition of protein synthesis (through elevated PERK pathway activation) induces sleep, suggesting that levels of protein translation may be a direct determiner of behavioral state (Methippara et al., 2009). This is the first evidence that PERK may be relevant to behavioral regulation outside the context of stress, aging, and disease.

Using pharmacological and transgenic approaches, we demonstrate that the PERK pathway is required for sleep. Pharmacological inhibition of PERK reduced sleep in two animal models: *Drosophila melanogaster* (also known as the common fruit fly) and *Danio rerio,* or zebrafish. Furthermore, transgenic knockdown of PERK pathway signaling in neurons suppressed sleep in *Drosophila* while neuronal overexpression of PERK significantly increased sleep amount. Finally, we demonstrate that PERK activity in a small subset of wake-promoting neurons is sufficient to alter *Drosophila* sleep and wake behavior and change the release of a wake-promoting neuropeptide. These results illustrate that a signaling pathway responsible for suppressing protein translation in the cell is necessary to promote sleep and provide the first evidence of a mechanistic link between protein synthesis and sleep regulation in the brain.

Results

PERK pathway activation is associated with wakefulness

Since it is well-established that sleep deprivation leads to ER stress (Shaw et al., 2000; Naidoo et al., 2005; Naidoo et al., 2007) and activation of the PERK pathway across species (Naidoo et al., 2008) (Brown et al., 2014), we sought to further characterize the effect of wake on PERK pathway activity. As mentioned previously,

PERK phosphorylates the alpha subunit of eukaryotic initiation factor 2 (eIF2α) (Pestova et al., 2008) in order to halt new protein synthesis at ER ribosomes. Thus, phosphorylated eIF2α (peIF2α) levels are a proxy for PERK pathway activity. To confirm previous findings, we first examined the levels of expression of peIF2α)following sleep deprivation. We observed that in *Drosophila,* 6 hours of sleep deprivation increases the expression of peIF2α compared to undisturbed controls (**Figure 1A**), which recapitulates previously published data (Naidoo et al., 2008). We also examined changes in peIF2α at the beginning and the end of the daytime to see whether normal wakefulness across the day correlates with any changes in PERK pathway activity. We find that peIFα are elevated at the end of the day (ZT10) compared to the beginning to the day (ZT0) (**Figure 1B**). Thus, activation of the PERK pathway appears to be a molecular correlate of wakefulness in the fly.

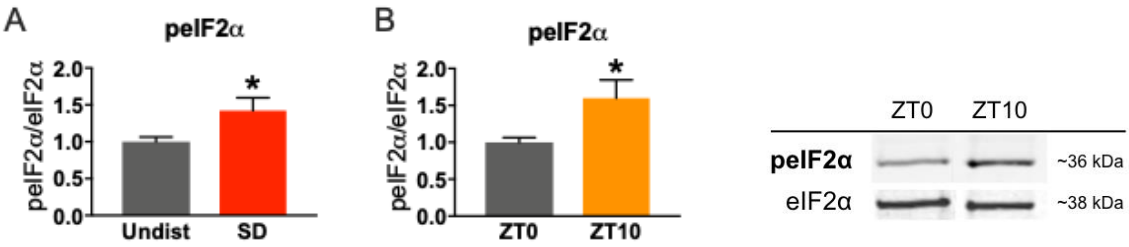


Figure 1. Activation of the PERK pathway is associated with wakefulness in *Drosophila*
(A) Quantification from western blot analysis of eIF2α expression. peIF2α expression is increased after 6 hours of sleep deprivation (SD) at the end of the night compared to undisturbed controls (Undist) *(N=9, *P<.05)* (B) Quantification from western blot analysis of peIF2α expression in flies collected at the beginning of the lights-on period (ZT0) compare to the end later part of the day (ZT10). peIF2α expressing is higher near the end of the waking day compared to the start of the day *(N=9, *P<.05).* Representative peIF2α and eIF2α signal shown on the right.

Pharmacological inhibition of PERK reduces sleep

To begin to investigate the role of PERK signaling in sleep, we examined the effect of pharmacological inhibition of PERK on sleep in adult wildtype *Drosophila*

melanogaster. We compared sleep in wildtype flies that were administered a PERK inhibitor to flies treated with vehicle. GSK2606414 is a small molecular inhibitor of PERK (Axten et al., 2012). When administered at a concentration that has previously demonstrated therapeutic effects in a TDP-43 toxicity in *Drosophila* (Kim et al., 2014), we found that GSK2606414 significantly reduced nighttime sleep **(Figure 2A and 2B)**. Analysis of sleep architecture in flies fed vehicle or GSK 2606414 revealed that the drug did not significantly affect sleep architecture (**Figure 2C and Figure 2D**). We conducted ribosomal profiling of brain tissue from flies treated with vehicle or GSK0606414 and found that administration of GSK2606414 increases the expression of actively translating polysomes in the brain **(Supplemental Figure 1**). We also conducted a sleep deprivation experiment to determine whether inhibiting PERK would affect the homeostatic response to sleep loss. We found that GSK 2606414 significantly blunted the amount of recovery sleep the following morning after 6 hours of sleep deprivation at the end of the night (**Supplemental Figure 2).**

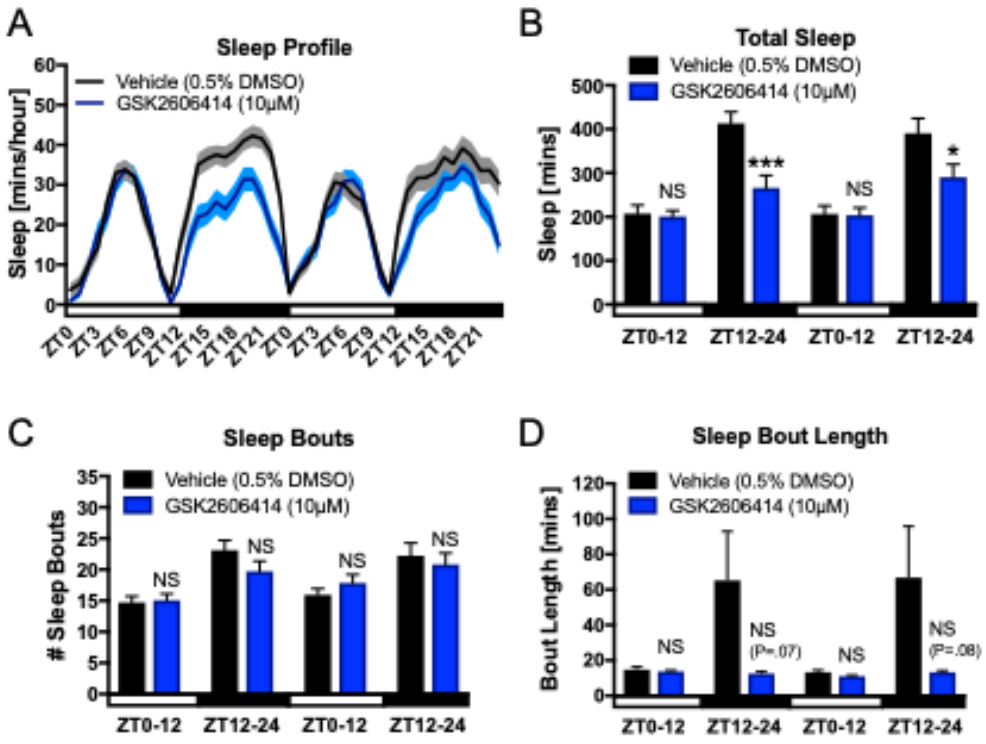


Figure 2. Pharmacological inhibition of PERK with the inhibitor GSK 2606414 inhibits sleep in *Drosophila*
(A) Sleep profile of flies treated with GSK 2606414 or vehicle (0.5% DMSO). The PERK inhibitor GSK 2606414 suppresses nighttime sleep in *Drosophila (N=42, shaded area represents SEM)* (B) GSK 2606414 significantly reduces sleep at night *(***P<.001, *P<.05)* (C) GSK 2606414 does not significantly affect sleep bout number (D) GSK 2606414 does not significantly affect sleep bout length.

In addition to GSK 2606414, we also conducted oral administration of the eIF2B activator ISRIB. Similar to GSK2606414, ISRIB represses PERK pathway activation in order to enhance protein translation. Administration of ISRIB significantly decreases sleep at night, similar to GSK 2606414 (**Figure 3A and 3B**). Sleep architecture is not significantly changed following ISRIB administration (**Figure 3C and 3D**). Thus, two different pharmacological interventions that reduce PERK pathway activity by different mechanisms both reduce sleep.

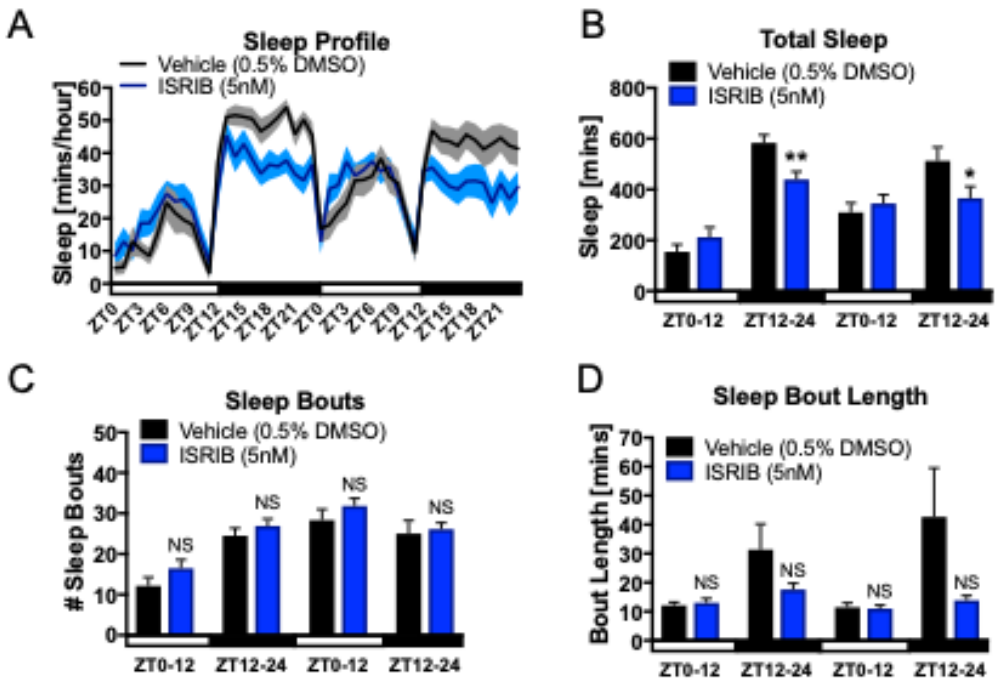


Figure 3. Pharmacological administration of the eIF2B activator ISRIB inhibits sleep in *Drosophila*

(A) Sleep profile of flies treated with ISRIB or vehicle (0.5% DMSO) *(N=14, shaded area represents SEM)* (B) Quantification of daytime and nighttime sleep. ISRIB reduces sleep at night (N=14, ***P<.01, *P<.05)* (C) ISRIB does not significantly affect sleep bout number *(N=14)* (D) ISRIB does not significantly affect sleep bout length *(N=14)*

To validate our pharmacological intervention of PERK pathway activity, we also examined the effects of GSK 2606414 in the *Danio rerio,* or zebrafish, model. Like *Drosophila*, zebrafish exhibit diurnal sleep rhythms that can be recorded and analyzed with video tracking software (Prober et al., 2006). Here, single-housed zebrafish larvae are monitored in single well and drugs can be administered directly into the water while sleep is being measured. Administration of the PERK inhibitor recapitulated the sleep reductions observed in the fly. Larval zebrafish administered GSK0606414 exhibit significantly reduced sleep, both during the day as well as at night **(Figure 4).**

