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Genetic and Anatomical Regulation of Sleep in *Drosophila*: A Role for the Mushroom Bodies, Circadian Neurons, and the Gene, *Clock*, in Sleep Promotion

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ABSTRACT

Genetic and Anatomical Regulation of Sleep in *Drosophila*: A Role for the Mushroom Bodies, Circadian Neurons, and the Gene, *Clock,* in Sleep Promotion

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After nearly 100 years of research, the function of sleep is unknown, prompting the desire to examine its regulation in a simpler model organism. In 2000, *Drosophila* was described as a novel model system to investigate sleep. These early studies defined features of normal sleep in *Drosophila*, and presented evidence that sleep was regulated genetically, since mutations in circadian genes affected the quantity and quality of fly sleep. However, it was unknown at the start of this thesis research whether sleep was a neuroanatomically regulated behavior, and actively promoted by the brain, as in mammals. To examine this possibility, we undertook an anatomical screen whereby various regions of the fly brain were examined for a sleep-regulatory function. The data presented here support a role for the *Drosophila* mushroom bodies (Chapter 2,3) and circadian circuitry (Chapter 3,4) as sleep regulatory regions important for sleep promotion, and a potential role for specific regions of the mushroom bodies and/or central complex in wake-promotion (Chapter 3). We also examined the role of the circadian gene *Clock* in sleep by assessing multiple mutant alleles, the contribution of genetic background to the phenotype, and the specificity of the *Clock* mutant sleep phenotype (Chapter 4). Importantly, we were able to rescue components of the *Clock* sleep phenotype by rescuing *Clock* function within mushroom body and circadian neurons (Chapter 4).

These data provide the first evidence that sleep is neuroanatomically regulated in flies, as in mammals, and may be both actively promoted and inhibited. The data further suggest a

genetic and anatomical link between circadian and sleep regulatory regions in the fly brain through *Clock*. Together, these data suggest that searching for genes expressed in the mushroom body, or *Clock* target genes might be a successful strategy to use in gene discovery, and to ultimately uncover the function of sleep.

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LIST OF ABBREVIATIONS

5-HT	5-hydroxytryptophan
ABL	average sleep bout length
bHLH	basic helix-loop-helix
cAMP	cyclic adenosine monophosphate
CC	central complex
CI	sleep consolidation index
Clk	*Clock*
CREB	cAMP response element binding protein
cry	*cryptochrome*
CS	Canton S (special)
CT	constant temperature
CT	circadian time
cyc	*cycle*
DA	dopamine
DD	constant darkness
dDAT	*Drosophila* dopamine transporter
DN	dorsal nuerons
DPM	dorsal paired medial cells
EEG	electroencephalograph
ET	enhancer trap
GABA	gamma-aminobutyric acid

GAL4	yeast transcription factor
GAL80	GAL4 inactivating yeast transcription factor
GFP	green fluorescent protein
Hid	*head involution defective*
HU	hydroxyurea
KC	Kenyon cell
LD	light dark (entrained conditions)
LN	lateral neuron
LPN	lateral posterior neuron
LSL	long-sleeper
MB	mushroom body
METH	methamphetamine
NREM	non-REM (rapid eye movement) sleep
PDF	pigment dispersing factor neuropeptide
per	*period*
PI	pars intercerebralis
PKA	protein kinase A
R	rebound
REM	rapid eye movement sleep
RT-PCR	reverse transcriptase polymerase chain reaction
S	sleep
SCN	suprachiasmatic nucleus
SD	sleep deprivation

Shi	*shibire*
SOG	subesophageal ganglion
SPZ	sub-paraventricular zone
SSL	short-sleeper
SWS	slow wave sleep
TC	temperature cycling
TGUC	tubulinGAL80^{ts}; UAS*Clk*
tim	*timeless*
TNT	tetanus toxin
UAS	upstream activating sequence
UH	UAS-*Hid*
UST	UAS*shibire*^{ts}
VLPO	ventral lateral preoptic area
W	wake
WT	wild-type
yw	*yellow white*
ZT	zeitgeber time

Dedicated to the fields,

woods, lakes, and ponds of Carleton, NS.

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CHAPTER 1

Introduction

Why do we need to sleep? How can some people feel refreshed after four hours of sleep while others require ten? Why do we learn poorly if we are tired? Or fail to remember something that we've learned without proper sleep? The answers to these questions have remained elusive, in part because the means for dissecting complicated behaviors such as sleep have only become available in the past century. Until the early 1900's, we did not even know the basis for inheritance, however, with the discovery of the gene, and the ensuing explosion of molecular biology, we have now been given the tools to begin to understand our own behavior.

Behavior is the end result of a seemingly incomprehensible series of interactions between genes, molecular pathways, cells, and neuronal networks. However, much progress has been made in the genetic/anatomical dissection of complex behaviors. The first single gene to be linked to behavior was *period*, a central component of the molecular feedback loop required to sustain behavioral rhythmicity under constant conditions (Konopka and Benzer, 1971). The discovery of *period* was critical, in that it convinced the scientific community that a behavior as complex as the daily circadian pattern of locomotor activity could be severely disrupted by mutating a single gene. Since this finding, many more genes have been linked to specific behaviors, including circadian rhythms (Review: Allada et al., 2001), courtship (Review: Manoli et al., 2006), learning and memory (Waddell and Quinn, 2001), and alcoholism (Review: Guarnieri and Heberlein, 2003). Until very recently, researchers have not attempted to dissect the genetic regulation of sleep, and thus, the function of sleep has remained a mystery.

Sleep is a behavior that everyone can understand on a personal level since it is something we experience daily, and that nearly everyone has had a problem with at some point in their lives. The 2005 NSF "Sleep in America" poll cites that 75% of people report experiencing a sleep problem one night or more a week, 40% obtain less than 7hr sleep a night on weekdays, and 26% of people polled say that they only receive a good nights sleep a few nights a month. Of these people, 62% report daytime sleepiness a few times a week, and 46% miss work or make errors during work (NSF, Sleep in America Poll, 2005). These statistics are enlightening, and suggest that not only is it difficult for us to get enough sleep as a society, but that this translates into potentially dangerous situations in the workplace. The American Academy of Sleep Medicine cites that more than 50 million people suffer from chronic sleep disorders, including chronic insomnia, obstructive sleep apnea, parasomnias, restless legs syndrome, narcolepsy, and circadian sleep disorders such as delayed and advanced sleep phase syndrome (AASM Online). People who report having at least one serious medical condition also report a higher incidence of sleeping less than 6hr a night, experiencing poor sleep quality, insomnia, and daytime sleepiness, suggesting a possible link between sleep and general health and well being (NSF, Sleep in America Poll, 2005). Given the number of people suffering from poor sleep, it is not surprising that over 43 million prescriptions for sleep aides were written, and sales of two popular sleep aides Ambien, and Ambien CR totaled 1.5 billion dollars in 2005 (San Francisco Chronicle, March 3rd, 2006).

Sleep, or lack thereof, is a major health concern, for the reasons listed above, and therefore an area of intense research. Obviously, if a drug or therapy could be developed to improve the quality of life for people experiencing problems with sleep, or if sleep efficiency could be improved in an otherwise healthy person, freeing more of that person's time for family

or work, this would be one of the most important scientific breakthroughs of all time. However, despite the importance of sleep, and despite the intense research already dedicated to sleep, we are unclear as to its ultimate function, and how sleep is accomplished at the genetic, molecular, or cellular level. We do know that sleep is a complicated behavior that may ultimately involve the action hundreds of genes, in every cell within the brain, and an extraordinary level of coordination between neuronal circuits.

As a means to begin to understand the complex regulation and function of sleep we focused our experiments on the fruit fly, *Drosophila melanogaster*, a recently described model organism for sleep research. At the start of this thesis research, nothing was known about the anatomical regulation of sleep in *Drosophila*, and very little was known about the genetic regulation of sleep in *Drosophila* or mammals. We thought that the elucidation of sleep function would be greatly aided by identifying sleep-relevant tissues in the fly, providing a focus point for the future search for sleep regulated molecular pathways within these tissues. A majority of the work presented in this thesis attempted to answer the question: what neuroanatomical areas regulate sleep in the fly?

Examining the contribution of single genes to sleep can be a successful strategy to uncover the regulation and function of sleep. We focused our experiments on a sleep regulatory candidate gene *Clock,* a transcription factor involved in generation of circadian timing (King et al., 1997; Allada et al., 1998) with a “short-sleep” phenotype (Naylor et al., 2000; Hendricks et al., 2003a). We hoped that by verifying the role of this gene in sleep regulation, and by placing its action within sleep-regulatory tissues, we could then begin to assay exactly *how* this gene influences sleep amount. The remainder of the work presented in this thesis attempts to answer

the question, does *Clock* promote sleep, and if so, where in the brain is it acting to perform this function?

Sleep Definition, Function, Regulation; Sleep in Drosophila

Sleep is defined as a behavioral state involving: 1) behavioral quiescence, 2) a specific sleeping posture, 3) a specific sleeping site, 4) an increased arousal threshold, 5) rapid reversibility (as opposed to coma), and perhaps most importantly, 6) homeostatic regulation (Tobler, 2000). Many important biological processes in animals are homeostatically regulated, including temperature, thirst, and feeding, which points to the importance of sleep as a basic requirement for survival. Like other homeostatic processes, there is a sleep "set point", whereby an increase in sleep need will eventually reach a level where it will trigger the behavior (sleep) required to return to a rested state. If restoration is prevented by sleep deprivation, sleep need will continue to accumulate. When sleep is eventually allowed to occur, it will result in "sleep rebound", which is reflected by an increase in sleep duration or sleep intensity. Sleep intensity can be measured quantitatively by the time required to initiate asleep (sleep latency), the number of brief awakenings, and slow wave activity, explained below.

In mammals, sleep can also be defined by changes in electrical activity of the brain, or electroencephalography (EEG) (Harvey et al., 1937; Blake et al., 1939). As revealed by EEG, sleep is comprised of two primary states – rapid eye movement (REM) sleep, and non-rapid eye movement (NREM) sleep. REM sleep is primarily characterized by muscle atonia, dreaming, and high frequency theta waves (Aserinsky and Kleitman, 1953; Jouvet et al., 1959). NREM sleep is characterized by muscle relaxation, and low frequency brain waves, including delta

waves, or “slow waves” (<4 Hz), which are a marker for sleep homeostasis, since they are more frequent at sleep onset and dissipate with time spent sleeping (Review: Steriade, 2000).

Two Proposed Functions for Sleep: Restoration and Memory Consolidation

A major unanswered question in science is: what is the function of sleep? Since sleep has not typically been examined using a genetic strategy, the best indications of the function of sleep have been obtained by examining the physical and behavioral consequences of sleep loss. Most theories for why we sleep focus around the idea that sleep serves a restorative function, although what exactly is restored by sleep varies depending on the theory. These theories can be grouped into two main categories: a) tissue restoration, and b) memory consolidation/ neuronal function.

Sleep and Tissue Restoration

The tissue restoration theory suggests that our brain and body restores substances depleted during wake, or eliminates toxins that accumulate during wake. This notion was based on the observation that rats died after 2-3 weeks of chronic total sleep deprivation (Reviewed in; Rechtschaffen and Bergmann, 1995), which is nearly equivalent to depriving the same rat of food. The reason for eventual death was never satisfactorily explained. It was noticed however that rats dramatically increased their food intake, but lost weight and experienced increased heat loss, heartbeat, and energy expenditure. Additionally, the rats became disheveled and sickly looking, developed digestive ulcers, skin lesions, and hair loss, despite maintaining the same amount of grooming activity; and furthermore host defense systems appeared to break down (Review: Rechtschaffen and Bergmann, 1995). Interestingly, *Drosophila* has also been shown to die after chronic sleep deprivation totaling 60-70 hr (Shaw and Franken, 2003). The fact that sleep deprived rats suffer from a loss of thermoregulation suggests a link between sleep and the

regulation of metabolism. Additionally, in mammals, sleep duration is correlated with metabolic rate, since smaller mammals with higher metabolic rates tend to sleep more than larger mammals (Zepelin and Rechtschaffen, 1974).