Figure 4. Pharmacological inhibition of PERK inhibits sleep in *Zebrafish*
A) Sleep profile of larval zebrafish over the course of two days and nights. The PERK inhibitor GSK2606414 increases locomotor activity and decreases sleep during the day and night in zebrafish larvae. B) Both sleep bout length and sleep bout number are reduced by drug administration. Vehicle (n=102) GSK2606414 (n=114)

Neuronal knockdown of the PERK pathway reduces sleep

Since pharmacological administration of a PERK inhibitor acts on PERK in all cell types without any spatial specificity, we subsequently utilized a genetic approach to assess the role of PERK in the brain on sleep behavior. We expressed transgenic UAS PERK RNAi in neurons using a neuronal synaptobrevin (nSyb) GAL4 driver and found expression of PERK RNAi in neurons significantly reduced sleep (**Figure 5A and Figure 5B**). Analysis of sleep architecture shows that genetic knockdown of PERK in neurons reduces both the number and length of sleep bouts per day (**Figure 5C and 5D**). We also overexpressed a murine form of GADD34, or Growth arrest and DNA damage-inducible protein (also known as Protein Phosphatase 1 Regulatory Subunit 15A), a phosphatase that dephosphorylates peIF2α. We used the same neuronal driver to see if another manipulation that reduces levels of phosphorylated eIF2α in the brain would recapitulate the effect of PERK knockdown. Indeed, overexpression of mGADD34 also significantly reduces sleep amount compared to parental controls. (**Figure 5E and Figure 5F**). mGADD34 overexpression did not consistently affect sleep architecture throughout the experiment but on the second day of recording we observed decreases in

sleep bout number and bout length (**Figure 5G and 5H**). These results suggest that knockdown of phosphorylated eIF2α reduces sleep in *Drosophila* and provides further evidence that the PERK pathway is required to promote sleep.

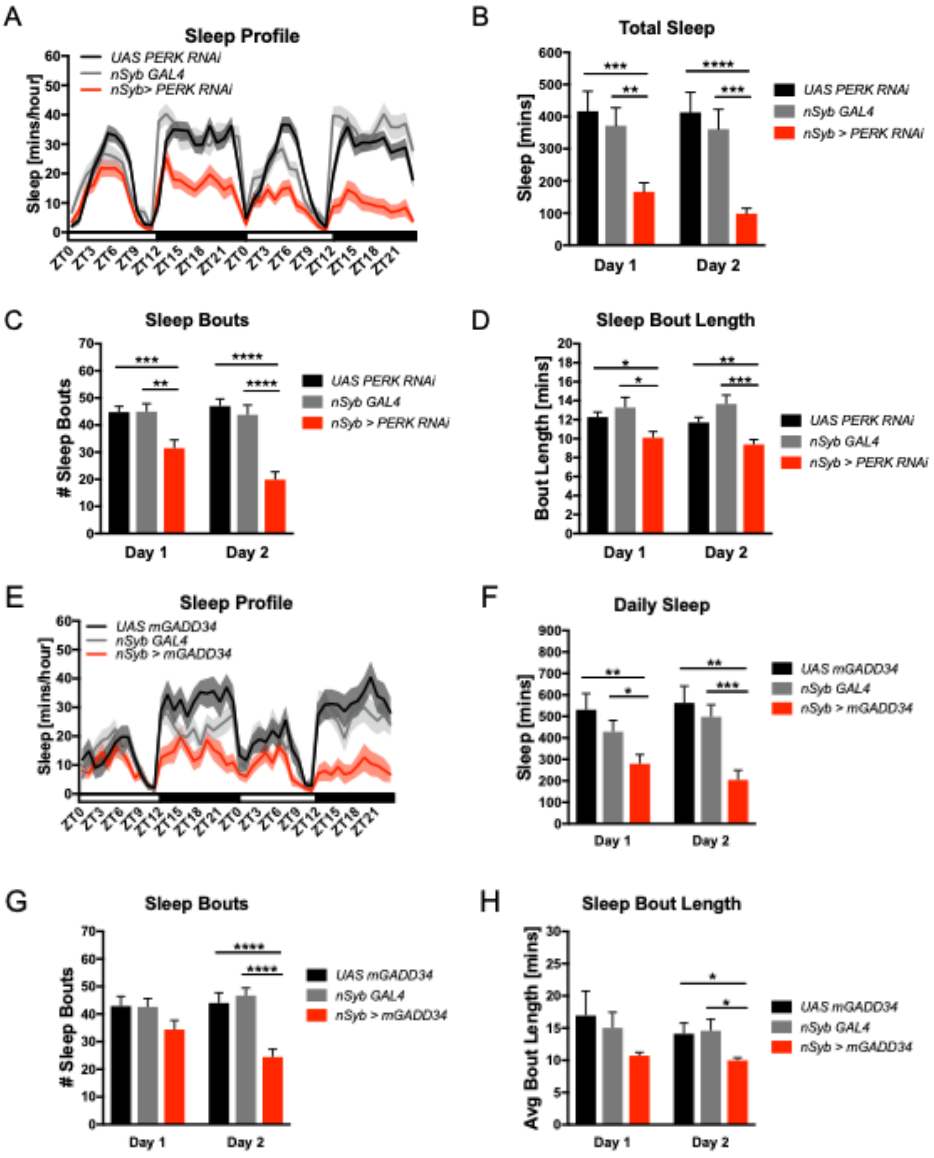


Figure 5. Genetic inhibition of peIF2α reduces sleep
(A) Sleep profile of flies expressing PERK RNAi in nSyb-expressing neurons compared to uncrossed parental controls. Expression of PERK RNAi in neurons reduces sleep (B) Quantification of daily sleep amounts. Total sleep is significantly reduced following PERK RNAi expression in neurons *(*P<.05, **P<.01,***P<.001)* (C) The average number of sleep bouts is reduced in flies expressing PERK RNAi in neurons *(**P<.01,***P<.001, ****P<.0001)* (D) PERK RNAi expression in neurons decreases average sleep bout length per day *(*P<.05, **P<.01,***P<.001)* (E) Sleep profile of flies expressing mGADD34 in nSyb-expressing neurons compared to uncrossed parental controls. Expression of mGADD34 in neurons reduces sleep (F) Quantification of daily sleep amounts. Total sleep is significantly reduced following mGADD34 expression in neurons *(*P<.05, **P<.01,***P<.001)* (G) The average number of sleep bouts is reduced in flies expressing mGADD34 in neurons *(****P<.0001)* (H) PERK RNAi expression in neurons decreases average sleep bout length per day *(*P<.05)*

Following constitutive expression of PERK RNAi and GADD34 overexpression in neurons, we sought to conduct a more acute genetic reduction in PERK. To gain both spatial and temporal specificity with our transgenic manipulations, we used the GAL4/UAS GeneSwitch system to restrict the expression of PERK RNAi to neurons in adulthood. This allows us to examine the effect of PERK knockdown independent of developmental effects from transgene expression. We expressed UAS PERK RNAi in neurons using an RU486-inducible neuron-specific GeneSwitch driver and found administration of RU486 in flies expressing PERK RNAi in neurons reduces sleep (**Figure 6A**). The effect on total sleep is less dramatic than what was observed with constitutive knockdown of PERK (**Figure 6B**) and sleep bouts were not significantly changed by RU486 induction of PERK RNAi (**Figure 6C**). However, we observe a significant reduction in average sleep bout length as a result of RU486-induced knockdown of PERK (**Figure 6D)**. Thus, acute knockdown of PERK in neurons reduces sleep and suggests that PERK is required to maintain sleep in *Drosophila.*

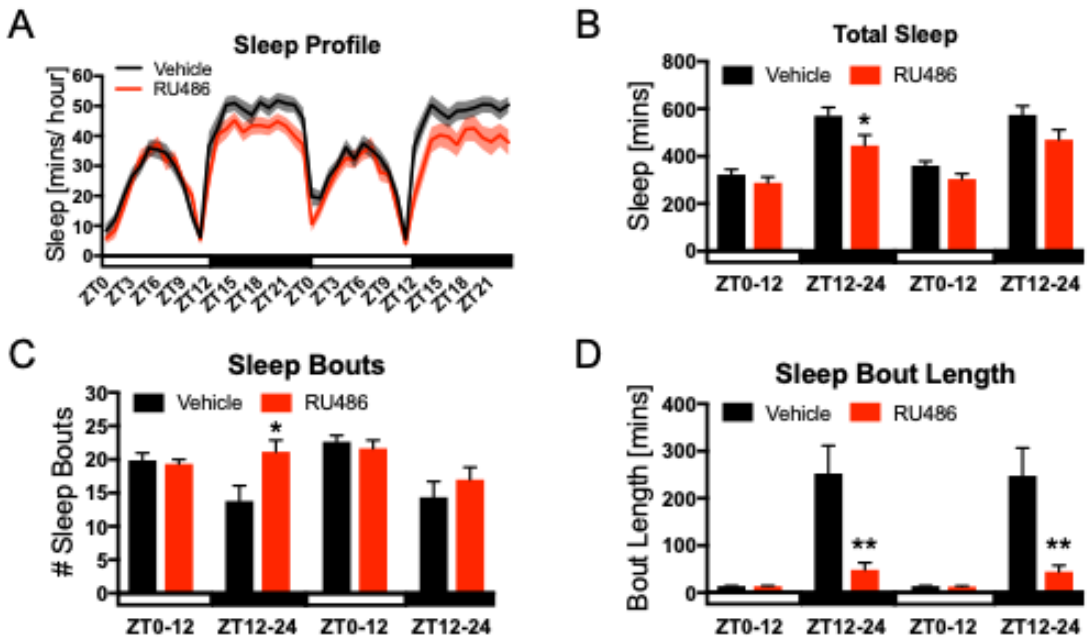


Figure 6. Brain-specific inhibition of PERK inhibits sleep in *Drosophila*
(A) Sleep profile of neuron-specific GeneSwitch > PERK RNAi flies administered either RU486 or vehicle *(N=28)* (B) Quantification of sleep during the day and night *(N=28, *P<.05)* (C) Number of sleep bouts are not significantly changed overall, through RU486 administration increased the number sleep bouts on the first night of the experiment *(N=28 *P<.05)* (D) Average sleep bout length is reduced at night after RU486 administration *(N=28, **P<.01)*

Overexpression of PERK induces sleep

The previous experiments demonstrate that PERK signaling is required for sleep but what remains to be seen is whether induction of PERK activity is sufficient to induce sleep in the fly. We overexpressed PERK in neurons using the nSyb GeneSwitch driver and found that induction of PERK significantly increases sleep amount (**Figure 7A and Figure 7B**). To control for the effects of RU486, we crossed the nSyb GeneSwitch line to w1118 flies, as a background control for the UAS dPERK line. RU486 alone did not change the sleep profile when administered to flies not carrying the UAS dPERK transgene (**Figure 7A**). Analysis of sleep architecture demonstrates that both sleep bout number and sleep bout length increase following RU486 administration (**Figure 7C and 7D**). We then took flies from the recording and placed them back on regular food for

three days and confirmed that after removal of RU486, sleep patterns return to normal (**Supplemental Figure 3**). This further demonstrates that the promotion of sleep we observed was due to PERK signaling.

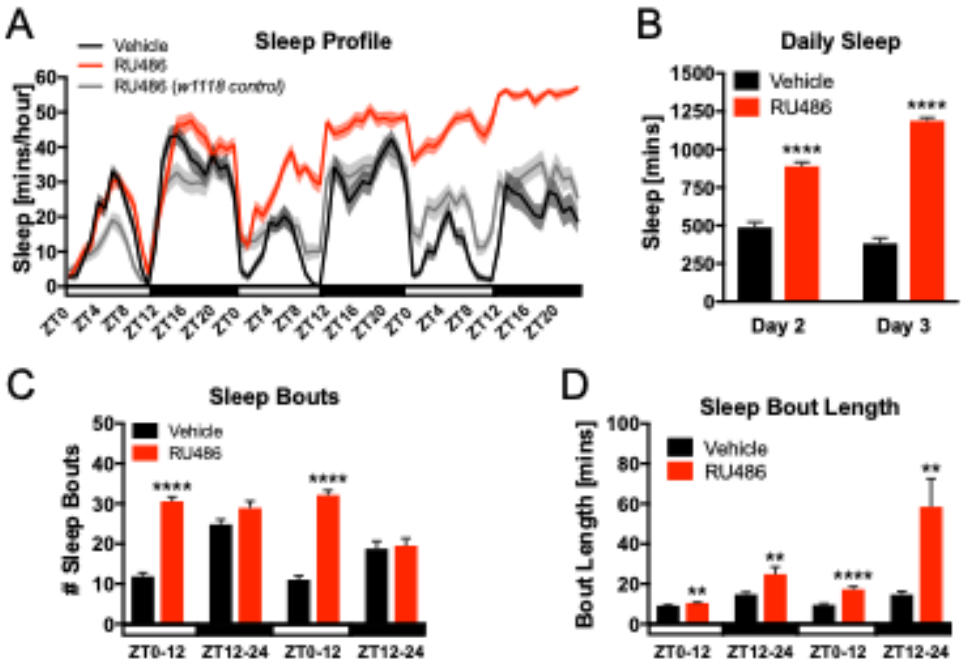


Figure 7. Neuronal overexpression of PERK induces sleep
(A) Sleep profile for nSyb GeneSwitch> dPERK flies on vehicle compared to RU486. The gray trace shows sleep amount in progeny of nSyb GeneSwitch and w1118 wildtype controls fed RU486. Sleep in these flies is not increased compared to vehicle-fed transgenic flies, suggesting that the effect of RU486 on sleep is specific to acute PERK overexpression. Successive days on RU486 food incrementally increases sleep time in flies in nSyb GeneSwitch> dPERK transgenic animals *(N=28)* (B) Quantification of sleep on the second and third days of RU486 administration. RU486 significantly increases daily sleep amount in nSyb GeneSwitch>dPERK flies *(N=28, ****P<.0001)* (C) RU486 administration increases the number of daytime sleep bouts *(N=28, ****P<.0001)* and (D) increases sleep bout length during both the day and night *(N=28, **P<.01, ****P<.0001)*

Mechanisms underlying PERK-mediated changes in sleep

Finally, we sought to explore the potential mechanisms through which modulation of PERK activity might affect sleep. Because the effects of PERK pathway inhibition on sleep appear to occur mostly during the night, we posited

that this might be modulated by changes in the translation of wake-promoting molecules. For example, PERK signaling may be required to put a brake on the translation of wake-active molecules and inhibiting the PERK pathway might lead to the continued translation of wake-active molecules into the night that could explain the significant nighttime sleep reductions we observe. Thus, we chose to assess the requirement of PERK in mediating sleep in a small circuit of the brain known to promtoe wake. We chose neurons expressing the neuropeptide pigment dispersing factor (PDF), which is known to promote wakefulness in *Drosophila* (Parisky et al., 2008) as a circuit-or-interest. Both the spatial and time-of-day expression of PDF is well-characterized; PDF is expressed in 8 neurons in either hemisphere of the brain that project to the medulla dorsal protocerebrum of the brain (Muraro and Ceriani, 2014). Levels of PDF are elevated during the day and its expression and release is significantly downregulated at the beginning of the night (Fernández et al., 2008) (Gorostiza et al., 2014) (Liang et al., 2016). We used PDF GAL4 to drive PERK RNAi expression in PDF neurons and found that it significantly reduced sleep relative to parental controls (**Figure 8A and 8B**). In contrast, overexpression of PERK in PDF neurons significantly increased sleep compared to parental controls (**Figure 8C and 8D**). Next, we examined the effect of PERK knockdown and overexpression on the release of PDF from the subset of PDF-expressing clock cells known as the small lateral ventral neurons (sLNvs) subset where significant time-of-day plasticity has been demonstrated (Fernández et al., 2008) (Gorostiza et al., 2014). We found that overexpression of PERK significantly reduces PDF

release at a timepoint where PDF release is relatively high during the day (ZT 2) and knockdown of PERK significantly increases PDF release when levels are low at night (ZT 14) (**Figure 8E**). This demonstrates that changes in PERK signaling in a small subset of wake-promoting neurons is sufficient to alter sleep and neuropeptide release.

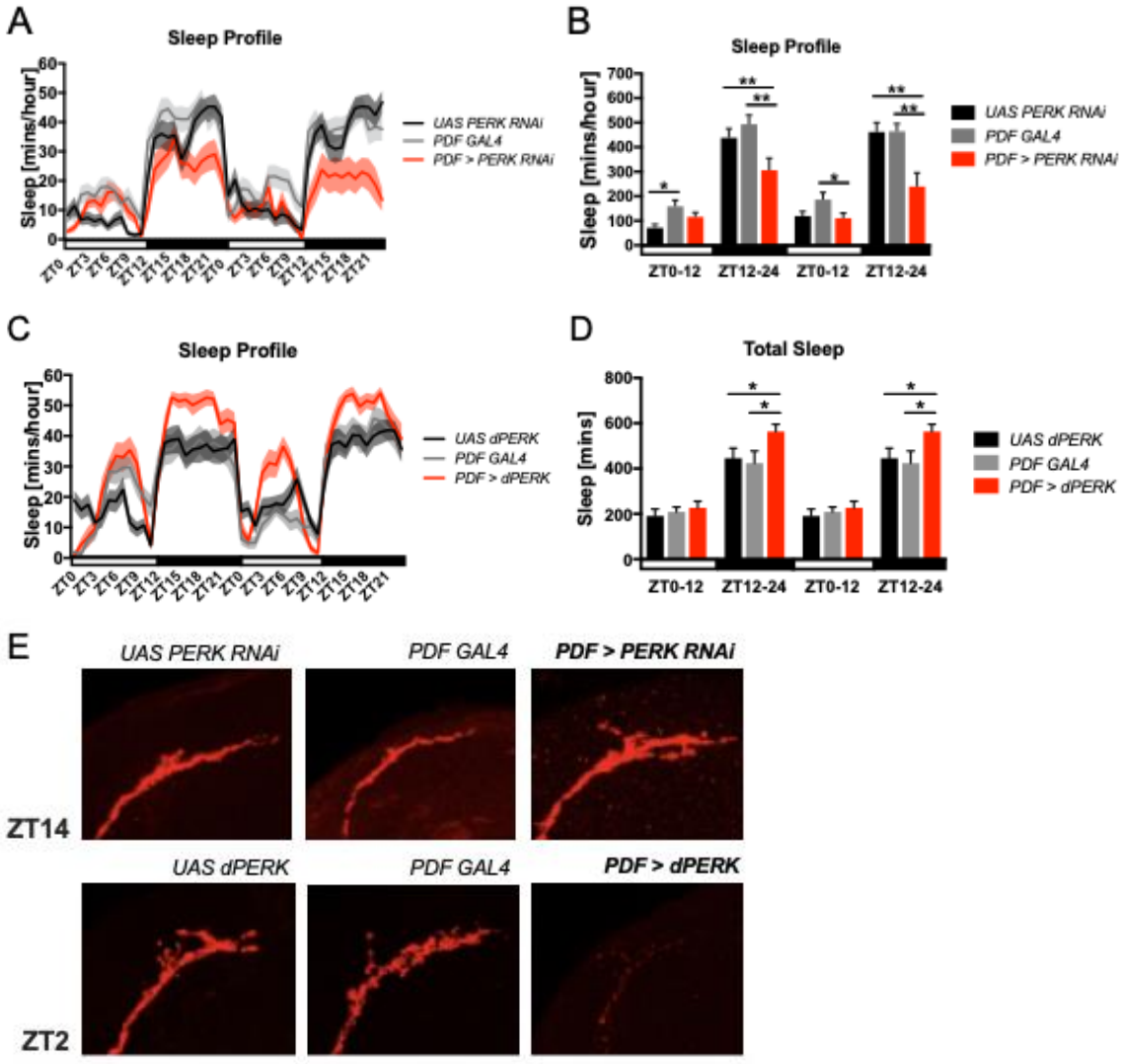


Figure 7. Genetic knockdown and overexpression of PERK in PDF neurons reduces and increases sleep and alters PDF release (A) 48 hour sleep profiles of flies expressing PERK RNAi in PDF neurons compared to parental controls.*(N=18, shaded area represents SEM)* (B) Quantification of daytime and nighttime sleep. Transgenic flies expressing PERK RNAi in PDF

neurons exhibit significantly less sleep than parental controls at night *(N=18, *P<.05, **P<.01)* (C) 48 hour sleep profiles of flies overexpressing PERK in PDF neurons compared to parental controls *(N=18, shaded area represents SEM)* (D) Quantification of daytime and nighttime sleep. Flies overexpressing PERK in PDF neurons exhibit significantly more sleep than parental controls at night *(N=18, *P<.05)* (E) Representative confocal images of sLNvs projections in transgenic flies and parental controls. For PERK knockdown experiments, brains were collected at ZT2 (upper panels). For PERK overexpression experiments, brains were collected at ZT14 (lower panels). At ZT2, PERK overexpression in PDF neurons significantly suppresses PDF release compared to parental controls. At ZT14, PERK knockdown in PDF neurons significantly increases PDF release compared to parental controls. (α-PDF, 1:1000; AlexaFluor 594 1:500).

Discussion

In the following study, we identify a sleep-promoting role for the PERK pathway, a critical regulator of protein synthesis in the cell. Our data is consistent with previous work demonstrating that pharmacological enhancement of PERK increases slow-wave sleep in rats (Methippara et al., 2008). Together, these data suggest that levels of protein biosynthesis are an important determining factor in establishing behavioral state. Moreover, since previous literature has shown that the UPR is activated by sleep deprivation, our results reveal a bidirectional relationship between sleep and proteostatic signaling in the brain. Because the UPR is traditionally understood to be a response to stress and disease states, our finding that PERK regulates a daily behavior like sleep provides a new perspective on the role of the UPR in young and healthy cells.

One of the outstanding debates about sleep function is whether sleep is necessary to enhance or suppress protein translation in the brain. Early work addressing this question identified increases in synapse number and size in the *Drosophila* brain across wake (Bushey et al., 2011). In mammals, the sheer volume of synaptic connections renders a global analysis of sleep-mediated changes more technically challenging. In parts of the motor cortex or the somatosensory cortex in mice, there is evidence of both decreases in synapses (de Vivo et al., 2017) as well as increases in synapses (Yang et al., 2014) during sleep. In particular, synaptic growth occurred in the

motor cortex during sleep following a motor learning task (Yang et al., 2014). This points to the possibility that depending on the brain region or on the specific tasks that occur in wakefulness, both decreases or increases in translation could occur in different circuits. In our study, we observed that inhibiting PERK signaling most consistently had the effect of suppressing sleep during the nighttime. One possible interpretation of this result is that the physiological pattern of PERK activation is such that it is not required during the day to such an extent at night. In other words, the physiological dynamics of protein synthesis may be such that PERK is most strongly activated by the accumulation of wake-active proteins and that during normal wakefulness there are higher levels of global synthesis occurring as a result of elevated energy demand in the brain. Our findings that PERK activation is elevated at the end of the day would support this theory. Thus, it is possible that wakefulness is associated with net increases in protein translation that are reduced during sleep. Further analysis will be required to address this possibility, in particular an analysis of PERK activation across more timepoints and within different cell types (to determine whether highly active circuits might be more relevant to PERK-mediated sleep) will be useful in the future.