Sleep and Memory Consolidation

A second theory that has received a significant amount of attention recently is based on the hypothesis that sleep aides in memory formation. This hypothesis is based primarily on the observation that cognitive and behavioral impairments result following a period of sleep deprivation. There is a considerable amount of controversy over which particular stage of sleep (NREM/REM) is most important for memory consolidation, what types of memory are consolidated, or if sleep is necessary for memory consolidation at all (See Discussion in Oct 1, 2005 issue of Sleep between Stickgold (for) and Siegel (against)).

Many of the first experiments investigating a link between sleep and memory focused on a role for REM sleep. The accumulated body of literature from these experiments suggests an important role for REM sleep in memory consolidation, as REM sleep duration increases following intense learning in rats, cats, humans and mice, and REM sleep deprivation prevents proper memory consolidation (For a non-biased review of the literature see Benington and Frank, 2003). These conclusions have not gone unchallenged however (Siegel, 2001; Vertes, 2004). The primary opposing concerns focus around the observations that most researchers have only found procedural memories (memory of skills and procedures) to be affected by REM sleep loss, that the timing of a REM sleep increase following learning is quite variable, and many deprivation procedures are stressful, which may itself increase REM sleep. In addition, people taking some types of antidepressant drugs that suppress REM sleep are still able to form memories (Siegel 2001; Vertes, 2004). Vertes (2004) proposes that rather than memory consolidation, REM sleep

may serve to "prepare the brain for recovery from sleep" and to "maintain minimum requisite levels of CNS activity throughout sleep".

Despite these criticisms, the accumulated evidence in support of a role for sleep in memory consolidation, especially memory enhancement, is difficult to ignore (Reviews: Benington and Frank, 2003; Stickgold, 2005; Walker and Stickgold, 2006). Newer reports have found an association between sleep and enhancement of declarative memory (conscious facts, learned knowledge) (Gais and Born, 2004), addressing the criticism that sleep only served to consolidate procedural memories. NREM sleep, and slow wave sleep (SWS) in particular is emerging as an important component of the link between sleep and memory consolidation. Recent evidence suggests that this occurs at a cellular level. Neuronal spiking patterns observed in the hippocampus and cortex during learning are replayed during slow wave sleep, in a similar sequence, a process which has been proposed to aid in transfer/consolidation of short-term memory from the hippocampus to long-term memory storage in the cortex (Ji and Wilson, 2007).

While it is agreed that memory formation requires synaptic plasticity, resulting in strengthening of synaptic connections (synaptic potentiation), it is currently unclear as to whether memory enhancement during sleep occurs through a process resembling synaptic potentiation, or synaptic depression (Review: Benington and Frank, 2003; Walker and Stickgold, 2004). Most genetic evidence suggests that sleep is primarily a time of synaptic depotentiation, since genes known to increase synaptic plasticity are up-regulated during wake, and down-regulated during sleep (Cirelli et al., 2004). Because it may seem counterintuitive that sleep is both a time of memory consolidation and a decrease in synaptic plasticity, the synaptic homeostasis hypothesis (Tononi and Cirelli, 2003) has been proposed as a way to clarify how memories may actually be strengthened during sleep through synaptic depotentiation. The

synaptic homeostasis hypothesis suggests that low frequency waves observed throughout the brain during SWS function to *globally* depotentiate synapses that are potentiated during wake/learning. Rather than weakening memories however, this may actually serve to reduce the synaptic "signal to noise" ratio, therefore giving the strongest synapses the most weight (Tononi and Cirelli, 2003). In support of this hypothesis, low frequency delta waves (<4Hz) are increased locally in particular brain regions required to learn a task after training during sleep, and the intensity of SWS in this region is correlated with enhanced task performance following sleep (Huber et al., 2004a). This effect can be mimicked by artificially depotentiating synapses with low frequency stimulation during wake using transcranial magnetic stimulation (Huber et al., 2007). Importantly, inducing SW activity using stimulating electrodes during sleep enhances memory consolidation on a word-pair association task (Marshall et al., 2006).

Sleep is Regulated by Homeostatic and Circadian Processes

Before we can begin to answer the question of why we sleep we should first understand how sleep is regulated. For instance, if sleep only occurred following a large meal we might conclude that the function of sleep was to digest large meals. While this does not appear to be the case (although sleep and feeding are related), sleep tends to last for a particular length of time each night, and begin and end at the same time of day, which may in some way be linked to its function.

Two models have been proposed to explain how sleep amount and timing are related, the Two-Process model (Daan et al., 1984), and the Opponent Processes model (Edgar et al., 1993), where the two-processes (sleep amount and sleep timing) were termed Process S and Process C. Process S, or sleep amount, is the homeostatic component of sleep, which increases with time

spent awake, and dissipates with time spent asleep. Sleep need can be thought of as the gradual accumulation/dissipation of some "sleep need" factor, whether that be an actual molecule, a change in the cellular properties of sleep regulatory brain regions, or some other biochemical process. Process C, or sleep timing, is the circadian component of sleep. Circadian (about a day) rhythms are endogenously generated ~24hr rhythms that dictate the appropriate timing of everything from hormone secretion, to appetite, to body temperature, and sleep/wake state, with respect to the external environment. Under constant conditions, these rhythms have a period of approximately 24hrs, however, they can be synchronized (or "entrained") to the 24hr day by information from the environment, primarily light. Process C determines the proper *timing* of sleep, and consolidates sleep into a single long, continuous bout. This aspect is an extremely important feature of sleep, since in mammals and many other species, restorative sleep is only possible if it occurs in a consolidated bout.

The two-process model was based primarily on the observation that homeostatic sleep rebound in response to sleep deprivation (Process S) persists in animals lacking a circadian rhythm of sleep timing (Process C) (Review: Achermann, 2004). It suggests that the circadian rhythm in some way sets the "threshold" for the occurrence of sleep. In this model, sleep does not generally occur until the homeostatic sleep drive has surpassed a pre-set circadian threshold, and waking is initiated when sleep drive has dissipated to the circadian threshold for wake (Figure 1.1A).

The opponent processes model on the other hand suggests that the anatomical location of the circadian pacemaker in mammals, the suprachiasmatic nucleus (SCN) (Moore and Eichler, 1972; Stephan and Zucker, 1972) opposes Process S by releasing an "alerting factor" – this

factor counteracts sleep need, so it is highest when the animal is the sleepiest, to maintain wakefulness. Around bedtime, the alerting factor drops sharply, allowing sleep regulatory regions responsible for the generation of Process S to initiate sleep, and stays low until sleep need has dissipated (Figure 1.1B). This model was proposed after SCN lesions in monkeys resulted in increased amounts of sleep, in addition to abolishing all behavioral and hormonal rhythms (Edgar et al., 1993), and has further been supported by SCN lesions in the mouse (Easton et al., 2004). There is debate about whether the opponent process model is correct, since SCN lesions in rats result in no overall change in sleep (Mistlberger et. al, 1987) or a very slight (~4%) increase in sleep (Mendelson et al., 2003).

On the surface, these models appear quite different, but in reality, both imply that cells within sleep regulatory centers must be able to integrate both sleep need and sleep timing signals to determine the proper amount of sleep and translate this integrated signal into cellular output. In both models, this could occur through the integration of patterns of synaptic activity or hormone release from the SCN in combination with increase of "sleep factor x." One way to incorporate the two models would be to suggest that the SCN-generated "opposition signal" is stuck in "sleep" mode following SCN lesion or at the "upper circadian threshold" as defined by the two-process model. Clearly, more research needs to be done to decide in favor of either model or a new model, and will be aided by elucidating the anatomical and genetic regulation of sleep.

Drosophila is a Model System to Study Sleep

Since we are still unsure as to what the function of sleep is, or even how sleep need and circadian rhythms are integrated to determine sleep amount, it suggests that perhaps a "simpler" model

system may be useful in studying these processes. The elucidation of other complicated behaviors has been aided by research using model organisms, and the model organism of choice for the past ~40 years has been *Drosophila melanogaster*. The impact that *Drosophila* research has made on the field of circadian rhythms has been invaluable, (Review: Allada et al., 2001), and *Drosophila* research has uncovered genes underlying human behaviors such as learning and memory (Review: Waddell and Quinn, 2001), courtship (Review: Manoli et al., 2006), aggression (Review: Robin et al., 2007), alcohol response (Review: Guarnieri and Heberlein, 2003), human disease (Review: Leyssen and Hassan 2007), and olfaction (Review: Jefferis and Hummel, 2006), to name a few.

In 2000, two papers were published which introduced *Drosophila* as a model system to study sleep (Hendricks et al, 2000; Shaw et al., 2000). It had been noted for many years that the fruit fly experienced prolonged periods of immobility resembling mammalian sleep. Hendricks et al. and Shaw et al. were the first to show by a variety of methods that flies were actually sleeping during periods of inactivity. First, flies exhibited a species-specific sleep posture and location, assuming a prone position near food when singly housed, and away from food, an area of social interaction and activity, when housed in groups (Hendricks et al., 2000). Second, both groups demonstrated that arousal thresholds were increased during periods of immobility (Hendricks et al, 2000; Shaw et al., 2000), which lead to the definition of a unit of sleep as any period of immobility lasting >5 minutes (Shaw et al., 2000). Third, and perhaps most important, if flies were deprived of sleep during a normal time of inactivity, they quickly recovered lost sleep during a normally active period (homeostatic sleep rebound) (Hendricks et al, 2000; Shaw et al., 2000). Importantly, flies exhibit shared molecular sleep/wake mechanisms with mammals, since

flies responded similarly as mammals to drugs used to modulate sleep such as antihistamines (increased sleep), and caffeine (decreased sleep) (Hendricks et al, 2000; Shaw et al., 2000).

Since homeostatic sleep regulation is such an important feature defining sleep, Huber et al. (2004b) further examined this behavior in *Drosophila*. They independently confirmed that after 5 minutes of immobility, arousal threshold was increased, and reached a plateau, defining 5 minutes of immobility as a time unit of sleep. Importantly, they showed that arousal threshold was further increased following sleep deprivation. As in mammals, they confirmed that the amount of sleep rebound in flies is correlated with the length of sleep deprivation, and also that sleep rebound has a circadian component, with most rebound confined to the next normal sleep phase. A crude measure of sleep fragmentation (average sleep bout length) had previously been described in flies under normal sleep/wake conditions (Hendricks et al., 2003a). Huber et al. developed a more sophisticated measurement of sleep intensity, which incorporated both the number of brief awakenings and average sleep bout length. They confirmed that sleep intensity (reduced sleep fragmentation) was increased during the normal sleep phase of the fly, compared to the waking phase, and also showed that sleep intensity was increased following sleep deprivation (Huber et al., 2004b).

Together these experiments validated using the fly as a model system to study sleep behavior. The following sections will describe what is currently known about the anatomical and genetic regulation of sleep in flies and mammals. An emphasis will be placed on fly literature, where available.

Anatomical Regulation of Sleep

As mentioned, we believe that the elucidation of sleep function will ultimately be aided by the identification of sleep-regulatory tissues in the fly. The following sections will describe the different types of sleep-regulatory tissues in mammals, and will present information on three candidate sleep-regulatory regions in the fly brain, based on their behavioral functions.

Mammalian Sleep is Actively Promoted and is Regulated by Multiple Neurotransmitter Systems

While a detailed description of mammalian neuroanatomy is beyond the scope of this thesis, the basic principles are likely to be conserved between mammals and *Drosophila* and will be addressed in brief.