Within this work we provide evidence that PERK signaling within a small number of neurons in the brain is sufficient to alter sleep behavior. Our finding does not exclude the possibility that PERK regulation of sleep occurs within multiple brain circuits and cell types. In fact, given its broad role in regulating global protein synthesis in the cell, we would posit that this is likely to be the case. It the future, analysis of sleep effects of PERK signaling in various cell types and brain regions will be important to determine whether cellular specificity PERK regulation of sleep exists. However, these results do suggest that on a global level in the brain, downregulation of protein translation may be a key molecular correlate of sleep.

Both sleep dysfunction and sustained UPR activation have been implicated in the pathophysiology of numerous neurodegenerative diseases. Recent studies have found that pharmacological modulation of PERK signaling has therapeutic benefits in rodent models of frontotemporal dementia and prion disease where overactivated PERK signaling comorbid with the traditional hallmarks of disease (Moreno et al., 2013) (Radford, et al., 2015) (Halliday et al., 2017). Thus, the finding that PERK is relevant to sleep — a behavior that may modulate disease risk — marks a critical juncture in our understanding of mechanisms underlying sleep dysfunction and neurodegenerative disease risk. Aside from disease pathogenesis, it has also been suggested that the UPR plays a critical role in aging. High levels of UPR activation are evident across multiple tissue types in old animals (Martínez et al., 2016). In this study, experiments were conducted in non-aged adult flies when the UPR still operates in an adaptive manner. In young animals, baseline levels of UPR activity are low (Brown et al., 2014). However, with age, chronic ER stress occurs along with age-related sleep disruptions (Brown et al., 2014). Thus, investigation of proteostatic regulation of sleep in ways similar to this study may provide greater insight into how to prevent or treat age-related diseases in which sleep disturbances are a risk factor or common feature. Additionally, approaching sleep as both a prodromal marker and modifiable risk factor for various diseases could be used to better diagnose and treat illness in the future. As such, the results from this study have important implications for our understanding of the molecular mechanisms underlying sleep and wake and may link sleep and other biological processes in the brain that are modulated by the UPRR.

Experimental Procedures

Animals

Wildtype *Drosophila melanogaster* in the following study are in the White Canton-S 10 genetic (wCS10) background strain, which was originally a gift from Ronald Davis (Scripps Research Institute, Jupiter, FL). UAS PERK RNAi (#42499), and neuron-specific GeneSwitch (#40265) fly lines were purchased from the Bloomington Stock Center at Indiana University and outcrossed 7 generations into the wCS10 background in our lab before being crossed for behavioral experiments. PDF GAL4 was originally a gift from John Zimmerman and outcrossed 7 generations into the wCS10 background. All other fly lines were maintained in the w1118 background. UAS PERK RNAi with Dicer2, UAS dPERK, and UAS mGADD34 were a generous gift from Hyung Don Ryoo (New York University). The nSyb GAL4 driver (#48590) was purchased from Bloomington Stock Center and the nSyb GeneSwitch GAL4 was a gift from Amita Sehgal (University of Pennsylvania). Flies were maintained in an environmental room at 25°C in a 12:12 hour light:dark cycle on standard dextrose media (UPenn Cell Center). Females flies were used for all experiments.

Drosophila *and zebrafish sleep assays*

Female files were collected separately under CO_2 anesthesia after eclosion and allowed to grow to adulthood (5-7 days of age) before recording. Individual flies were placed in 65mm x 5mm tubes containing dextrose media. For sleep experiments requiring pharmacological interventions or RU486 administration, locomotor tubes contained dextrose food was prepared with either vehicle, drug, or RU486. Rest and

activity was recorded by video as described previously (Zimmerman et al., 2008). Sleep is defined as 5 or more minutes of continuous inactivity. Zebrafish sleep assays were conducted as previously described (Prober et al., 2006). Briefly, individual zebrafish larva (5 dpf) are placed in separate wells within a 96-well plate. Recordings are conducted in a temperature-controlled chamber using an infrared camera which captures movement during both the day and night.

Drug administration

For *Drosophila* sleep assays, GSK 2606414 (Tocris Bioscience) and ISRIB (Tocris Bioscience) was prepared in 50% dimethyl sulfoxide (DMSO) and incorporated into the dextrose media at a final concentration of 10μM GSK 2606414 and 5nM ISRIB. The final concentration of DMSO vehicle is 0.5%. Mifepristone (RU486, Sigma Aldrich) was prepared in 80% ethanol (EtOH) and incorporated into dextrose media at a concentration of 100μM and final vehicle concentration of 0.8% EtOH. Wildtype flies, transgenic crosses, and parental control lines were placed in locomotor tubes on drug/RU486 or vehicle between 5-7 days of age at least 24 hours before the start of sleep recording.

Fly head preparation and Western blotting

Flies were sacrificed over dry ice and protein was extracted using a standard cell lysis buffer (10mM Tris-HCl, 1mM EDTA, 10% Glycerol, 1% Triton-X, 150mM NaCl) containing SIGMAFAST™ Protease Inhibitor Cocktail (Sigma-Aldrich) and Halt™ Protease Inhibitor Cocktail (ThermoFisher Scientific). Protein homogenates from whole flies were loaded (one fly per well) on sodium dodecyl sulfate (SDS) polyacrylamide gels (10% Tris-HCl) and then transferred to nitrocellulose membranes (Bio-Rad) and blocked

in Odyssey® TBS Blocking Buffer (LI-COR). Membranes were incubated with rabbit anti-phospho-eIF2α (Ser51) polyclonal antibody (1:1000, Cell Signaling) and mouse anti-eIF2α (L57A5) (1:1000, Cell Signaling), 1:1000. antibody. The membranes were subsequently incubated with with goat anti-rabbit IRDye®800RD (1:10,000, LI-COR) and donkey anti-mouse IRDye®680RD secondary antibodies (1:10,000, LI-COR). Protein expression was detected and analyzed using the Odyssey® Infrared Scanner (LI-COR).

Immunohistochemistry and quantification of immunofluorescence

Drosophila dissection and immunostaining of whole brains was conducted as previously described (Wu and Luo 2006). Antibodies to PDF (PDF C7) and elav (Rat-Elav-7E8A10 anti-elav) (expression not shown here) were obtained from the Developmental Studies Hybridoma Bank (University of Iowa). Anti-mouse AlexaFluor594 anti-rat AlexaFluor 488 (ThermoFisher Scientific) were used to visualize protein expression. PDF-positive neuronal projections in the dorsal protocerebrum were visualized at 126x magnification on the Leica TCS SP5 confocal microscope and images were collected in 3μM z-stacks. Individual z-stack images were analyzed using ImageJ. Quantification of fluorescently labeled synaptic projections was calculated using % area as previously described (**Serrano-Pozo et al., 2011**).

Reverse transcriptase PCR and product quantification

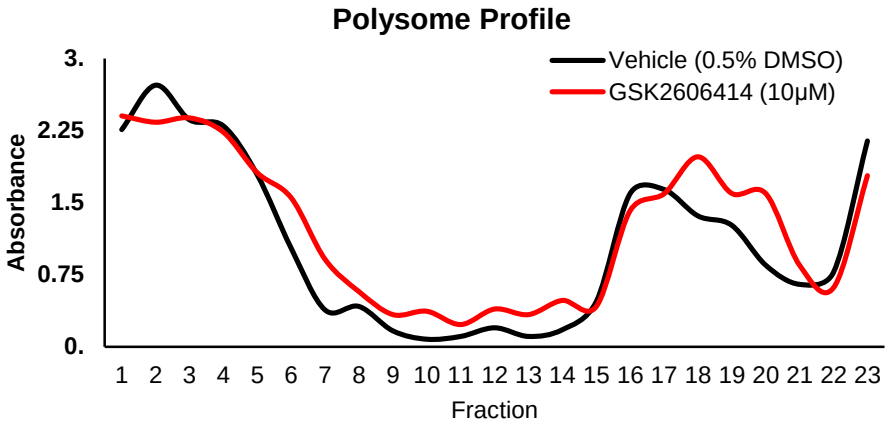
RNA was extracted from snap-frozen fly heads using the RNeasy Mini Kit (Qiagen). RNA concentrations were measured on the NanoDrop 2000 (ThermoFisher Scientific) and quantitative real-time PCR was conducted using the TaqMan® RNA-to-Ct™ 1-Step Kit (ThermoFisher Scientific) with the following primers: PERK

(Dm02137033_g1). RpL32 (Dm02361909_s1), Act5C (Dm02361909_s1) and GAPDH (Hs02758991_g1) (ThermoFisher Scientific).

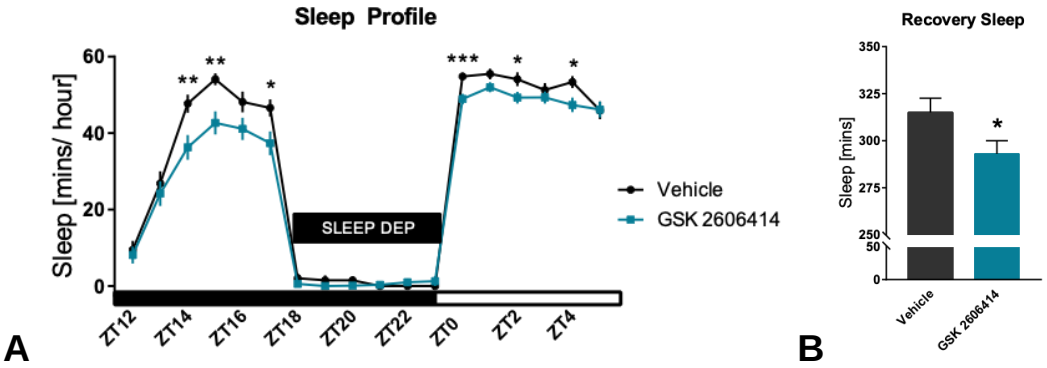
Statistical analysis

Student's T-Test was conducted to compare drug and vehicle groups in the sleep assays and IR-quantified protein concentrations. Holm-Hidak correction for multiple comparisons was applied when relevant. For larval zebrafish behavior, D'Agostino & Pearson omnibus normality test was performed to assess normality. Non-parametric Mann-Whitney test was performed to assess statistical significance between treatment groups. Statistical analysis was conducted using GraphPad Prism software (La Jolla, CA, USA).

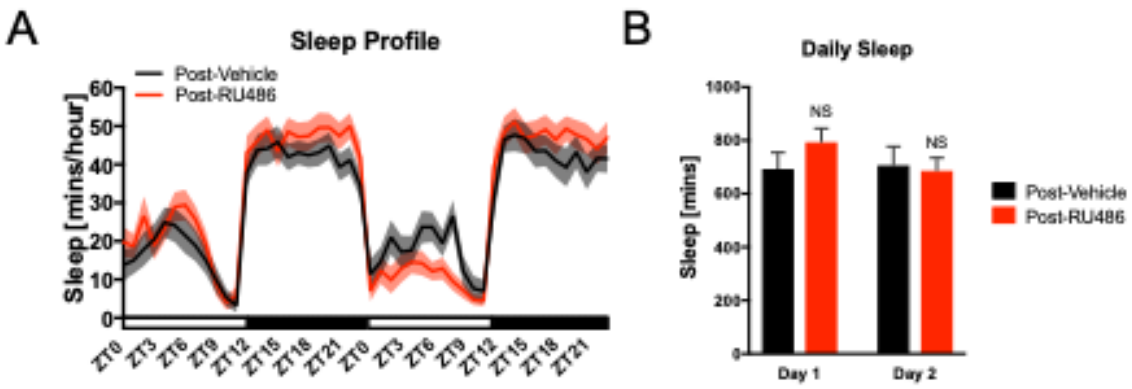
Supplemental Figures



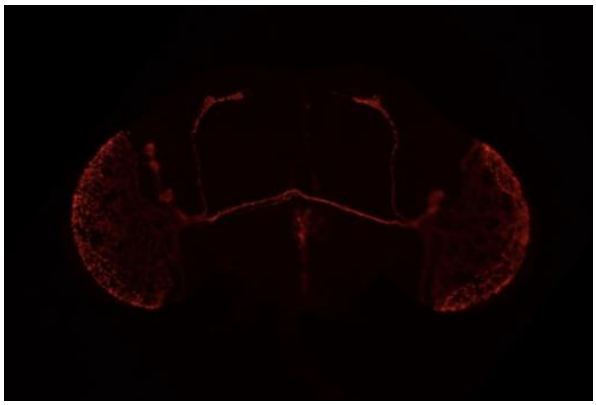
Supplemental Figure 1. GSK0606414 alters the translational profile of the brain. The expression of actively translating polysomes is higher following GSK 2606414 administration.



Supplemental Figure 2. Inhibition of PERK suppresses the homeostatic response to sleep loss in *Drosophila*
A) GSK 2606414 suppresses rebound sleep after 6 hours of sleep deprivation *(N=47 per group, *P<.05, **P<.01, ***P<.001)* B) Sleep during the 6 hours after deprivation (ZT0-ZT6) is reduced after GSK 2606414 administration *(*P<.05)* GSK2606414 – SLEEP DEP, N=47 per group



Supplemental Figure 3. Sleep patterns return to normal levels after RU486 is removed. (A) Sleep profiles for transgenic flies (nSyb GeneSwitch > PERK) after three days on normal dextrose medium after the end of the sleep recording where they were administered food containing vehicle (0.8% EtOH) or RU486 (100uM). (N=19) (B) Quantification of daily sleep amounts (N=19)



Supplemental Figure 4. PDF is expressed in a subset of lateral ventral neurons that have projections into the medulla and the dorsal protocerebrum.
Immunohistochemical analysis of PDF confirms its known expression pattern in the *Drosophila* brain.

CHAPTER 4

GENERAL DISCUSSION

This dissertation work has identified two novel mechanisms of molecular sleep regulation in *Drosophila melanogaster.* In **Chapter 2**, we demonstrated that Homer and DmGluRA interactions are required to promote sleep while in **Chapter 3** we identify a sleep-promoting role for the PERK Pathway of the Unfolded Protein Response. These findings highlight a functional relationship between sleep and two important processes in the brain: synaptic plasticity and cellular proteostasis. These studies have important implications for future work in the fields of neuroscience and sleep as they raise new questions about the molecular underpinnings of behavior in the brain. Here, we discuss the broader implications of the data and suggest some important lines of inquiry for further investigation.

The biological purpose of sleep

A common thread linking much of sleep research together is the continued search for the biological purpose of sleep. No other complex behavior is so pervasive in nature and yet comparatively so poorly understood. Following millions of years of evolution, humans have evolved to spend a third of our lives “unplugged” from the external world at the expense of many other biological drives, suggesting that sleep serves some critical biological function. The negative health consequences that humans experience after insufficient sleep provide strong empirical evidence that sleep is indispensable to health and well-being. As discussed in **Chapter 1,** the wide range of processes that are negatively impacted by sleep loss seem to highlight the fact that sleep likely serves multiple biological functions, just a couple of which include metabolism and immune

function (Benington and Heller, 1995) (Gamaldo et al., 2012), as previously discussed. Continuing scientific research appears to support this notion. Because we show that Homer and PERK promote sleep, it suggests that the processes they regulate are functionally relevant for sleep. Homer proteins are a critical component of synaptic plasticity, as evidenced by many different experimental investigations. For example, Homer and mGluR interactions are necessary for long-term depression (Ronesi and Huber, 2008) and endocannabinoid-mediated synaptic plasticity in the hippocampus (Roloff et al., 2010). As discussed in **Chapter 1**, there is strong evidence that synaptic plasticity is modulated by sleep. Thus, Homer regulation of sleep provides further support for the notion that sleep and plasticity are functionally connected. Along the same lines, PERK regulation of sleep highlights a relationship between sleep and protein homeostasis. This association has gained significant attention in recent years since sleep has been identified as a risk factor for neurodegenerative disease. Neurodegenerative diseases are characterized by severe aggregation of misfolded proteins in the brain, and thus serve as an extreme example of protein dyshomeostasis. If PERK directly regulates sleep, this suggests that sleep is driven by cues of proteostatic stress that could underlie the relationship between sleep loss and disease. Importantly, though Homer and PERK seem to act in entirely disparate cellular processes, there is considerable evidence that synaptic plasticity and proteostasis are closely linked. The most obvious connection between these two processes is that synaptic plasticity is dependent on changes in protein synthesis (Kang and Schuman, 1996) (Huber et al., 2000) (Buffington et al., 2014). Importantly however, the endoplasmic reticulum is responsible not only for protein synthesis for also for the highly localized transport of synaptic receptors to their membrane compartments in response to neuronal activity (Aridor et al., 2004). Thus, there is crosstalk between signals at the

synapse and translational processes in the ER and the shared role of Homer and PERK in promoting sleep suggest they may be mechanisms that link synaptic plasticity and cellular proteostasis.

At its core, sleep remains a fascinating phenomenon in evolutionary history. The goal of the studies described here has been to investigate the involvement of molecular signaling pathways in sleep regulation that could provide greater insight into how sleep is organized at the cellular level. Understanding what molecules are required for sleep across species is an endeavor that bears positive impact not only in our understanding of sleep, but in understanding all other processes that depend on this mysterious daily process to function.

Sleep and calcium signaling

Homer proteins are critical regulators of calcium signaling in the cell (Worley 2007) and it will be important to consider the implications of calcium dynamics in sleep regulation in the future. Calcium is a fundamental and essential ion in the cell that is critically important for the maintenance of cell signaling (Brini et al., 2013). In much of neuroscience research, increases in intracellular calcium levels are a broadly-accepted proxy for neuronal activity (Lin and Schnitzer, 2016). However, the intracellular role of calcium beyond its association with cell membrane depolarization is less commonly investigated in the context of behavioral regulation. Calcium itself acts as a second messenger and regulates processes such as gene transcription (Berridge, 1998), neurotransmitter release (Südhof, 2012), and memory (Tanaka et al., 2008). In mammals, various lines of evidence have shown that calcium is necessary for sleep regulation. In rats, T-type calcium channels in the thalamus are required for slow waves during sleep (David et al., 2013). Furthermore, it has been shown that calcium-

dependent hyperpolarization regulates the duration of sleep in mice; sleep time is significantly reduced in mice carrying genetic impairments in calcium-dependent kinases as well as calcium channels (Tatsuki et al., 2016).

Homer couples mGluR to IP3 receptors, which are the primary channel for calcium release from the endoplasmic reticulum (Tu et al., 1998). Thus, IP3-mediated calcium release could be a mechanism underlying the relationship between Homer, DmGluRA, and sleep. Previously it has been shown that Homer1a expression - which reduces coupling to Homer1 to group I mGluRs in mammals - suppresses intracellular calcium release (Kammermeier and Worley, 2007). This suggests that coupling of Homer and mGluRs affects calcium signaling in ways that may be critically relevant to sleep. Thus, our findings on Homer and DmGluRA mediated sleep regulation in *Drosophila* highlight a need to further understand how neuronal calcium regulation in the brain may regulate behavioral processes including sleep.

Sleep and proteostasis

As previously discussed in **Chapter 1 and Chapter 3**, healthy cellular function requires homeostatic regulation of transcription and translation, protein folding, protein chaperones, and controlled protein degradation. The finding that PERK promotes sleep suggests that disruptions in protein homeostasis may serve as an intracellular signal for sleep. In particular, this work has demonstrated that phosphorylation of eukaryotic initiation factor 2α (eIF2α) is a key integrator of sleep regulation in the cell. This is supported by previous work in rodents demonstrating that maintaining phosphorylation of eIF2α with the drug salubrinal increases slow wave sleep (Methippara et al., 2009). While changing PERK activity in these studies allows us to directly probe the effect of protein translation levels on sleep, an area of future investigation may be to analyze how

levels of protein misfolding directly impact sleep. We would predict that enhanced protein misfolding would alter sleep and wake states. One way to disrupt protein homeostasis would be to to examine the sleep effects of various mutant alleles that include protein misfolding such as superoxide dismutase (SOD). Because mutations that disrupt protein folding are implicated in many neurodegenerative diseases, these pathways have long been studied in the context of disease. However, we believe that it will be useful to generate/examine mutations that cause relatively less dramatic changes in protein folding and see how they affect signaling via pathways such as PERK as well as sleep behavior. This may allow us to probe changes in ER stress signals in ways that are closer to physiological levels that that observed in traditional neurodegeneration models. In these mutants, assessing the effects of PERK inhibition or activation will provide further insight into the mechanisms driving sleep as they relate both to protein misfolding and UPR activation.

An issue that was not addressed in this dissertation but that is highly pertinent to the study of the UPR is the effect of age on UPR pathway activity and protetostatic regulation in the cell. It has been suggested that the UPR plays a critical role in aging and neurodegeneration, and high levels of UPR activation are evident across multiple tissue types in aged animals (Martínez et al., 2016). In this study, we examined the relationship between PERK signaling and sleep in young, adult flies. At this age, the UPR still operates in an adaptive manner where baseline levels of UPR activity are low and cellular stress induced a level of response that is capable of returning the cell to homeostasis. However, work in our lab has previously shown that age is associated with chronic ER stress and UPR activation (Brown et al., 2014). Furthermore, these cellular changes are associated with age-related sleep disruptions (Brown et al., 2014). Thus, in a healthy animal, activation of the UPR is associated with functional proteostatic

regulation while aged animals exhibit high levels of prolonged ER stress that may further indicate differential regulation of these pathways on behavior compared to young animals. Since sleep quality declines with age, it is important to consider how cellular signaling pathways become dysregulated over time, as this may allow us to better understand the biology of aging and degeneration.

Understanding how Homer and PERK alter cell signaling and synaptic homeostasis

A shared feature of Homer and PERK signaling is that these signaling pathways are activated by extended wakefulness. Our work has demonstrated that these two pathways promote sleep, but we do not address their role in modulating neuronal activity or synapse formation. An important line of inquiry in the future will be to understand how Homer and PERK signaling affect neuronal output. In mammalian systems, the role of Homer and metabotropic glutamate receptor signaling on cellular excitability has been explored in cell culture (Kammermeier and Worley, 2007) and DmGluRA signaling has been studied using heterologous systems (Raymond et al., 1999), but to date there has not been any *in vivo* analysis of DmGluRA signaling in the *Drosophila* central nervous system, though there is evidence that DmGluRA regulates synaptic potentiation NMJ (Bogdanik et al., 2004). In the fly, wake is associated with elevated local field potentials in the brain (van Swinderen et al. 2004). In the context of our dissertation work, we would predict that loss of Homer and DmGluRA binding, which we demonstrated is associated with increased wake, causes higher baseline levels of neuronal activity in the brain. This will be an interesting topic to address in the future.