The brain is not quiescent during sleep, in fact, many areas that are active during wake are also active during sleep, resulting in overlapping functions for many of the nuclei and neurotransmitter systems during both states. Sleep regulatory neurons in the mammal can be grouped into four main classes based on their primary functions: sleep/wake regulatory, sleep promoting, sleep state stabilizing, and sleep timing.

Sleep/wake regulatory neurons consist of cholinergic, noradrenergic, and serotonergic neurons in the brainstem, cholinergic neurons in the basal forebrain, and histaminergic neurons in the hypothalamus. Together, these brainstem and hypothalamic projections project diffusely throughout the entire cortex. These systems fire in a state-specific manner, firing rapidly during wake, slowly during NREM sleep, and infrequently or not at all during REM sleep, and are thought to generate the proper neurochemical environment for which sleep to occur (Review:

Saper et al., 2001 and references within). I have classified these neurons as “sleep/wake regulatory” since their activity is not specific to sleep.

At least some sleep promoting functions are performed by a small nucleus within the hypothalamus, the ventral lateral preoptic nucleus (VLPO) (Sherin et al., 1996). GABA/galanin (inhibitory) neurons of the VLPO contact, or terminate in the vicinity of, all major sleep/wake regulatory neurons (Sherin et al., 1998; Steininger et al., 2001). The VLPO is the only area of the brain so far shown to be specifically active during sleep, which has been verified both by immediate early gene expression immunohistochemistry (a marker of recent neuronal activation), and electrophysiological recording (Sherin et al., 1996; Szymusiak et al., 1989; Szymusiak et al., 1998). Importantly, sleep/wake regulatory regions can also influence the activity of the VLPO, since they provide reciprocal inhibitory connections onto this area (Chou et al., 2002). The existence of reciprocal connections between sleep/wake regulatory regions and the VLPO suggests the possibility of an anatomical “flip-flop” switch (Saper et al., 2001), since VLPO firing would inhibit sleep/wake regulatory neuron firing, and vice versa. Strong firing in either direction would favor one state or the other, with few transitions. However, the brain must be able to switch between sleep and wake at least twice a day, and this is thought to be accomplished by increasing homeostatic or circadian drive to sleep, tipping the switch towards sleep, where it will remain until sleep drive is reduced (Saper et al., 2001).

A group of neurons not thought to contribute to either homeostatic or circadian sleep drive may provide a “stabilizing” effect on the sleep circuit. Neurons in the lateral hypothalamus that express the neuropeptide, orexin, innervate all regions of the sleep/wake regulatory system as well as the VLPO (Peyron et al., 1998), providing excitatory input to these areas, which presumably activates sleep/wake regulatory regions and inhibits the VLPO (Hagan et al., 1999;

Methippara et al., 2000; Brown et al., 2001). Orexin is proposed to stabilize the flip-flop switch, preventing transitions between sleep and wake. This hypothesis is supported by the observation that animals with defects in orexin receptor function (Lin et al., 1999), the orexin peptide itself (Chemelli et al., 1999), or orexin levels (Nishino et al., 2000) exhibit the sleep disorder narcolepsy, which is characterized by rapid transitions between wake and sleep, particularly REM sleep.

As previously mentioned, sleep timing is accomplished by neurons within the suprachiasmatic nucleus (SCN), a small nucleus in the hypothalamus containing the circadian pacemaker, which generates the endogenous rhythms responsible for regulating many physiological processes (Moore & Eichler 1972, Stephan & Zucker 1972). Every SCN cell contains the inhibitory neurotransmitter GABA (Buijs et al., 1995), however they also contain a variety of non-neurotransmitter molecules, including neuropeptides and cytokines (Swaab et al., 1975; Kramer et al., 2001; Cheng et al., 2002; Kraves and Weitz, 2006). These or other SCN-secreted factors appear to be involved in maintaining rhythms of locomotor activity, since transplanting intact SCN tissue enclosed in a semi-permeable membrane restores locomotor rhythmicity in an SCN ablated animal (Silver et al., 1996). Importantly however, not all rhythms are restored, including neuroendocrine rhythms, suggesting that synaptic outputs are required to regulate these rhythms (Lehman et al., 1987; Meyer-Bernstein et al., 1999). Perhaps surprisingly, the SCN has very few direct synaptic outputs to sleep regulatory regions, and instead, probably works to regulate sleep timing by indirect, multi-synaptic pathways, or humorally (Fuller et al., 2006). The primary synaptic output of the SCN is to the subparaventricular zone (SPZ) (Watts et al., 1987), which then projects to the dorsal medial hypothalamus (DMH). Lesions of both the SPZ (ventral portion) and DMH disrupt the sleep/wake rhythm (Lu et al., 2000; Chou et al.,

2003). The DMH sends dense projections to both the lateral hypothalamus, the location of orexinergic neurons, and the VLPO (Chou et al., 2003), which might provide a mechanism for how the SCN regulates the timing of sleep. Despite the fact that the SCN does not have direct synaptic connections to sleep areas, these areas influence SCN neuron activity. Superimposed onto spontaneous SCN firing activity, which tends to fire the most during the light phase, regardless of the nocturnal/diurnal preference of an animal (Meijer et al., 1997; Meijer et al., 1998) is a separate electrical firing pattern that is correlated with sleep/wake state. SCN neurons fire rapidly during waking, REM sleep, and NREM sleep deprivation, and less during NREM sleep and REM sleep deprivation (Deboer et al., 2003). Taken together this suggests that changes in SCN activity are regulated by sleep/wake state. The fact that deprivation also alters SCN firing rates suggests that the SCN may respond to homeostatic sleep need.

Candidate Sleep Regulatory Regions in Drosophila

Prior to this thesis research, there were no anatomical regions linked to sleep generation in *Drosophila*, leaving open the possibility that sleep was not even regulated by the brain in this species. There was however an indication that *Drosophila* exhibit state-dependent brain activity patterns (Nitz et al., 2002). Local field potentials were recorded in live behaving flies during waking and sleeping, as defined by periods of 5 minutes or more of immobility (Shaw et al., 2000; Huber et al., 2004b). Recorded spike-like potentials (an indication of neuronal activity) were increased during waking, and diminished during sleep, suggesting that electrical activity in the waking brain differed from the sleeping brain. Local field potentials are disrupted when brain activity was reduced genetically, indicating that they were not movement related artifacts. While this study found a correlation between sleep/wake state and brain activity, it did not imply an

active role for the brain in promoting sleep in *Drosophila*, and did not attempt to assign any particular structure to sleep regulation (Nitz et al., 2002).

Until the past several years, the fly was used mostly as a genetic or developmental model, and the brain was largely ignored. Recently, researchers have become interested in dissecting neural circuitry regulating complex behaviors, and have been successful, due in part to the much simpler central nervous system of the fly. The fly brain contains neurons (approximately 200,000) with axons and dendrites, organized into loose nuclei/ganglia with discrete projections, and neuropil regions, consisting of tightly packed neurites. The brain is organized into three segmental neuropil levels; these are the tritocerebrum, the deutocerebrum, and the protocerebrum. While it is unclear exactly what the functions of each neuropil region are, the protocerebrum is probably concerned with higher order processing functions (Flybrain Atlas).

Drosophila neurotransmitters tend to be expressed in a small number of neurons with wide distribution patterns (Monastirioti, 1999). Many of the same neurotransmitters that have been shown to influence sleep in mammals including serotonin, dopamine, histamine, GABA, glutamate, and acetylcholine are also present in *Drosophila*. *Drosophila* however uses one unique neurotransmitter, octopamine and unlike mammals is not thought to use noradrenaline (Review: Monastirioti, 1999; Heisenberg, 2003).

The following sections will focus on three neuroanatomical regions/networks containing several features that suggest that they may perform sleep regulatory functions in *Drosophila*. The first is the network of circadian neurons, whose functions make them ideal candidates to regulate sleep timing, and/or influence sleep amount. The second and third regions are structures whose roles in activity regulation and learning and memory imply that they may also contain sleep-

regulatory functions, especially given the links between memory and sleep, as previously discussed.

The Circadian Neuronal Network in Drosophila may Regulate Sleep Timing

Similar to mammals, *Drosophila* contain neurons dedicated to the regulation of circadian behavior. While a link between these neurons and sleep timing has not been demonstrated, the details of how the circadian system generates rhythmic behavior is fairly well understood. The circadian system in *Drosophila* is a collection of eight cell groups, totaling about 150 neurons in number (Figure 1.2). Circadian neurons in the fly are defined by whether or not they express the PER protein (Ewer et al., 1992), and are named by their positions in the brain. There are three groups of lateral neurons, which line the border between the optic lobes and central brain, the ventral lateral (small and large – sLNv, lLNv), and dorsal lateral (LNd). There are also three groups of dorsal neurons, within the dorsal protocerebrum of the brain. These are named dorsal neuron groups 1, 2, and 3 (DN1 – divided into anterior and posterior subsets, DN2, DN3). A seventh group, the lateral posterior neurons (LPN), also express PER, and may be involved in some aspects of rhythmicity, including entrainment to temperature (Yoshii et al., 2005; Shafer et al., 2006). An eighth group consists of a single neuron that is considered an LNv, but does not express PDF (explained below).

Circadian neurons can be grouped into distinct functional units (For reviews see Chang et al., 2006; Taghert et al., 2006). The LNvs express the neuropeptide pigment dispersing factor (PDF), which is the only known circadian output molecule at this time (Renn et al., 1999b), although there is some evidence that the neuropeptide IPNamide might be a circadian output molecule of the anterior DN1 cells (Shafer et al., 2006). The sLNvs are the true “pacemaker”

cells, driving rhythmicity under constant darkness conditions (Stoleru et al., 2005). Under LD entrained conditions, circadian behavior in flies can be divided into two distinct phases, known as “morning” and “evening” behavior. The daily rhythm in behavior under LD conditions is characterized by anticipation to the Dark>Light transition in the morning, followed by a startle response to lights on, a “mid-day siesta”, most pronounced in male flies, anticipation to the Light>Dark transition, followed by a startle response to lights off. Flies are a diurnal species, and so, most of their daily activity occurs during the light phase, and most of their sleep occurs during the dark phase (reversed at higher temperatures). The sLNv are thought to drive morning behaviors, and the LNd, some DN1 cells, and the PDF(-) LNv to drive evening behaviors (Grima et al., 2004; Stoleru et al., 2004). The single PDF(-) LNv along with the DN1 may also be responsible for driving rhythmicity under constant light conditions, suggesting that these cells comprise a second, separate oscillator (Murad et al., 2007).

Currently, it is not entirely clear how the clock regulates timing of sleep/wake behavior. Since circadian cells themselves do not appear to employ neurotransmitters for cellular communication, at least not serotonin, dopamine, or histamine (Hamasaka et al., 2006), one hypothesis is that circadian cells release neuropeptides that work to consolidate the sleep/wake cycle via action on down stream target tissues. One possible candidate is PDF, since mutations in PDF (Renn et al., 1999b), or misexpression of PDF, targeted to PDF(-) cells, disrupt locomotor activity (Helfrich-Forster et al., 2000). PDF receptors are found in a region of the brain containing a number of neurosecretory cells, the pars intercerebralis (Lear et al., 2005), and thus, might affect locomotor activity by modulating activity/ promoting release of hormones in this region. A second neuropeptide, neuropeptide F, has recently been found to be expressed in circadian cells, specifically, the LNd, where it is proposed to play a role in regulating the

sexually dimorphic locomotor activity observed between males and females (Lee et al., 2006). It will be interesting to examine whether this peptide also regulates the sexually dimorphic aspects of sleep in flies (Huber et al., 2004b).

sLNvs, LNds, and DNs send projections to the dorsal brain (Review: Hall, 2003, 2005), however, direct motor output regions are not concentrated in this area, raising the question, how do circadian neurons generate rhythmic locomotor outputs? It is possible that the circadian system in flies regulates rhythmic sleep/wake activity through multisynaptic pathways, as may be the case in mice (reviewed previously). Potential targets of the circadian system include the mushroom bodies and central complex, regions of higher order processing, reviewed next.