In addition to neuronal activity, one of the outstanding questions regarding sleep is how sleep affects synapse number, size, and strength. There remains much debate

about whether the sleep is primarily necessary to prune or build synapses in the brain. As discussed in **Chapter 1**, the synaptic homeostasis hypothesis posits that synaptic downscaling is a key feature of sleep. This hypothesis is support by data in flies and mice which demonstrate that transcripts of synaptic proteins are reduced during sleep (Bushey et al., 2011) and that electron microscopy of synapses in the somatosensory cortex and motor cortex of the brain are reduced during sleep (de Vivo et al., 2017). However, there is also evidence of synapse formation following learning in the brain, demonstrating that synaptic growth is also a feature of at least some neurons during sleep (Yang et al., 2014). It will be interesting to determine if Homer and PERK signaling impact synapse formation or morphology in the *Drosophila* brain. A possible approach to interrogate synaptic changes in the brain would be to use a transgenic approach such as expression of fluorescently labelled Bruchpilot (Brp)-Short in the brain (Mosca and Luo, 2014) in order to visualize active zones in different brain regions. The benefit of this approach is that different cell-type drivers could be used to asses changes in different brain regions or across different cell types. A general prediction regarding Homer and PERK may be that loss of signaling from either pathway would result in enhanced active zone expression that would correlate with increases in wakefulness.

Technical limitations

Two major limitations of examining *Drosophila* sleep using video analysis as described here is that 1) measurements of sleep are dependent on changes in locomotor activity and 2) flies must be individually housed for the duration of the sleep recordings. While inactivity is a proven and reliable measure of sleep in *Drosophila*, the most definitive measure of sleep would require direct electrophysiological recording of brain activity. This remains a technically challenging approach, but would also provide

useful information about the dynamics of cell and circuit activity as they relate to wake and sleep in the fly brain. Additionally, as discussed in **Chapter 1**, separation of individual flies into separate locomotor tubes for sleep analysis introduces confounds of social isolation. Our lab has shown that social isolation leads to ER stress and sleep disruptions (Brown et al., 2017) which must be considered in sleep experiments that are conducted over multiple days. In our experiments, we limit our recordings to 2-3 days under normal light/dark conditions whenever possible to limit this confound. However, even in the absence of known cellular stress markers, it is possible that sleep analysis of a single-housed fly for even a single day would appear different than that of a socially enriched animal. This concept applies not only to sleep studies, but to all types of behavioral research, as any stress directly cause by the experimental paradigm is a potential confound to the observed results. Building new analytical tools that would allow for analysis of group behavior (while still tracking individual animals separately) will be an important endeavor for behavioral research moving forward.

In addition to the technical limitations of our behavioral analysis, a significant limitation that applies to all *Drosophila* research is the limited availability (relative to the commonly used mouse model) of highly specific, high quality antibodies that can be used for immunohistochemical analysis of protein expression. One of the questions we would like to answer is what the subcellular location of Homer and DmGluRA is in the brain. It is likely that Homer and DmGluRA are co-expressed at the postsynaptic density, but confirmation is still required. Without the proper antibodies, we were unable to conduct immunohistochemistry to visualize Homer and DmGluRA in the whole brain, which would allow us to address questions about where Homer and DmGluRA are specifically expressed in the cell and how their expression and location may change

across behavioral state. Thus, both the cellular and subcellular localization of Homer and DmGluRA in the *Drosophila* central nervous system remains unknown.

Finally, in humans and other mammals, sleep is divided into distinct stages: non-rapid eye movement (NREM) and REM sleep. Both NREM and REM exhibit their own electrophysiological signatures and corresponding changes in muscle tone and eye movements (Carley and Farabi, 2016). Unlike mammals, *Drosophila* do not exhibit REM sleep. In this way, using the *Drosophila* model limits our analysis of sleep to a single sleep state. In the context of our findings, the role of Homer and PERK signaling in sleep regulation may have NREM and REM specificity in mammalian systems that will be important to address in higher order model systems.

Follow-up experiments

While we have discussed some of the future directions and outstanding questions from our studies throughout this dissertation, here, we propose specific experiments that would provide an appropriate follow-up to this body of work and address some of the emergent inquiries on DmGluRA, Homer, and PERK signaling.

One of the future directions of our investigation of DmGluRA- and Homer-mediated regulation of sleep is to determine whether DmGluRA and Homer signaling have circuit-specific effects on sleep and activity. Notably, the behavioral observation that DmGluRA genetic knockdown disrupts light-mediated sleep distribution strongly suggests that DmGluRA is acting in a light-responsive circuit (such as the lateral ventral clock neurons) in order to regulate sleep. We would thus propose a GAL4 screen to drive DmGluRA RNAi and Homer RNAi in known sleep-regulatory cells and brain circuits to identify cells where these molecules are required for sleep, with an initial focus on

light-responsive circadian circuits. Changes in sleep as a result of RNAi expression in different cell types of brain regions will provide the spatial information required to further understand how DmGluRA and Homer regulate sleep.

As discussed in a previous section of this chapter, Homer proteins are required for modulating calcium signaling (Worley 2007), which suggests that disrupting DmGluRA and Homer associations at the synapse may have an impact on calcium signaling in the cell and that these changes may underlie the effects on sleep that we observed in the CRISPR mutant fly. In addition to mGluRs, Homer proteins also bind to IP3 receptors on the ER membrane (Tu et al., 1998) that regulate the release of ER calcium stores into the cytoplasm upon activation and acutely depolarize the cell. We would propose an experiment to determine whether Homer-DmGluRA mediated sleep regulation involves IP3 signaling by knocking down *IP3* in the CRISPR genotype. If IP3 signaling mediates the effect of the CRISPR mutation on sleep, we would not observe any additional sleep changes following *IP3* knockdown compared to the original CRISPR binding mutant. To expand this inquiry along the same lines, we also propose knocking down *Homer* in the CRISPR genotype to determine the extent to which Homer regulates sleep through its interaction with DmGluRA. We would expect that if Homer promotes sleep solely through DmGluRA interactions, sleep following *Homer* knockdown in the CRISPR genotype would not be significantly changed relative to sleep in the CRISPR mutant as observed in this study.

As mentioned in the previous section, we were not able to conduct a complete analysis of Homer and DmGluRA expression in our studies due to the limited availability of robust and specific antibodies, especially for DmGluRA. For this reason, while we were able to show that Homer expression in the brain does not change as a result of the

CRISPR=mediated DmGluRA binding site mutation, we could consistentlyl generate a measurable signal using the DmGluRA antibody to determine whether this was also the case for DmGluRA protein expression in the CRISPR mutant. Thus, it follows this technical limitation that we also could not characterize of the subcellular expression patterns of DmGluRA and Homer in the *Drosophila* brain, even though this remains an important and outstanding question. Higher order systems express multiple mGluR and Homer subtypes in different subcellular compartments so it seems highly plausible that DmGluRA and Homer, being the sole variants in the fly, may be expressed differentially depending on cell type or neural circuit identity. An experimental approach in the near future may be to generate a transgenic GFP-tag on DmGluRA in both the wildtype fly and in the CRISPR genotype to characterize the subcellular location of DmGluRA. In the wildtype flies, we may use this approach to determine the synaptic location of DmGluRA under normal conditions. Then, by examining DmGluRA expression in the mutant fly using the same GFP-tag approach, we may also determine if DmGluRA localization changes as a result of Homer binding suppression in the CRISPR mutant. This would provide information about the potential role of Homer in mediating DmGluRA synapse localization and expression.

In addition to investigating the effect of DmGluRA-Homer binding loss on receptor expression, it will also be important to characterize changes in reception function and signaling as well. Previously, loss of DmGluRA has been shown to affect synaptic potentiation at the neuromuscular junction (Bogdanik et al., 2004). Given the tractability of recording from neurons in the NMJ (which are larger than central nervous system neurons and therefore more easily accessible to electrophysiological recordings) a practical experiment to follow-up our sleep experiments would be to record the electrophysiological response of neurons in the NMJ of flies carrying the DmGluRA

binding site mutation in response to extended stimulus trains as previously described (Bogdanik et al., 2004). It will be interesting to determine whether loss of Homer binding affects the baseline physiological response of DmGluRA at the NMJ. Ideally, this experiment should eventually be conducted in the central brain, though such an approach is much more technically difficult. Understanding the electrophysiological consequences of Homer/DmGluRA binding changes will not only provide insight into sleep regulation but will generate critical and fundamental information about mechanisms underlying glutamatergic neurotransmission regulation.

Finally, given that sleep is important for learning and memory and mGluR and Homer signaling have been implicated in learning and memory in mammalian systems (Riedel and Reymann, 1996) (Simonyi et al., 2005) (Mahan et al., 2012) we would propose investigating whether DmGluRA and Homer protein interactions are required for earning and memory in *Drosophila.* It has been shown that DmGluRA is necessary for learning in a courtship conditioning assay in *Drosophila* (Schoenfeld et al., 2013) and thus we would propose conducting a courtship conditioning assay on the DmGluRA-Homer binding mutant to determine whether the interaction between DmGluRA and Homer proteins is necessary for *Drosophila* learning behavior. If the CRISPR binding mutant exhibits impairment in learning and memory, it would suggest that DmGluRA and Homer binding are necessary to modulate learning in the fly.

Similar to the questions raised by our investigation of DmGluRA and Homer signaling in the fly, one of the main lines of inquiries that follows our investigation of PERK-mediated sleep regulation is whether the effects of PERK on sleep are cell-specific or circuit-specific. We would propose that a first set of follow-up experiments should determine whether different cell types mediate the effects of PERK on sleep. We

would express PERK RNAi in neurons (using neuonal *elav* or *nSyb* drivers) and glial cells (using drivers such as *repo* or *alrm* GAL4) to determine whether PERK is required for cell in certain cell types. Along the same lines, an RNAi screen of multiple sleep-relevant (and non-sleep relevant circuits) in the brain would provide information about whether regional specificity exists in PERK regulation of sleep. It will be interesting to determine whether PERK signaling regulates sleep exclusively through circuits that are necessary for sleep/wake or whether modulation of sleep by PERK is a general phenomenon that can occur in any cell type or circuit. These experiments could provide a foundation to uncover unique neural maps of sleep regulation that may be hitherto unknown.

In the discussion section of **Chapter 3**, we discussed the notion that the association between PERK activation and wakefulness could be explained by the possibility that extended wakefulness may be associated with enhanced energy demands that require increases in protein synthesis. However the experiments described in this dissertation do not directly address the relationship between energy, metabolism, and PERK signaling. Recently, Stahl et al., 2017 demonstrated that sleep is associated with reduced metabolic rate in *Drosophila* using a system that measures levels of CO2 production and sleep simultaneously in individual flies. Using this approach, we would propose to directly measure both sleep behavior and metabolic rate in flies under various conditions of PERK modulation. Based on the findings in this dissertation, would predict that knocking down PERK activity in the fly – which increases wakefulness – would be associated with enhanced CO2 production in *Drosophila*. These experiments would illuminate a currently unexplored relationship between UPR signaling and metabolic demand in the fly. We would postulate that enhanced metabolic demand would be associated with increases in neuronal activity as well. Thus, we may also seek

to measure calcium levels using genetically encoded calcium indicators to determine whether neuronal activity is changed and where these changes may occur as a result of alterations in PERK signaling.

Finally, while we found that acutely altering PERK signaling (either by pharmacological administration or RU486-mediated transgene induction) significantly affects sleep amount, our analysis of PDF neuropeptide release was conducted using a constitutive PDF GAL4 driver to knockdown and overexpress PERK. Therefore, it will be important to conduct a similar experiment using an inducible GAL4 driver to determine whether the observed circuit-specific effect of PERK on sleep and PDF neuropeptide release is the result of any developmental effects occurring in response to the loss or induction of PERK signaling. To address this, we propose using a PDF GeneSwitch GAL4 (Depetris-Chauvin et al., 2011) in order to express PERK RNAi or overexpress PERK in the same PDF circuit but only during adulthood. Transgenic crosses would be placed on regular medium until adulthood upon which RU486 administration would be conducted to induce transgene expression. Analysis of both sleep and neuropeptide release following RU486 administration in adult transgenic flies would allow us to determine whether the effects on sleep behavior and PDF release occur independent of developmental changes in PERK transgene induction or whether modulating PERK signaling in adulthood produces effects that are different from those seen in our experiments using a constitutive GAL4 driver. Lastly, we would propose further experiments to characterize other changes in the PDF neurons and in related circuits that may occur as a result of changes in PERK signaling. For example, if PDF neuropeptide release is altered in response to changes in PERK, this may affect other cells that innervated by PDF neurons, such as the PLP neurons which express the neuropeptide allatostatin (AstA) (Chen et al., 2016). It will be critical to determine

whether changing PERK signaling in a group of cells within a circuit produces changes in downstream cells. For example, it is possible that reduced or enhanced PDF release on PLP neurons may leads to changes PDF receptor expression at postsynaptic targets. If this is the case, altering PDFR expression to rescue the effects of PERK knockdown or overexpression would demonstrate a requirement for transmission between those synaptic contacts in PERK regulation of sleep.

Understanding the balance of sleep and wake

Humans and many other species are unable to survive without sleep. In the lab, sleep deprived rats die after two weeks (Rechtschaffen et al., 1983) and after multiple days of sleep deprivation, the Pacific beetle cockroach exhibits increased mortality (Stephenson et al., 2007). In humans, a rare disease known as fatal familial insomnia causes early death (Manetto et al., 1992). An inherent part of investigating sleep is to also delve into the biological limits on wakefulness. The empirical evidence exists that demonstrates that animals cannot sustain long periods of uninterrupted wake, but much remains to be understood about why humans and other species evolved this way. Both studies described here highlight signaling pathways in the cell that are modulated by extended wakefulness and subsequently promote sleep. This suggests that Homer and PERK may be part of a combination of molecular cues in the cell that encode or limit the extent of wakefulness that a cell can sustain before sleep becomes necessary.

The results of our studies suggest that molecules in the cell may provide a signal for sleep that determines the limit of wakefulness in the cell and brain. While it is not surprising that different species exhibit different patterns of daily sleep, there is interesting evidence that the amount of wake that an organism can sustain varies even

within the same species. For example, *Astyanax mexicanus* is a species of fish that inhabits highly divergent environments, with some populations living in rivers while other populations stay in dark caves (Duboué et al., 2011). The blind cavefish populations of *Astyanax mexicanus* have evolved to lose their eyes and also sleep for very short amounts of time (Duboué et al., 2011). This demonstrates an evolutionary adaptation to the dark, food-scarce environment of the caves and suggests that environment plays a large role in determining sleep patterns. It seems likely that the type of waking activities dictated by the environment have a key role in determining the balance of sleep and wake for any given organism over time. Different environments and survival demands require divergent activity and input from various neuronal circuits which may ultimately determine the type/amount of sleep that is necessary for any given animal. There is a limited amount of work that has been done looking at how different types of waking behavioral tasks might differentially affect sleep. This will be a meaningful and important line of inquiry moving forward. Related to this work, examination of the upstream wake-active (and potentially task-specific) signaling pathways that may act on Homer and PERK will allow us to better understand the molecular signals that encode the cellular transition from wake to sleep.

Conclusion: Sleep from flies to humans

In this dissertation, we employed the fruit fly as an animal model to understand the molecular and neurobiological regulation of sleep. Understanding the biology of sleep has important implications for human health and disease, and the *Drosophila* model is well-placed to uncover functional mechanisms bridging the two processes. There is significant genetic conservation between *Drosophila* and mammalian systems; approximately 60% of human disease genes are conserved in the fly (Fortini et

al., 2000). Thus, interrogation of gene function as it relates to behavior in ways such as those described here may elucidate disease-relevant biology that could inform treatment in the clinic in the near future.

Sleep dysfunction is a common comorbidity of many neuropsychiatric disorders and neurodegenerative diseases. Some specific examples include Autism Spectrum Disorders (Devnani and Hegde, 2015), schizophrenia (Kaskie et al., 2017), Alzheimer's disease (Musiek et al., 2015), and Parkinson's disease (Knie et al., 2011). Notably, Homer and PERK have been implicated or examined for their therapeutic potential in many of these disorders and diseases (Wang et al., 2016) (Folsom et al., 2015) (Soler et al., 2018) (Stutzbach et al., 2013) (Chen et al., 2013) (Mercado et al., 2018). Homer and PERK therefore highlight the clinical relevance of molecular regulators of sleep, and the importance of understanding how sleep is regulated in the brain. Furthermore, it is possible that therapies that improve sleep might concurrently improve outcomes for some of these disorders and diseases characterized by sleep disturbances.

Given the strong association between poor sleep and negative health outcomes, it is imperative that greater attention be paid to our biological need for sleep. Nowadays, it appears that we live in a world which dramatically undervalues sleep. Modern society is replete with external influences that negatively impact sleep. The high prevalence of shift work, international travel, electronic devices, and even dietary habits are just some examples of environmental influences that disrupt sleep (Shochat, 2012). Insufficient sleep has become so ubiquitous that in 2014, the Centers for Disease Control and Prevention (CDC) declared it to be a public health epidemic. Some studies have sought to quantify the financial cost of this epidemic, estimating that poor sleep accounts for billions of dollars in losses each year worldwide (Hafner et al., 2016) (Vincent et al., 2018). The fervent public interest in sleep in recent years appears to be widespread

acknowledgment of what scientists have long observed to be true: that sleep is one of the most important and indispensable behaviors for health and well-being.

This work has identified two critical regulators of sleep at the cellular level. Prior to this dissertation, an overwhelming range of research had already demonstrated the vast complexity of sleep regulation in the brain. These findings fill in just a small portion of the uncompleted puzzle of sleep, and much work remains to tackle this mysterious phenomenon in nature.

APPENDIX

Genetic loss of *DmGluRA* reduces lifespan and sleep in aged flies

Loss of *DmGluRA* reduces lifespan

Since sleep/wake disruptions are known to negatively impact health, investigated the lifespan effects of *DmGluRA* knockdown in *Drosophila.* We conducted a survival assay of null *DmGluRA* mutants and wildtype flies and found that loss of *DmGluRA* is associated with a reduction in both median and maximum lifespan (**Figure 1a**). The Log-rank test to assess differences in the survival curves between genotypes indicates that the reduction in survival is statistically significant in null *DmGluRA* flies compared to wildtype flies. Average lifespan was reduced by more than 20% in null *DmGluRA* mutants compared to wildtype *Drosophila* (**Figure 1b**). Thus in addition to altering normal sleep/wake patterns, loss of *DmGluRA* impacts the aging process of the fly.

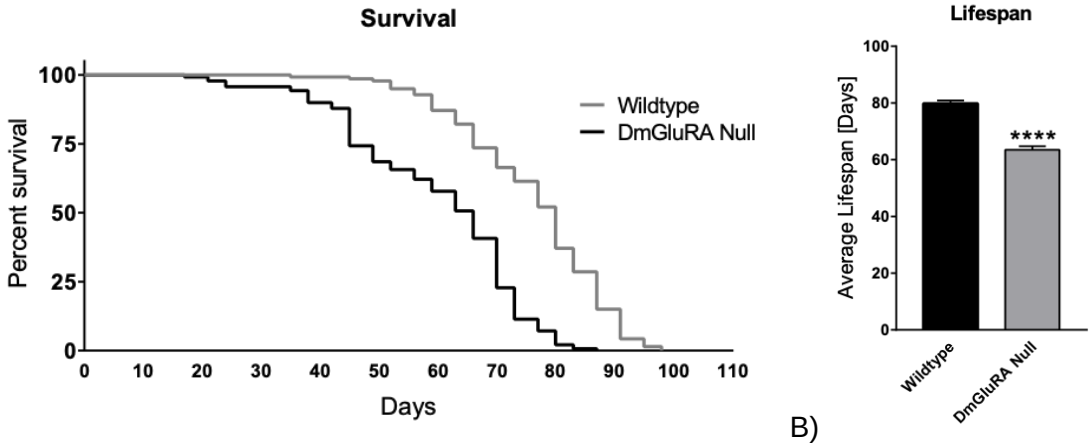


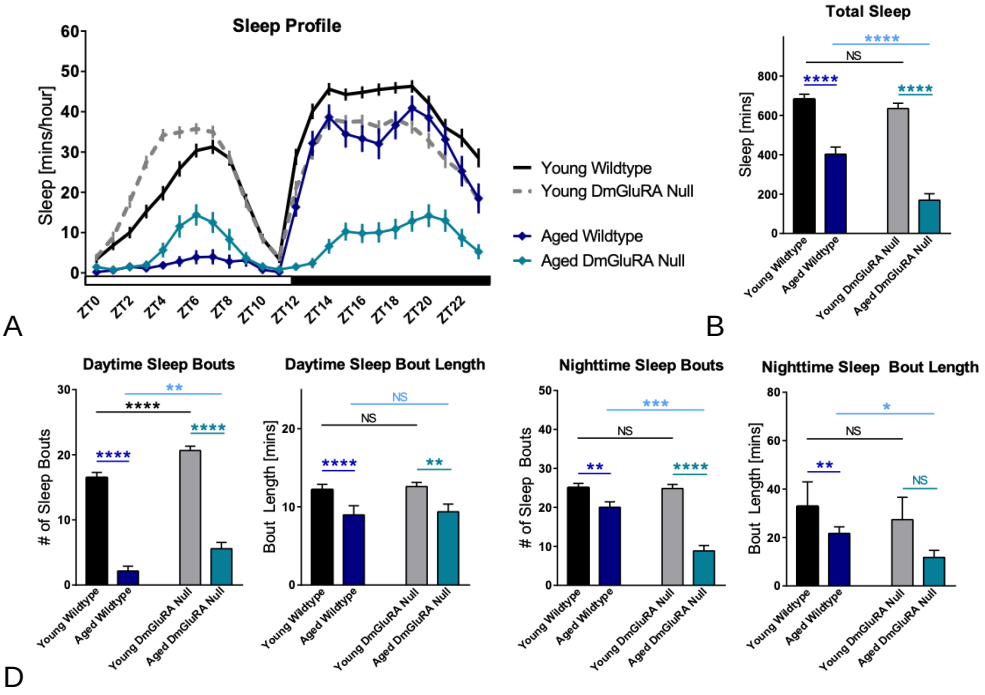
Figure 1. Null *DmGluRA* mutants have reduced lifespan

(A) Survival curves of wildtype flies and null *DmGluRA* mutants. Null *DmGluRA* mutants exhibited a shorter median lifespan compared to wildtype controls (Wildtype median age = 80 days; *DmGluRA* null median age = 66 days). Log-rank (Mantel-Cox) test was performed to determine statistical significance of difference between survival curves *(N=140, P<.0001)* (B) Average lifespan is significantly reduced in null *DmGluRA* mutants compared to wildtype flies *(N=140 per genotype, error bars represent SEM) (P<.0001)*

Loss of *DmGluRA* exacerbates age-related sleep loss

Following our observation that loss of *DmGluRA* reduces lifespan, we sought to determine if sleep in null *DmGluRA* mutants undergoes any age-specific changes that are different from wildtype flies. We examined sleep behavior in aged, one month old wildtype flies and null *DmGluRA* mutants since it has been found that during this time, *Drosophila* exhibit behavioral changes that are indicative of a shift to senescence (Carey et al., 2006). At one month of age, both wildtype and null *DmGluRA* mutants exhibit both daytime and nighttime sleep loss relative to young flies in each genotype (**Figure 2a**). Notably, while young null *DmGluRA* mutants and wildtype flies exhibit no overall difference in daily sleep amount (**Figure 2b**), aged null *DmGluRA* mutants sleep significantly less during the 24 hour day in comparison to wildtype flies (**Figure 2b**) and the effect size of age-related sleep loss is greater in null mutants(**Figure 2b**). This difference in sleep appears to be due to a dramatic reduction in nighttime sleep, since aged null *DmGluRA* mutants sleep more during the day than wildtype flies (**Figure 2a**). Thus, aging is associated with both daytime and nighttime sleep loss as previously shown (Brown et al., 2014), but the loss of *DmGluRA* appears to exacerbate this effect, primarily due to a large reduction in sleep during the night.