The Mushroom Bodies are a Sleep Regulatory Candidate Region

The mushroom bodies (MB) are one of the most prominent neuropil formations in the fly brain (Figure 1.3A). The MB structure is formed by the axonal projections from a cluster of ~2500 cell bodies/hemisphere, called "Kenyon cells (KC)", which are located in the dorsal protocerebrum. Dendrites from KC neurons form the "calyx", and KC axons travel in a large fiber bundle called the peduncle, which branches into 5 anatomically distinct lobes, termed the α, α', β, β', and γ lobes. β, β' and γ lobes project medially, and the α and α' lobes project vertically. All MB neurons arise from 4 neuroblasts/hemisphere, which develop into four clonal units. Each single clone gives rise to neurons comprising all 5 lobes, in a developmentally programmed sequence. *Drosophila* has 5 main developmental stages: embryo, 1st instar, 2nd instar, and 3rd instar (larval stages), pupa, and adult. γ lobe neurons are born during the 1st larval instar, followed by the birth of α'/β' neurons in the late 3rd instar, and lastly, the α/β neurons, which are born only after pupation (Lee et al., 1999b). Dendrites from neurons forming the five MB lobes segregate within

the calyx in a complicated pattern determined by clonal origination and birth order (Lin et al., 2007). While the functional significance of this is currently unknown, it suggests that inputs onto MB KC dendrites can be anatomically segregated with the result of influencing the activity of neurons in specific MB lobes. The MB were considered to be a homogeneous structure, but following these recent anatomical studies this can no longer be considered probable.

The most obvious input to the MB is from antennal lobe projection neurons, where they make cholinergic synapses onto the KC's and calyx (Oleskevich, 1999). MB neurons also receive inputs from two large paired cells called the dorsal paired medial (DPM) cells, which arborize extensively over the MB lobes (Waddell et al., 2000). Inputs to the MB, and MB extrinsic (output) neurons are difficult to characterize, partly due to the fact that it can be difficult to distinguish pre and post-synaptic specializations in *Drosophila* neurons (Review: Strausfeld and Meinertzhagen, 1998). However, one detailed anatomical analysis concluded that MB efferent neurons serve to both connect the MB lobes to each other, and to other neuropil regions within the protocerebrum (Ito et al., 1998). This study also concluded that there are differences between the output patterns of the vertical (α/α') and medial (β/β', γ) lobes. Medial lobes tend to contact extrinsic MB neurons throughout the entire lobe, whereas the vertical lobes contact extrinsic neurons primarily at the head, and not throughout the shaft of the lobe. Also, the vertical and medial lobes contact extrinsic neurons with projection patterns to different areas of the protocerebrum – the medial lobes interact with cells projecting to the inferior medial protocerebrum (impr), and the vertical lobes interact with cells projecting to the superior medial and superior lateral protocerebrum (smpr, slpr). Interestingly however, all MB extrinsic neurons project to anterior regions of these three neuropil regions (imp, smp, slp), suggesting a "MB-linked" neuropil region in the fly brain (Ito et al., 1998).

Despite the fact that MB inputs and output pathways are not clearly defined, their neurotransmitter receptor profiles indicate that their activity may be modulated by a variety of different neurotransmitters. MB neurons receive dense innervation from dopaminergic projections, and express receptors for many types of neurotransmitters, including octopamine, acetylcholine, GABA, glutamate, and dopamine (Review: Monastirioti, 1999; Cayre et al, 1999; Su and O'Dowd, 2003). Of note, investigators have had difficulty determining the neurotransmitter identities of intrinsic mushroom body neurons themselves, and so, this remains unknown (Cayre et al, 1999).

One function of the mushroom bodies that makes them a candidate sleep-regulatory region is their role in activity regulation. Specifically, the mushroom bodies are proposed to suppress activity. Activity was assayed by placing flies in small tubes, and measuring an activity "count" each time the fly crossed the center of the tube. Under these conditions, flies walk back and forth from one end of the tube to the other, rarely stopping in the middle or turning around (Martin et al., 1998). Total activity was increased in flies with mushroom body lesions and in mushroom body developmental mutants, and in flies in which the mushroom bodies were selectively inactivated (Martin et al., 1998). Specifically, mushroom body defects appear to inhibit the termination of walking bouts (Martin et al., 1998; Helfrich-Forster et al., 2002). The authors noted in their discussion that since MB inactivation did not influence the speed of walking, or the number of walking bouts, just the length of the walking bout itself, that the MB might "exert their influence by regulating arousal" (Martin et al., 1998). Interpreted slightly differently, this could mean that the MB normally promote sleep, and increased walking is an indirect effect of being awake for a longer period of time.

Another function of the MB that makes them a candidate sleep region is their role in regulating learning and memory, since memory consolidation, and/or synaptic remodeling is considered to be one of the primary functions of sleep (Benington and Frank, 2003). The mushroom bodies have long been associated with a role in learning and memory, including olfactory learning, courtship conditioning, context generalization in visual learning, and spatial learning (Reviews: Zars, 2000; Siwicki and Ledewsky, 2003; Davis, 2005; Keene and Waddell, 2007). Many of the genes required for long-term memory formation (Review: Waddell and Quinn, 2001) are expressed at high levels in the MB (Table 1, Keene and Waddell, 2007). Flies with developmental defects, flies in which the MB have been chemically ablated, and flies in which normal MB cellular function has been disrupted fail to learn olfactory discrimination tasks (Heisenberg et al., 1985; deBelle and Heisenberg, 1994; Connolly et al., 1996). The MB were first shown to be sufficient for memory formation when memory function in the mutant, *rutabaga*, was rescued by expressing a wild type *rutabaga* gene only within the MB, which the authors attributed primarily to rescue within γ lobe neurons (Zars et al., 2000).

MB activity within particular lobes, and DPM neuronal activity, appears to be differentially required for memory acquisition, consolidation (a process presumably involving synaptic potentiation and remodeling) and storage of short term or long-term memories (Quinn and Dudai, 1976; Folkers et al., 1993; Tully et al., 1994). Output from MB α'/β' lobes is required during memory acquisition and consolidation (Krashes et al., 2007). DPM neuron output also appears to also be required to consolidate memories, possibly by modulating α'/β' lobe activity (Keene et al., 2004, 2006; Yu et al., 2005; Krashes et al., 2007), since when DPM output is blocked, the ability to form long-term memories is blocked (Waddell et al., 2000). Stable memory traces appear to be stored within MB α/β lobe neurons, since α/β lobe output is

required to retrieve memories, but is not required during memory acquisition or consolidation (Dubnau et al., 2001; McGuire et al., 2001; Schwaerzel et al., 2002). α lobes specifically might store LT memories (Pascual et al., 2001; Yu et al., 2006). Despite original hypotheses (Zars et al., 2000), γ lobe neurons do not appear to be required for olfactory memory formation or retrieval (Dubnau et al., 2001; McGuire et al., 2001; Schwaerzel et al., 2002; Isabel et al., 2004), however, they may be required for courtship conditioning (Manoli et al., 2005).

Together, the evidence that the MB contain receptors for a variety of neurotransmitters found to regulate sleep/wake state in mammals, regulate activity levels, and are required for memory formation and storage, suggests that the MB may be a sleep regulatory region in the *Drosophila* brain.

The Central Complex is a Sleep Regulatory Candidate Region

The central complex (CC) is unique in the *Drosophila* brain in that it is the only neuropil structure that spans the midline, suggesting by structure and position alone that it might be involved in hemispheric coordination (Review: Strauss, 2002). It is comprised of four major structures, the ellipsoid body, noduli, fan shaped body, and protocerebral bridge (Figure 1.3B). Two primary neuronal classes are found in the CC, large field and small field neurons. Large field neurons arbourize in one or more layers of a specific CC structure, and provide connections to more distant, non-CC structures. Small field neurons mostly form small subunits within CC structures, and a minority may provide connections between CC structures, and/or to non-CC structures (Renn et al., 1999a). The four CC structures probably do not contain functionally homogeneous neuronal subtypes. For instance, the ellipsoid body (EB) contains classes of

neurons called ring, or "R" neurons, whose dendrites segregate into distinct regions based on R class (Renn et al., 1999a), similar to what is seen in the MB calyx (Lin et al., 2007).

The central complex receives inputs through large-field neurons from most areas of the brain, and communicates within the structures via small field neurons. There are no obvious prominent tracts from sensory or motor areas, which might help to determine its function, although there is evidence that the CC receives direct inputs from the optic lobes and antennal lobes (Hanesch et al., 1989) and from widely branching neurons in the protocerebrum (Hanesch et al., 1989). The main output of the CC appears to be an accessory structure called the "ventral bodies" (Hanesch et al., 1989), a structure with as yet unknown function.

Similar to the MB, CC activity appears to be in a position to be modulated by a number of different neurotransmitter systems. The CC is innervated by dopamine and octopamine, and the ellipsoid body in particular is densely innervated by serotonin, and expresses octopamine receptors (Review: Monastirioti, 1999). Unlike the MB, antibody staining has been successful in identifying the neurotransmitter identities of CC neurons. R-type ellipsoid body neurons are GABAergic, and neurons of the fan-shaped body and noduli are cholinergic (Hanesch et al., 1989), suggesting that these groups of neurons perform different functions.

As with the MB, one function of the CC is to regulate activity. Flies with disrupted CC function show the opposite activity phenotype compared to flies with disrupted MB activity. Flies with central complex developmental mutations, and flies in which CC neurons are selectively inactivated show reduced amounts of activity (Strauss et al., 1992; Strauss and Heisenberg, 1993; Martin et al., 1999). While flies with disrupted MB exhibited longer walking bouts (Martin et al., 1998), flies with disrupted CC walked normal speeds, and had the same number of walking bouts as controls, but walked in shorter bouts. Two substructures of the

central complex were identified as being particularly important in activity regulation; a set of neurons linking the protocerebral bridge, ellipsoid body, and noduli, and a group of large-field neurons projecting to the fan shaped body. These neurons are particularly interesting because they arborize near the α lobe of the mushroom bodies, and may provide an anatomical link between the MB and CC (Martin et al., 1999).

The central complex has also recently been assigned a function in learning and memory. Since the central complex receives visual inputs, Liu et al. (2006) examined the role of the CC in visual memory. They found that developmental defects in the CC resulted in flies with impaired visual pattern memory. Also, *rutabaga* (olfactory learning and memory mutant) flies failed to form visual pattern memories. They rescued visual pattern memory in *rutabaga* mutants by expressing the wild type *rutabaga* gene in a very specific set of large-field neurons in the upper portion of the fan shaped body (which they termed F5 neurons). These are the same neurons that were previously mentioned which arborize near the MB α lobe. They went on to show that a separate set of neurons in the fan shaped body (F1) are required for the formation of another component of visual pattern memory (Liu et al., 2006).

The central complex can be considered a sleep-regulatory candidate region for the same reasons that the mushroom bodies were considered. Like the MB, the CC contain receptors for a variety of neurotransmitters found to regulate sleep/wake state in mammals. Since the ellipsoid is GABAergic, it may function to inhibit wake-regulatory tissues, similar to the VLPO in mammals, or even to inhibit sleep-promoting tissues. The CC regulate activity levels, but rather than normally suppressing activity, like the MB, they are thought to do the opposite, and enhance activity. And, like the MB, the CC are required for memory formation and storage, although the

MB is involved primarily in olfactory memory, whereas the CC is involved in visual memory storage.