Analysis of sleep architecture in aged flies revealed that with age, both wildtype and null mutants have fewer and shorter sleep bouts during both the day and night compared to flies at one week of age (**Figure 2d**), with the exception that average nighttime sleep bout length in null *DmGluRA* mutants is not significantly changed with age (**Figure 2d**). In contrast to sleep architecture, changes in wake architecture across age in both genotypes demonstrate that aged flies exhibit fewer and longer wake bouts that contribute to the overall increase the total wake time (**Figure 2e**). We also examined sleep in both wildtype and null *DmGluRA* mutants at 6 weeks of age. Here, we observed that activity peaks in the transitional periods between day and night became blunted (**Figure 3**), similar to what has been previously demonstrated in 2 month old flies (Koh et al., 2006). Since it is well established that locomotion decreases significantly with age (Koh et al., 2006; Carey et al., 2006), we believe that low levels of activity make it more difficult to interpret sleep at this age.



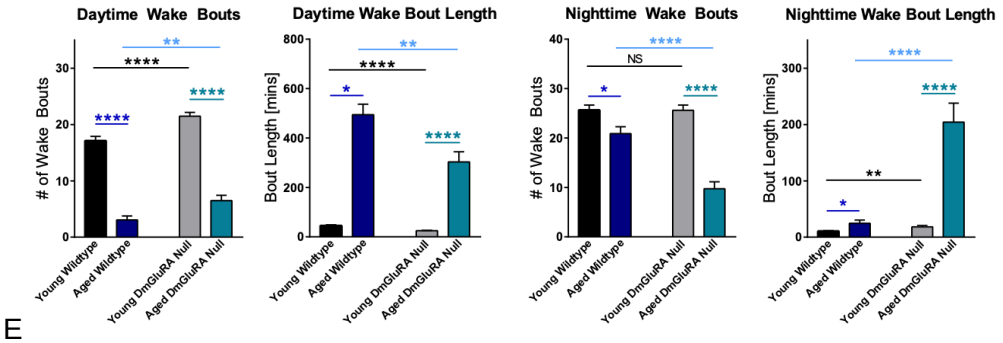


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Figure 2. Age-related sleep loss is exacerbated in null *DmGluRA* mutants
(A) Sleep profile of wildtype and null *DmGluRA* mutants at one week and one month of age (*N≥27 per group, error bars represent SEM)* (B) Sleep per 24hr day. Young one week old null *DmGluRA* mutants do not exhibit differences in total sleep per day compared to wildtype flies. At one month of age, total daily sleep amounts are significantly reduced in null DmGluRA mutants compared to wildtype flies. Total daily sleep is reduced with age in both wildtype and null DmGluRA mutant flies (*N≥27)* (C) Total daytime sleep and nighttime sleep are reduced with age. Both wildtype and null *DmGluRA* mutants display significant daytime and nighttime sleep loss at one month of age compared to one week old flies. Across genotypes, null *DmGluRA* mutants sleep more during the day and less at night at both one week of age and on month of age compared to wildtype flies (*N≥27)* (D) Both sleep bout number and sleep bout length become reduced with age in wildtype flies. In null *DmGluRA* null mutants, day and night sleep bout number is reduced with age and average daytime sleep bout length is shorter. At one month of age, null *DmGluRA* mutants have fewer sleep bouts and shorter average sleep bouts at night compared to wildtype flies. (*N≥27)* (E) Wake becomes more consolidated with age. Aged flies exhibit fewer daytime and nighttime wake bouts and average wake bouts are longer. Null *DmGluRA* mutants display a much greater increase in the average length of wake bouts at night null at night compared to wildtype flies *(N≥27) (*P<.05, **P<.01, ***P<.001) ****P<.0001)*

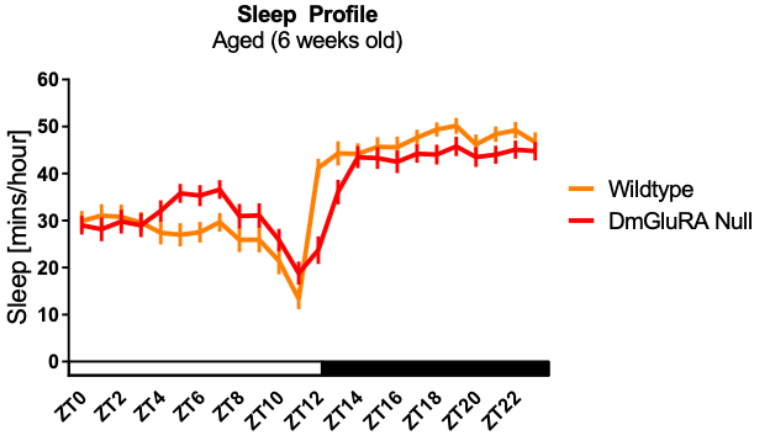

Figure 3. 6 week old flies have blunted activity in the transition between lights on/off
Daily sleep profile for wildtype wCS10 and null DmGluRA mutants over the course of a 72 hour sleep experiment. Null *DmGluRA* mutants exhibit higher levels of sleep during the day and lower levels of sleep during the night relative to wildtype flies *(N=27, error bars represent SEM)*

Discussion

We demonstrate here that loss of the *Drosophila* metabotropic glutamate receptor *DmGluRA* reduces lifespan and accelerates age-related sleep loss in the fly. Increased sleepiness during the day and an inability to sleep at night are common features of sleep in the elderly (Reviewed in Cooke and Ancoli-Israel, 2011) and it has been previously reported that aged *Drosophila* have sleep patterns that are less restricted to the night and more evenly dispersed across the 24 hour day/night cycle

It is interesting that null *DmGluRA* mutants are short sleepers, but only when older. In young flies, loss of *DmGluRA* did not alter the total sleep over the 24 hour day (**see Chapter 2**). However, aged flies without *DmGluRA* sleep significantly less than aged wildtype flies. In both wildtype and mutants, sleep amount is less than that seen in older flies. This suggests that loss of *DmGluRA* accelerates age-related sleep loss, rather than directly regulating sleep amount at all ages of development. Thus, it may not merely be sleep fragmentation or short sleep in early life that determines lifespan, but also the proper timing and distribution of sleep across the day that may be important for the aging process. The association between short sleep in aged null *DmGluRA* flies and a reduction in the average lifespan is supported by evidence across species. In humans, short sleep duration has been strongly associated with increased mortality (though it should also be noted that abnormally long sleep duration produces the same association) (Reviewed in Grandner et al., 2010) and a previous study on short-sleeping *Drosophila* mutants also found an association between higher wake amounts and increased mortality (Bushey et al., 2010). Certainly, what remains to be determined is whether sleep can be causally linked to aging outcomes and lifespan. For example, in the context of our current findings, it will be important to determine whether

manipulations that increase sleep in aged null *DmGluRA* mutants could rescue the negative effects on lifespan.

Here, aged flies were defined as flies that were one month old. Previous studies examining the aging effect on *Drosophila* sleep reported reduced sleep time and increased sleep fragmentation flies that were two months of age (Koh et al., 2006; Brown et al., 2014), or twice the age of the aged group in our study. For the purposes of this study, we chose to examine sleep in one month old flies in order to measure sleep as close to the onset of age-related changes as possible. At one month of age, behavioral senescence may be just beginning (Carey et al., 2008) and the rhythm strength of sleep has not diminished to the extent that is observed than at two months of age (Koh et al., 2006). Notably, at 6 weeks of age, we saw that null *DmGluRA* mutants had similar levels of sleep to wildtype flies (**Figure 3**). However, we believe that aging-induced reductions in locomotor behavior (Koh et al., 2006; Carey et al., 2006) may mask sleep differences between groups at more advanced ages. Thus, we believe that one month may represent a critical midlife time point where age-related changes in *Drosophila* behavior should be observed in future studies.

How might metabotropic glutamate receptor signaling modulate the aging process? Like all cells in the body, neurons are vulnerable to the effects of age, as many homeostatic processes in the cell degrade with time (Nikoletopoulou and Tavernarakis, 2012) (Vayndorf et al., 2016). In young animals, glutamatergic receptor expression and calcium signaling in neurons changes across sleep and wake states (Lanté et al., 2011) (Bushey et al., 2015), which suggests that sleep may be important for regulating neural excitability and maintaining synaptic homeostasis. In addition to mGluRs, glutamate binds ionotropic receptors present on many different cell types (Meldrum, 2000).

Ionotropic glutamate receptors such as AMPA receptors (AMPARs) and NMDA receptors (NMDARs) are transmembrane ligand-gated ion channels that mediate fast synaptic transmission (Traynelis et al., 2010). Analysis of mGluRs in cultured suprachiasmatic nucleus (SCN) neurons has demonstrated that mGluR activation inhibits ionotropic glutamate receptor-mediated calcium increases in the cell (Haak, 1999). Furthermore, mGluR activation leads to a stable reduction in the expression of AMPA receptors at the cell surface (Sanderson et al., 2011). Interestingly, a reduction in AMPA receptors at the membrane has been found to be a correlate of sleep (Lanté et al., 2011) and during sleep, mGluR signaling is necessary for memory consolidation (Diering et al., 2017). Thus, one interpretation for why loss of mGluR would exacerbate sleep loss is that without mGluR signaling, modulation of intracellular calcium and receptor trafficking is lost, leading to increased cellular excitability during periods when inhibitory modulation is required, such as during sleep.

These results have important implications for our understanding of processes that are mediated by mGluR signaling. For example, mGluRs – including *DmGluRA* – are critical for learning and memory (Schoenfeld et al., 2013) (Diering et al., 2017). Thus, mGluR dysregulation may underlie both sleep and cognitive impairments in old age, which is supported by data that higher expression of mGluRs is associated with better cognitive outcomes in aged rodents (Ménard and Quirion, 2012). In the periphery, *Drosophila DmGluRA* signaling is required for development of the neuromuscular junction (NMJ). Null *DmGluRA* mutants have been previously shown to exhibit altered NMJ morphology and changes in cellular excitability at the NMJ (Bogdanik et al., 2004). A limitation of the experiments described in this study is that examination of sleep in a genetic null does allow us to rule out changes in development or in peripheral signaling as contributors to the observed sleep phenotype. While we did not identify any changes

in baseline locomotor ability or activity that would indicate a behavioral contribution of peripheral mGluR signaling in null flies, we cannot definitively attribute the sleep effects to brain-specific mGluR signaling. Additionally, we must also consider whether changes in development of glutamatergic synapses as a result of *DmGluRA* knockdown might mediate sleep behavior. Future directions will be to examine the effects of conditional genetic knockdown of mGluR signaling in the brain. Furthermore, as previously discussed, mammalian systems express multiple mGluR subtypes, and different mGluR subtypes may have distinct roles in regulating sleep and aging. Future investigation will be necessary to address this. Finally, metabotropic glutamate receptors have long been considered for their therapeutic potential (Reviewed in Vaidya et al., 2013), and thus may represent an avenue for sleep therapy development in the future.

Experimental Procedures

Lifespan Assay

Female wCS10 wildtype and null DmGluRA female flies were collected under CO_2 anesthesia for the lifespan assay. At one week of age, 140 flies were collected per genotype and separated into groups of 20 flies placed in separate vials containing standard dextrose media. Survival was scored every 3 to 4 days when flies were switched to fresh vials of standard dextrose media.

Statistical Analysis

Student's t tests were used to compare sleep between genotypes. When relevant, analyses of daytime and nighttime sleep were performed separately. Statistical analysis of survival curves was conducted using GraphPad Prism software (La Jolla, CA, USA)

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