The Genetic Regulation of Sleep

Identifying sleep regulatory tissues will only be relevant if we can then look within these tissues for sleep regulated genes and molecular pathways. Conversely, genes that are thought to regulate sleep may actually be discovered to do so indirectly, if they are not expressed within sleep-relevant tissues. Therefore, it will be necessary to identify both sleep regulatory regions, and sleep regulatory genes, in order to ultimately understand the function of sleep. The following sections will present what we currently understand about the genetic regulation of sleep in mammals and flies, and will focus on the candidate sleep regulatory gene, *Clock*, in particular.

What is Sleep Need? Examining Sleep Regulated Genes

A sleep-regulatory gene can be considered either a gene whose function is to determine the proper timing of sleep (Process C), or a gene whose expression is linked to the accumulation of sleep need (Process S), or both. Relatively few genes have been definitively linked to sleep regulation, which is almost certainly due to the difficulty in defining what a “sleep gene” is. The first level of complexity in defining a sleep gene is to decide whether a sleep gene should be expressed 1) only during sleep, or, 2) in a homeostatic manner – increasing in expression as sleepiness increases, and decreasing after sleep is initiated (Figure 1.4). I would argue that both are probably sleep genes, even though in the case of gene #2, it will be expressed at equal levels halfway through the waking and sleep phases. Gene #1 might represent a gene whose expression

serves to accomplish the function of sleep, whatever that may be, and gene #2 might represent a gene whose function is to track the homeostatic component of sleep, or, sleep need. The expression levels of gene #1 would not be expected to increase during a period of sleep deprivation, whereas gene #2 would. In addition to satisfying either of these expression pattern profiles, disruption of a sleep gene should affect the initiation, maintenance, duration, or homeostatic regulation of sleep, and it should be expressed in tissues that require sleep.

The general strategy to examine the genetic regulation of sleep is two pronged. The first strategy is to compare the contribution of many genes in the brain to sleep regulation, and the second is to examine the contribution of single genes to sleep regulation.

Genome Wide Changes in Gene Transcription Reveal Potential Functions of Sleep

mRNA microarray has become the standard technique to examine genome-wide mRNA expression, and four papers (Cirelli et al., 2004; Cirelli et al., 2005b; Terao et al., 2006; Zimmerman et al., 2006) have recently used this technique to assay sleep/wake state dependent gene expression in mammals and flies. A number of microarray experiments have been published with the goal of finding circadianly regulated genes (Claridge-Chang et al., 2001; McDonald and Rosbash, 2001; Ceriani et al., 2002; Lin et al., 2002; Ueda et al., 2002), and while a few robustly cycling genes showed up in all papers, notably a new central pacemaker gene termed *clockwork orange* (Matsumoto et al., 2007; Kadener et al., 2007; Lim et al., 2007), there was otherwise very little overlap (Review: Wijnen et al., 2007; Keegan et al., submitted). The degree of individual gene overlap in sleep microarray experiments has not been rigorously examined, however, all four studies identified similar classes of sleep/wake regulated genes, as will be explained below.

Cirelli et al. examined gene expression in asleep (S), awake (W), and sleep deprived (SD) rat cortical and cerebellar brain tissue (Cirelli et al., 2004), and fly heads (Cirelli et al., 2005b). Terao et al. (2006) examined gene expression in S, SD and sleep rebound (R) rats in cortical, basal forebrain, and hypothalamic tissue. Despite the experimental differences, both groups reported a number of genes in common which were up-regulated during SD or W. Both groups found that during W/SD, genes involved in gene transcription, stress response, and excitatory neurotransmission were up-regulated in the rat. Cirelli et al. (2004) also found that genes involved in oxidative phosphorylation, energy metabolism, and plasticity and long-term potentiation were up-regulated during W, and that during S, genes involved in translation, membrane trafficking, hyperpolarization, and synaptic depotentiation were up-regulated. These results were mostly replicated by similar microarray experiments repeated in the fly, with the notable exception that genes involved in synaptic plasticity were not up-regulated during W (Cirelli et al., 2005b). While it can be predicted that genes involved in excitatory neurotransmission would be up-regulated during W, and those involved in inhibitory neurotransmission up-regulated during S, it is not entirely expected that genes involved in the stress response would be up-regulated during W. What this suggests is that waking might be a type of cellular stress, and sleep is required to alleviate that daily stress. This result in part supports the “restorative” theory of sleep function. Additionally, the results of Cirelli et al. (2004) support a role for sleep in memory consolidation/ synaptic plasticity/ depotentiation on a molecular level. Cirelli et al. (2004) found that some of the S/W regulated genes in the cortex were also regulated similarly by S/W state in the cerebellum. Since the cerebellum is not typically considered to be a “sleep area”, this suggests that S/W regulated genes are not unique to one brain tissue, but probably occur in many neurons. Conversely, Terao et al. (2006) found S/W

regulatory genes within the basal forebrain and hypothalamus which varied from those in the cortex, suggesting that specific regions might respond uniquely (on a molecular level) to S/W state.

In opposition to the previous three studies, Zimmerman et al. (2006) found that most genes were down-regulated with SD, including genes involved in protein synthesis, neuronal excitability, calcium homeostasis, and *de novo* protein folding (chaperone proteins). These results in part seem to be contradictory to the rat/fly results of Cirelli et al. (2004, 2005b), who showed that genes involved in neuronal excitability were increased during W/SD. While these differences may be explained by differences in experimental procedures, Zimmerman et al. provide a novel interpretation of their data, and a re-assessment of the Process S model. They argue that instead of searching for molecules that increase in expression with time awake, perhaps we should look for molecules, or classes of molecules whose expression decreases with time awake. Their data suggests that there are mechanisms in place that act to limit wakefulness by actively turning off wake related genes, rather than actively promoting a different class of sleep related genes. This is a rather unique theory, and only time and further research will prove whether it is a valid possibility.

Examination of Sleep in Single Gene Mutants Reveal Potential Functions of Sleep

While it appears true that the expression of many genes are state-dependent, it is still unclear what the molecular events are that initiate transcription of these genes, or what the individual contribution of each gene is to sleep regulation. Researchers have used two general approaches to determine the role of single genes in sleep regulation; the first is to examine sleep phenotypes in animals in which candidate sleep-regulatory genes have been knocked out or disrupted in

some way, and the second is to randomly mutagenize the genome, and screen for single-gene mutants with disrupted sleep. Using these approaches, a number of molecular pathways have since been implicated in sleep, three will be covered here, including those for genes involved in learning and memory, neurotransmission, such as dopamine, serotonin and potassium, and circadian rhythm generation.

Learning/Memory and Sleep May Share Similar Molecular Mechanisms

The results of two papers examining the link between sleep and learning/ memory/ plasticity in flies support the hypothesis that these behaviors might be regulated by many of the same molecular pathways.

Activity of the transcription factor CREB (cAMP response element binding protein), as well as other components of the cAMP (cyclic adenosine monophosphate) signaling pathway, are required for long-term memory formation (Review: Waddell and Quinn, 2001). Additionally, CREB activity is circadianly regulated in mammals (Obrietan et al., 1999), and flies (Belvin et al., 1999) is expressed highly during wake (Cirelli et al., 1996; Zamboni et al., 1999), and is expressed in sleep-regulatory tissues in mammals (Capece et al., 1997; Zamboni et al., 1999). Hendricks et al. (2001) showed that CREB activity was inversely related to sleep, whereby low levels of CREB activity resulted in high levels of sleep, and high levels of CREB resulted in reductions in sleep. Additionally, CREB activity levels were increased following sleep deprivation, and sleep rebound was prolonged in flies where CREB activity was blocked. These results suggest that CREB activity may serve to maintain wakefulness, and it may serve a separate function, whereby its activity is required to accomplish the restorative functions of

sleep. This is consistent with two observed daily peaks of CREB activity in flies (Belvin et al., 1999).

Ganguly-Fitzgerald et al. (2006) examined the link between sleep and plasticity at both a behavioral and genetic level, by examining sleep following environmental enrichment/ impoverishment. It had previously been shown in mammals and flies that exposure to an enriched environment increased synaptic plasticity (Heisenberg et al., 1995; van Praag et al., 2000). In this experiment (Ganguly-Fitzgerald et al., 2006), sleep was examined in flies that were raised in either a socially enriched or socially impoverished, isolated environment. Surprisingly, flies that were raised in isolated environment slept less than those raised in a social environment, indicating that this effect may be due to reduced overall levels of synaptic plasticity. The effect of social enrichment on sleep was reversible, and the increase in sleep due to social enrichment was blocked in flies in which sensory perception (specifically vision and olfaction) was blocked, indicating that sight and smell are important mediators of social enrichment. Importantly for this discussion, mutations in genes affecting short-term and long-term memory, including many involved in the cAMP pathway, affected the ability to alter sleep based on social condition, especially when moving from an enriched to an impoverished environment. Also relevant to this discussion, Ganguly-Fitzgerald et al. found that sleep was increased following an intense learning procedure, and sleep deprivation abolished memory, as well as the learning-induced increase in sleep.

Genes Involved in Neurotransmission Regulate Sleep Amount

Sleep is regulated by multiple neurotransmitter systems in mammals. Genetic studies provide the means to examine the contribution of individual transmitters and their receptors on the overall regulation of sleep, including dopamine, serotonin, and potassium.

Many drugs used to maintain wakefulness are thought to work via dopaminergic mechanisms (Nishino et al., 1998). Andretic et al. (2005) showed that sleep could be modulated in *Drosophila* by administering the dopaminergic drug, methamphetamine (METH). METH administration dramatically reduced sleep amount, and reduced other sleep parameters such as sleep bout length, sleep bout number, and sleep latency. Flies fed a drug that inhibited DA synthesis showed the opposite phenotype, increased sleep, specifically during the light phase, when flies are normally most awake. METH also counteracted the effects of sleep deprivation, since flies fed METH following SD exhibited an attenuated amount of rebound sleep. A separate group (Kume et al., 2005) discovered a spontaneous mutant fly strain in their laboratory with high levels of locomotor activity, and mapped the mutation to the DA transporter gene, *dDAT.* A mutation in this gene would be expected to increase DA levels throughout the fly. They found that in addition to high levels of locomotor activity, *dDAT* mutants exhibited reduced levels of sleep, decreased arousal thresholds, and attenuated sleep rebound following deprivation. DAT knockout mice show similar phenotypes to *dDAT* mutant flies, including a reduction in sleep time, specifically NREM sleep, and increased wake bout durations (Wisor et al., 2001). Together, these results suggest that DA transmission is important for regulating sleep amount in flies and mice.

In mammals, serotonin (5-HT, 5-hydroxytryptophan) plays a complicated role in sleep regulation, but one suggestion is that 5-HT works by promoting, or more accurately, “dis-

inhibiting" sleep (Jouvet, 1999). Yuan et al. (2006) examined the role of 5-HT transmission in sleep regulation in flies. They found that 5-HT1A mutants had decreased sleep, decreased sleep consolidation, and decreased ability to rebound after sleep deprivation. They also demonstrated that increasing 5-HT levels pharmacologically resulted in increased sleep. Together, these results support a role for 5-HT in promoting sleep in the fly, as in mammals. They were able to localize these mutant effects to the mushroom bodies, a *Drosophila* sleep candidate area that was covered in detail previously (Yuan et al., 2006). While they could not completely rule out a role for two additional serotonin receptors (5-HT1B and 5HT2) in sleep regulation, they did not find a strong sleep regulatory role for either of these two receptors (Yuan et al., 2006). However, previous studies in the mouse had suggested that the 5-HT1B receptor was important for normal promotion of REM sleep, since REM sleep, and REM sleep rebound was reduced in 5-HT1B knockout mice (Boutrel et al., 1999).

Only one forward genetics screen performed with the goal of discovering sleep regulatory genes has been published in flies to date (and none in mice). Cirelli et al. (2005a) fed flies the mutagenizing agent ethylmethane sulfanate (EMS) and screened 6000 fly lines for mutations affecting sleep. In addition, they screened 3000 lines in which gene function was disrupted by insertion of a foreign DNA element, called a P element. Of these ~9000 mutant lines, 15 were found to exhibit reduced sleep amounts. One of the most severe mutants was termed *minisleep*, and was mapped to the α-subunit of a voltage dependent potassium channel that controls membrane repolarization after action potentials, *shaker (sh)* (Schwartz et al., 1988). *Sh* flies exhibited many of the features defining a short-sleeping fly, such as reduced daily sleep, reduced sleep consolidation, decreased arousal threshold, and reduced sleep intensity during rebound (but an equivalent amount of rebound compared to controls) (Cirelli et al., 2005a). It was recently

published that mutations in the β-subunit of the *shaker* channel, called *hyperkinetic (hk),* also result in a short-sleeping phenotype. Of note, it was also shown that both *sh* and *hk* mutants had learning and memory deficits (Bushey et al., 2007). Potassium conductance has also been shown to influence sleep amount in mammals, since potassium channel subunit Kv3.1/Kv3.3 double knockout mice are short-sleepers (Espinosa et al., 2004).

Genes Involved in Circadian Rhythm Generation Regulate Sleep Beyond a Role in Sleep Timing: A Focus on the Central Circadian gene, Clock

Given the connection between circadian rhythms (Process C) and sleep need (Process S), as previously discussed, it was a logical step to examine sleep in circadian mutants in both flies and mice, as a means to assay whether sleep and rhythms are interconnected at a genetic level. Perhaps surprisingly, many circadian genes were found to disrupt sleep amount, including the central circadian gene, *Clock* (Naylor et al., 2000; Hendricks et al., 2003a).

Before attempting to understand the role of *Clock* or other circadian genes in sleep, it is first necessary to explain the role that these genes play in generation of the circadian rhythm. The first circadian gene to be discovered (in fact, the first gene to ever be linked to behavior) was *period*, by Konopka and Benzer (1971). *Period* (*per*) was found using a forward genetics approach whereby the genome of *Drosophila melanogaster* was mutagenized, and flies containing mutations were screened for disruptions in circadian rhythms of "eclosion", the process of an adult fly emerging from its pupal case, which normally occurs in the early morning. Three separate strains with different mutations in *per* either eclosed at random times (per^{01}), or with a much shorter (per^{S}) or longer (per^{L}) period than normal flies. Years later, *per* was cloned (Jackson et al., 1986; Reddy et al., 1986), and found to resemble a basic helix-loop-

helix (bHLH) transcription factor, however, it lacked the HLH and DNA binding domains typical of a transcription factor. *per* mRNA and protein levels were shown to cycle with a roughly 24hr period (Siwicki et al., 1988, Hardin et al., 1990, Zerr et al., 1990), and PER protein was found to inhibit its own mRNA expression (Hardin et al., 1990). This provided a possible mechanism for generation of the molecular rhythm, although most likely through an intermediate transcription factor, with the ability to bind DNA. The most significant finding following the discovery of *per* was the identification of *timeless* (tim^{01}), another mutation which resulted in behavioral arrhythmicity, also found using a forward genetics approach in *Drosophila* (Sehgal et. al, 1994). Importantly, *per* and *tim* were found to interact molecularly (Zeng et al., 1996), and PER and TIM proteins to form heterodimers (Rothenfluh et al., 2000), however, *tim* also did not contain a DNA binding domain. This apparent contradiction was solved with discovery of the gene, *Clock*, a bHLH transcription factor containing a DNA binding domain, which was discovered as an arrhythmic mutant in a forward genetics screen, simultaneously in both flies (Allada et al., 1998), and mice (King et al., 1997), importantly, this was the first circadian gene to be cloned in mammals.

Clock is considered to be the genetic center of the circadian pacemaker. In addition to behavioral arrhythmicity, *Clock* mutations result in complete abolishment of *per* and *tim* transcription and cycling (Allada et al., 1998). *Clock* RNA and protein levels cycle with a ~24hr period (Bae et al., 1998; Darlington et al., 1998; Lee et al., 1998), and *Clock* activates transcription of *per* and *tim* by binding to E-Box sequences, a common binding site of bHLH transcription factors (Hao et al., 1997; Darlington et al., 1998). *Clock* forms a heretodimer with its binding partner, *cycle* (*cyc*), another bHLH transcription factor, as shown by immunoprecipitation and yeast two-hybrid interactions (Darlington et al 1998; Lee et al 1998;

Rutila et al., 1998; Bae et al., 1998, 2000). *Cycle* mutations also result in behavioral arrhythmicity, and reduced levels of *per* and *tim* mRNA and protein (Rutila et al., 1998), however, its own RNA and protein levels do not cycle (Darlington et al 1998; Lee et al., 1998; Bae et al., 1998, 2000). Taken together, this has lead to a model of circadian clock function as follows; CLK/CYC bind to and activate the transcription of *per*/*tim* mRNA*,* which leave the nucleus and are translated and phosphorylated, dimerize and re-enter the nucleus, where they then bind to and inhibit the action of CLK/CYC, thereby inhibiting their own transcription. This entire process takes approximately 24hrs to complete, and thus, forms the basis of the molecular circadian rhythm. Pacemaker cells relay this information to the rest of the brain via synaptic/hormonal outputs. A similar process exists in mammals, except that *cryptochrome* takes the functional position of *timeless*, and the mammalian ortholog of *cycle* is called *BMAL1* (Figure 1.5; Review: Allada, et al., 2001). In the past 10 years, many more circadian genes have been discovered in both flies and mice, and circadian rhythms have become one of the most comprehensively understood behaviors.

Sleep was first examined in *Clock* mutant mice (Naylor et al., 2000). Unexpectedly, sleep amount was significantly reduced in *Clock* mutants. In LD, *Clock* mutants exhibited a 1hr reduction in total sleep time as heterozygotes, and a 2 hr reduction in sleep time as homozygotes. On closer examination, this reduction was almost entirely due to a reduction in NREM sleep, during both the light and dark phases. In heterozygotes, this was due to NREM reduction during the dark phase, the normal wake period of the mouse. In constant conditions (DD), *Clock* homozygotes also showed a reduction in NREM sleep, however, the magnitude of this effect was diminished when compared to sleep under LD conditions (a reduction of 18% in LD compared to ~5% in DD compared to controls, as interpreted from Figure 2, Naylor et al., 2000). A reason for

this reduction in phenotype magnitude was not given, but suggests that light may in some way influence expression of the *Clock* phenotype.

Clock mutants also showed defects in sleep architecture and homeostasis (Naylor et al., 2000). During LD and DD, the decrease in NREM sleep in *Clock* mutants was due primarily to a reduction in NREM sleep bout length. Sleep bout number was not increased, and the number of brief arousals was not increased, which the authors interpret to mean that sleep quality was not reduced in *Clock* mutants, simply sleep amount. If it is true that sleep quality is preserved in *Clock* mutants, it could be predicted that they would compensate for sleeping less by increasing sleep intensity, as measured by SWS delta power, however this was not the case. This data suggests a disruption in the homeostatic accumulation of sleep need. To further examine the possibility that *Clock* mutants had a disrupted homeostatic sleep response, mice were deprived of sleep for 6 hrs, and recovery sleep was recorded for 12 hr. The amount of NREM sleep recovered following sleep deprivation was equivalent in controls and *Clock* mutants, however, since *Clock* mutants sleep less overall, and therefore lose less sleep during 6 hr of deprivation, the observed result might actually reflect a "hyper-rebound" in *Clock* mutants (my interpretation). Additionally, while there were no differences in REM sleep amount between *Clock* mutants and controls under baseline conditions, *Clock* mutants were less able to recover REM sleep after deprivation (Naylor et al., 2000).

Similar to the mouse, fly *Clock* mutants (Clk^{Jrk}) were shown to exhibit decreased sleep amounts, reduced sleep bout length, and disrupted sleep homeostasis. Hendricks et al. (2003a) examined sleep in Clk^{Jrk} mutants in both LD and DD and reported a significant decrease in sleep compared to controls, when sleep was measured in 30 minute intervals. In addition, they noted that both the number and duration of sleep bout lengths were reduced in Clk^{Jrk} mutants when

examined in 5 min bin detail in DD. To verify that the mutant sleep phenotypes were due to the *Clk*^{Jrk} mutation, and not due to the interaction between multiple genes in the fly (genetic background), they generated a fly in which the *Clk*^{Jrk} mutation could be induced throughout the fly by heat shocking in adulthood. While the number of flies is very low in this experiment, calling into question the validity of the result, they showed that hs-*Clk*^{Jrk} flies slept less following heat shock than control flies in DD. Finally, they examined sleep following sleep deprivation in DD, and indicate that they could elicit rebound in *Clk*^{Jrk} flies, but do not present the data (Hendricks et. al., 2003a). Shaw et al. (2002) examined rebound in *Clk*^{Jrk} flies and found that while control flies only recover ~30% of sleep lost during rebound, *Clk*^{Jrk} flies recover ~100%, resulting in a “hyper-rebound”.

Together, these results point towards a role for *Clock* in the normal homeostatic accumulation and/or dissipation of sleep need, since *Clock* mutants sleep less than normal, and have poor sleep recovery following deprivation. Rebound phenotypes are difficult to interpret, however, one possibility is that *Clock* is required to promote genes required for sleep maintenance. This is supported by the observation that the duration of sleep bouts is reduced, and sleep rebound is prolonged, in both *Clock* mutant mice and flies. If flies cannot maintain sleep, then prolonged sleep rebound may result, as the animal continuously initiates sleep in an attempt to reduce sleep need, but is unable. The interpretation of these experiments is complicated by the fact that Hendricks et al. (2003a) examined sleep in 30 min intervals, rather than 5 min intervals, an amount that has become accepted as the proper sleep “unit” in flies (Shaw et al., 2000; Huber et al., 2004b). Since *Clk*^{Jrk} flies are arrhythmic, only considering a sleep unit as one where flies are immobile for 30 consecutive minutes could miss a number of smaller, 5 min sleep intervals, and therefore under-score sleep amount. It will be necessary to examine sleep in 5 min intervals

in *Clock* mutant flies to observe the true nature of the sleep amount phenotype. In addition, the nature of *Clock* mutations in mice and *Clk*^{Jrk} flies are predicted to result in a dominant-negative phenotype (King et al., 1997; Allada et al., 1998). Results of dominant mutations can be difficult to interpret, since they may induce a phenotype that would otherwise not exist by interfering with other proteins. Thus, the function of *Clock* in sleep would be better understood by examining the phenotype of a null allele.

Mutations in the dimeric binding partner of *Clock*, *cycle* (*BMAL1* in the mouse) also severely disrupt sleep. *Cyc*^{01} loss of function mutations in flies result in decreased levels of baseline sleep, with reduced sleep bout lengths, similar to *Clk*^{Jrk} flies (Hendricks et al., 2003a). However, *cyc*^{01} mutants have sexually dimorphic responses to sleep deprivation, making these results more complicated to explain. Female *cyc*^{01} flies display a disproportionately large amount of sleep rebound, and *cyc*^{01} male flies do not rebound after sleep deprivation (Shaw et al., 2002; Hendricks et al., 2003a). Male *cyc*^{01} flies were also shown to have a dramatically reduced lifespan (Hendricks et al., 2003a), and female flies were found to be extremely sensitive to sleep deprivation, many dying after 10 hrs of total sleep deprivation, compared to no deaths in controls (Shaw et al., 2002), which may be due to an inability to efficiently reduce homeostatic sleep need in both sexes. *Clock* mutant mice and flies share similar sleep phenotypes, however, *BMAL1* male knockout mice demonstrate increased total sleep time (opposite from *cyc*^{01} flies), and similarly to *cyc*^{01} flies, decreased sleep consolidation, and an inability to rebound following sleep deprivation (Laposky et al., 2005). The difference in baseline sleep amount is difficult to explain, but may reflect differences in scoring methods between the two species, since in fact, sleep amount in *cyc*^{01} flies might be underrepresented in the Hendricks paper (2003a), as mentioned above.

Drosophila period (*per*) and *timeless* (*tim*), and their mammalian counterparts, also influence sleep, although to a lesser extent than either *Clock* or *cycle*. Fly *per*^{01} null mutations result in little to no effect on sleep (Hendricks et al., 2003a; Shaw et al., 2002). *Period*^{1}, *period*^{2} or *period*^{1,2} double knockout mice (mice have three functional *per* genes) result in noticeable, but almost negligible changes in sleep duration, sleep timing, sleep distribution, and delta power following SD (Kopp et al., 2002; Shiromani et al., 2004). It has recently been shown that a polymorphism in the *period3* gene affects sleep, but not circadian rhythms (Viola et al., 2007). Subjects homozygous for the *period3*^{5} allele exhibit increased slow wave sleep, increased slow wave activity during NREM sleep, and reduced cognitive impairment following sleep deprivation, but no alterations in circadian rhythms (Viola et al., 2007). Mutations in fly *timeless* (*tim*^{01}) result in normal levels of baseline sleep, but a complicated response to sleep deprivation, since *tim*^{01} flies do not respond to shorter amounts of sleep deprivation in DD (Shaw et al., 2002; Hendricks et al., 2003a), but do rebound following longer periods of deprivation (Shaw et al., 2002), or deprivation performed in LD (Hendricks et al., 2003a). There is a *timeless* ortholog in mice, however, the function of mouse *timeless* in the clock is unclear, and the circadian functional equivalent to *timeless* in the mouse clock appears to be served by the genes, c*ryptochrome*^{1} and c*ryptochrome*^{2} (Review: Allada et al., 2001). Unlike all other circadian mutants, c*ryptochrome*^{1,2} knockout mice demonstrate increased NREM sleep, increased sleep consolidation, and increased delta power (Wisor et al., 2002).

Despite some of the differences between mouse and fly sleep phenotypes, these results strongly suggest that circadian genes are involved in sleep regulation, including sleep amount, sleep consolidation, and response to sleep rebound, beyond simply dictating sleep timing. It was not entirely expected that a circadian gene would be involved in sleep regulation, and it is rather

complicated to explain these results based solely on either of the two previously proposed models of sleep regulation, the two-process and opponent process models. These studies raise a number of questions, the most pressing of which is, are sleep phenotypes due to loss of circadian genes within pacemaker tissues, or non-pacemaker/sleep-regulatory tissues, or both? *Clock* mRNA (King et al., 1997) and CLOCK protein (Houl et al., 2006) is widely expressed, in pacemaker and non-pacemaker tissues. In fact, fly CLK is expressed in the mushroom bodies (Houl et al., 2006), a sleep regulatory candidate region (previously discussed). McDonald et al. (2001) examined whole genome mRNA expression levels in *Clk^{Jrk}* mutant flies. As expected, they did not observe any cycling transcripts in the arrhythmic *Clk^{Jrk}* mutant background. However, they also identified a core of between 267-323 potential *Clock* targets gene, since their expression was either increased (80%) or decreased (20%) in a *Clock* mutant background. It is possible that disrupted transcription of these genes in pacemaker and/or non-pacemaker tissues could be responsible for the sleep phenotype observed in *Clock* mutant flies and mice.

Summary

Sleep is a complex behavior regulated by many regions in the mammalian brain and by a number of genes in flies and mammals. Only through close examination of the activity of these regions, and expression of sleep-regulated genes will we be able to uncover the function of sleep. We can claim to understand sleep only when it is understood in detail what the sleep need signal is, how it interacts with the circadian pacemaker, how it is sensed by sleep-regulatory regions and is translated into anatomical sleep output, and how sleep outputs result in a dissipation of the sleep need signal.

At the time I began my research, only four fly sleep papers had been published, two describing the model system (Hendricks et al., 2000; Shaw et al., 2000), one examining the role of CREB in sleep (Hendricks et al., 2001), and one examining the role of circadian genes in sleep regulation (Shaw et al., 2002). While these papers were the first to link two known molecular pathways to sleep regulation in flies, they did not comment on whether the effects of these genes were due to loss of function from the brain. In fact, it was still unclear whether sleep was a behavior that was controlled by the brain in the fly. While unlikely, it was possible that sleep in flies was what it looked like to the human observer – quiet resting with eyes open. We realized that to advance the model system, we had to show sleep was controlled by the brain in the fly, and that it was an actively promoted behavior, as in mammals. The discovery of a sleep-regulatory region would then provide us with a location to begin to dissect the molecular regulation of sleep, by focusing on genes expressed in that tissue during sleep and wake. To accomplish this, we employed a non-biased "neuroanatomy screen", whereby we screened regions of the brain for sleep-regulatory functions by observing sleep following inhibition of neural activity. We found that one area in particular, the mushroom bodies, promote sleep (Chapter 2). We also found that discrete regions of the mushroom bodies, and/or the central complex region might promote wake, although the contribution of these areas to wake regulation is not as clear (Chapter 3).

Another question that we were interested in pursuing was; why do mutations in circadian genes cause sleep phenotypes in mice and flies? The fly was uniquely positioned to answer these questions, since the tools were available to perform tissue-specific rescue, and therefore, examine the function of a circadian gene in both circadian and non-circadian tissues. We chose to further examine the role of *Clock* in sleep, since there was evidence both in the fly (Shaw et

al., 2002; Hendricks et al., 2003a) and mouse (Naylor et al., 2000) that mutations in *Clock* disrupted sleep. Interested in whether circadian neurons themselves were sleep-regulatory, we also examined the role of circadian neurons in sleep (Chapter 4). We confirmed that *Clock* mutants do indeed exhibit both sleep amount and sleep consolidation phenotypes, and were able to rescue components of the *Clock* sleep phenotype by returning *Clock* function to the mushroom bodies, and several uncharacterized circadian neurons. While this result does not clarify which tissues *Clock* functions within to promote sleep, it raises the possibility that *Clock* is required within non-pacemaker cells, perhaps the mushroom bodies for this function.

These studies provide a foundation for the identification of genes directly involved in the signal for sleep need. It is likely that the mushroom bodies contain the molecular machinery required to sense sleep need, since this tissue is required to generate sleep output. Also, since mutations in *Clock* disrupt sleep this suggests that *Clock* may be a central part of the molecular mechanism required to either sense sleep need, promote sleep, or both.

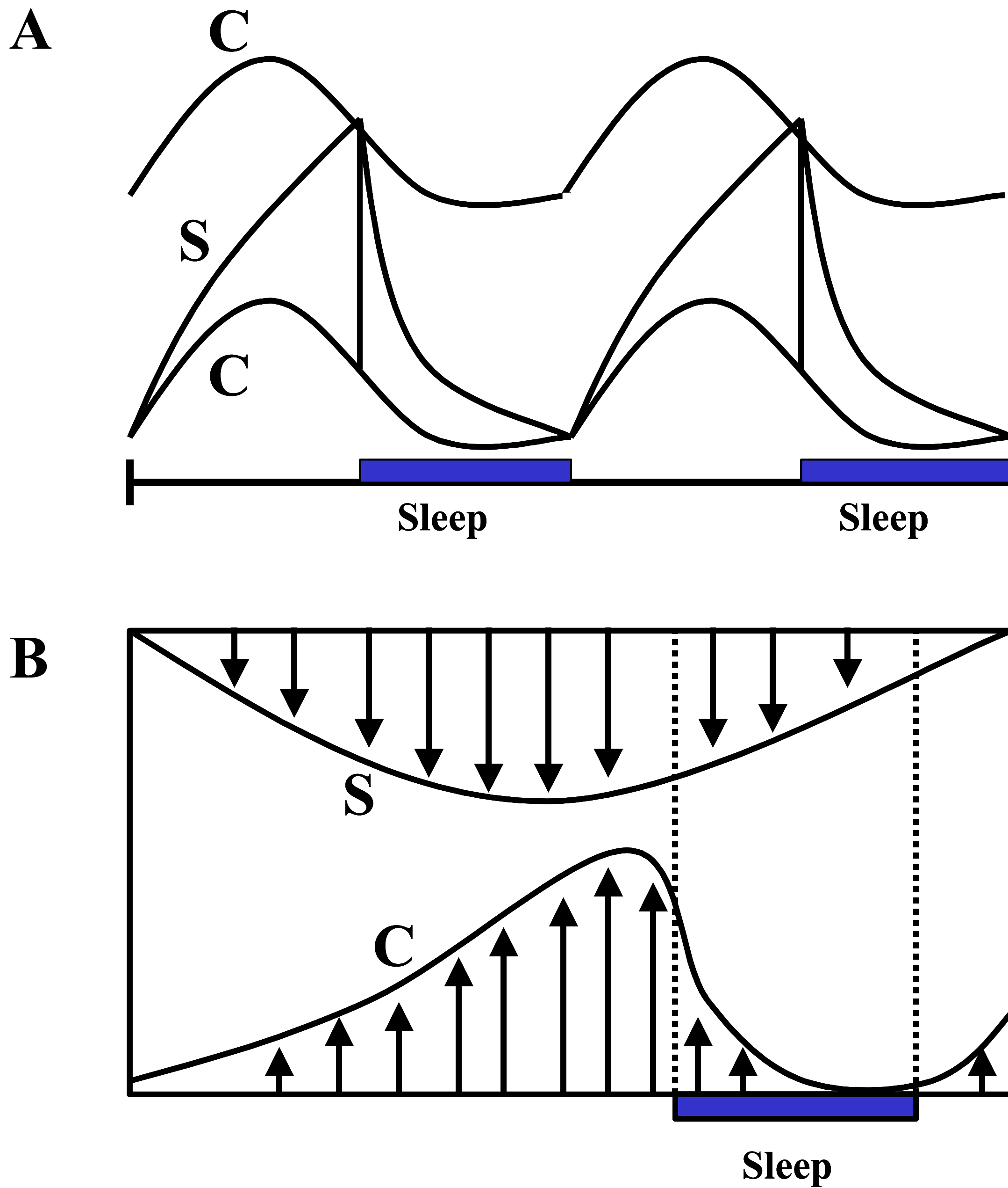


Figure 1.1 Two Theories of Sleep Regulation. (**A**) The two-process model (adapted from Edgar et al., 1993). (**B**) The opponent process model (adapted from Daan et al., 1984). Abbreviations: S = Process S (Sleep debt), C = Process C, circadian rhythm (**A**), Circadian alerting factor (**B**).

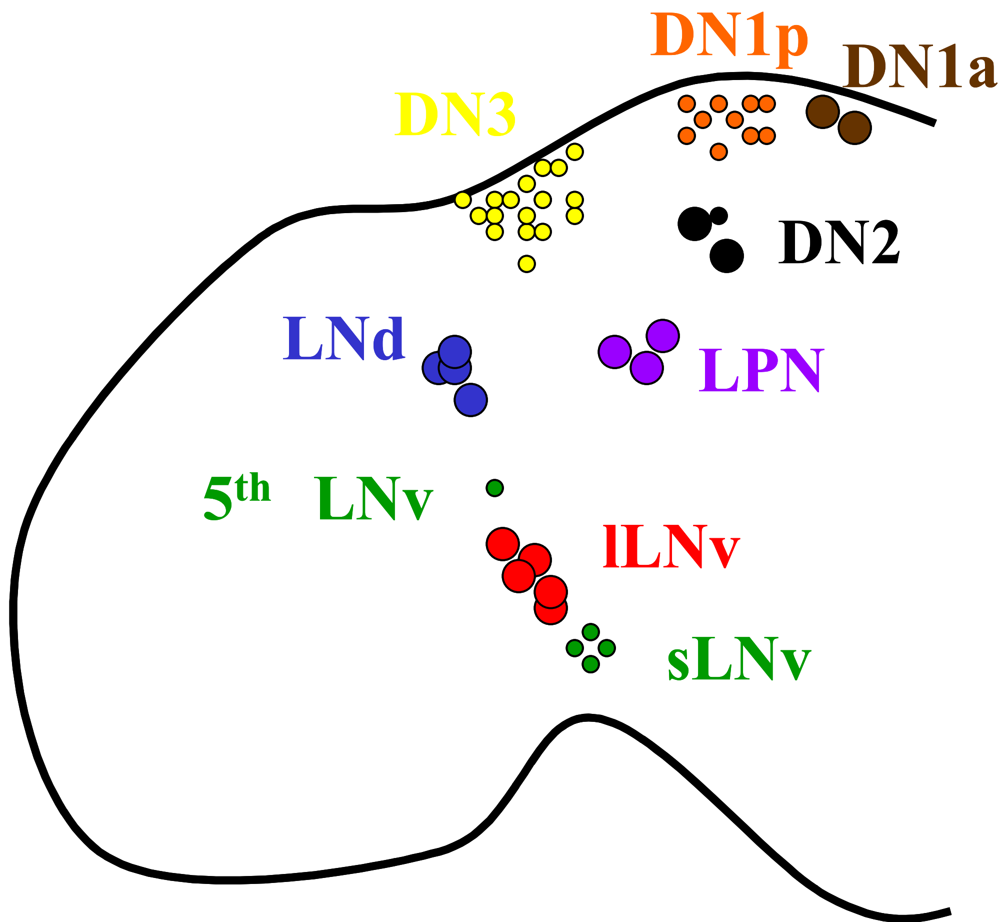


Figure 1.2 Groups Comprising the Circadian Cell Network. Eight groups of neurons define the circadian network including the small and large lateral ventral neurons (sLNv, lLNv), and a 5th atypical LNv, the dorsal lateral ventral neurons (LNd), the lateral posterior neurons (LPN), and three groups of dorsal neurons, the DN1 (anterior and posterior), DN2, and DN3 (adapted from Shafer et al., 2006).

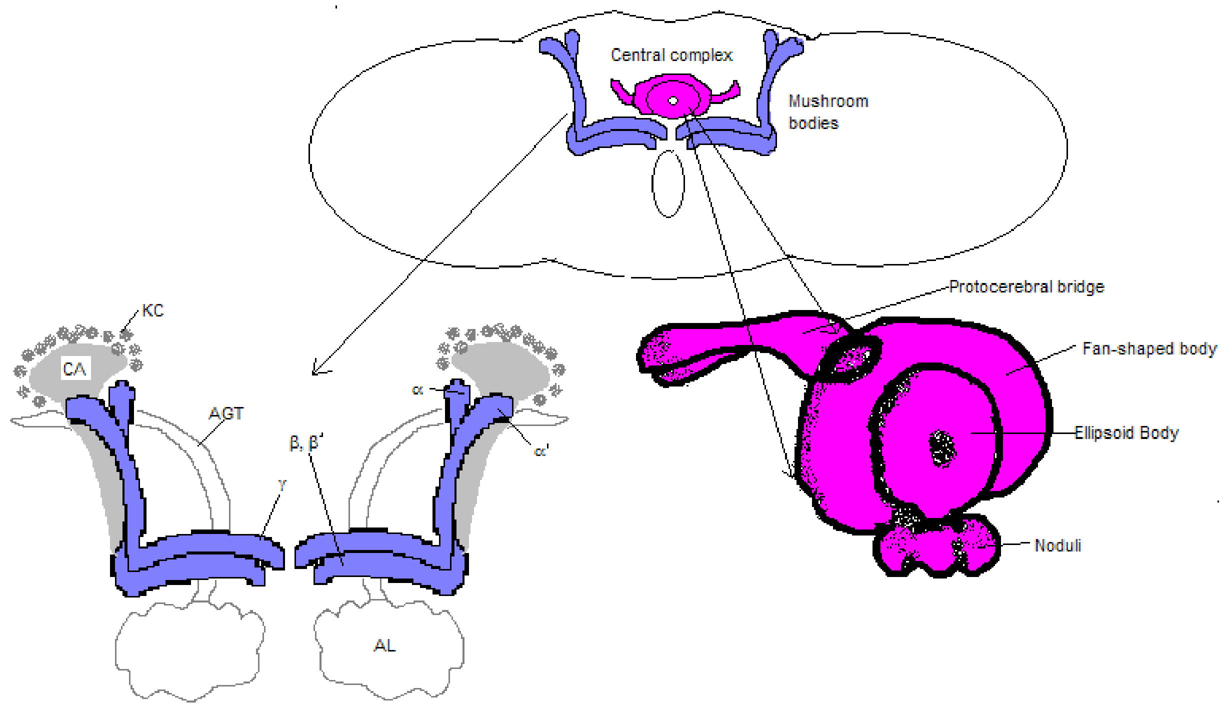


Figure 1.3 Schematic Representations of the Mushroom Bodies and Central Complex. (**A**) The bilateral mushroom bodies are shown here with the primary structures labeled. MB neurons = Kenyon Cell Bodies (KC), CA = calyx, α/α', β/β', γ lobes. MB accessory structures also shown including AL = antennal lobe, AGT = antennal glomerular tract. Schematic of the central complex including all main structures.

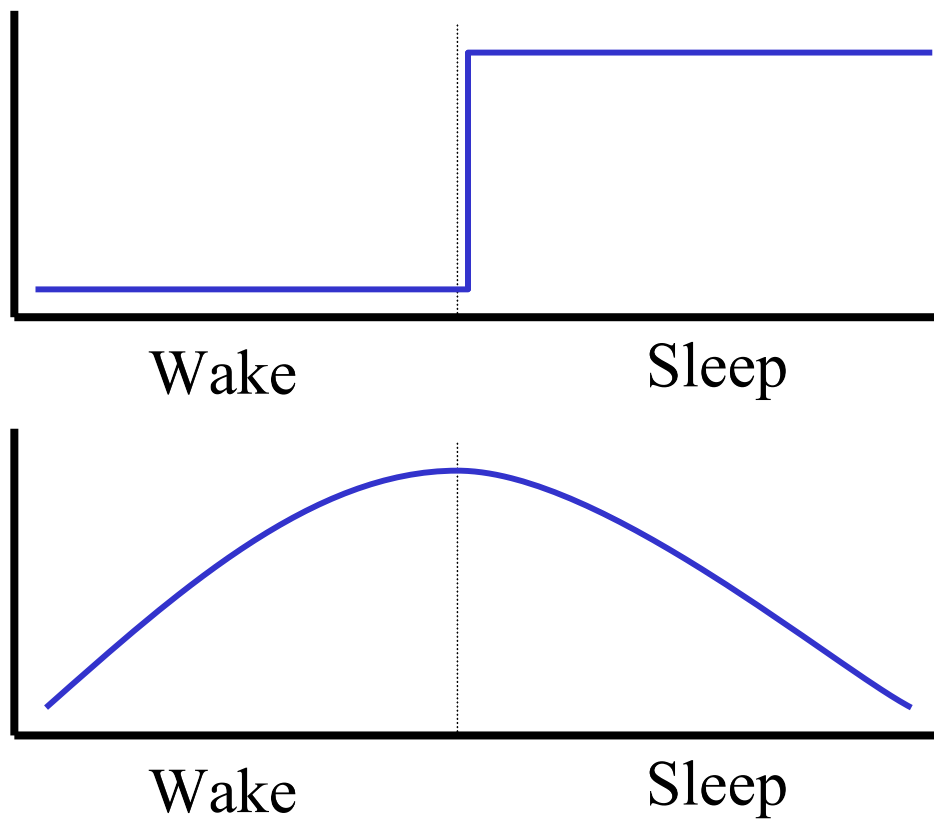


Figure 1.4 Two Possible Expression Patterns of a "Sleep Gene". Top: Type #1 – The expression of this gene is elevated during sleep only, reminiscent of a sleep output gene. Bottom: Type #2 – The expression of this gene follows a homeostatic pattern, it increases as sleep need increases, and decreases as sleep need dissipates.

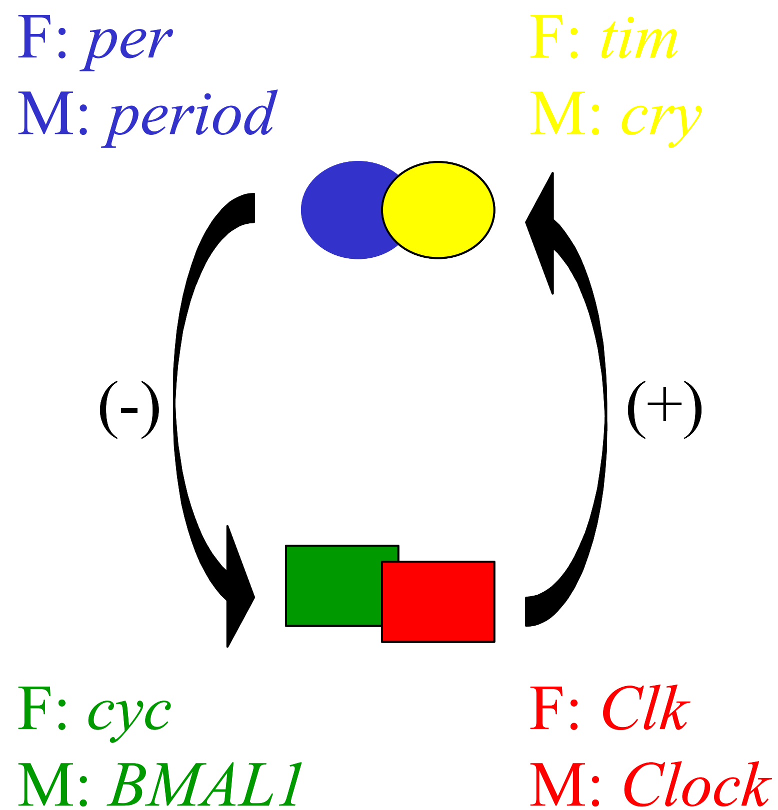


Figure 1.5 Comparison of Molecular Feedback Loop in Flies and Mice. See text for details (Adapted from Allada et al., 2001). Abbreviations: TIM = timeless, PER = period, CRY = cryptochrome, CLK = clock, CYC = cycle, BMAL1 = brain and muscle Arnt-like protein-1.

CHAPTER 2

A Dynamic Role for The Mushroom Bodies in Promoting Sleep in *Drosophila*

The fruit fly, *Drosophila melanogaster*, exhibits many of the cardinal features of sleep, yet little is known about the neural circuits governing its sleep (Review: Hendricks and Sehgal, 2004). We performed a screen of GAL4 lines expressing a temperature sensitive synaptic blocker *shibire*^{ts1} (Kitamoto, 2001) in a range of discrete neural circuits, and assayed sleep at different temperatures. We identified three short sleep lines at the restrictive temperature with shared expression in the mushroom bodies (MB), a neural locus central to learning and memory (Review: Davis, 2004). Chemical ablation of the MB also resulted in reduced sleep. These studies highlight a central role for the mushroom bodies in sleep regulation.

Evidence suggests that the fruit fly is a valid sleep model (Review: Hendricks and Sehgal, 2004). Flies exhibit long periods of immobility accompanied by increased arousal thresholds (Hendricks et al., 2000; Shaw et al., 2000; Huber et al., 2004b). These states are modulated by drugs known to regulate mammalian sleep including antihistamines and modafinil (Shaw et al., 2000; Hendricks et al., 2003b) and are correlated with brain activity (Nitz et al., 2002; van Swinderen et al., 2004). Fly sleep is homeostatically regulated, i.e., flies recover lost sleep, known as sleep rebound (Hendricks et al., 2000; Shaw et al., 2000; Huber et al., 2004b). Initial genetic analyses of fly sleep have implicated a variety of molecular pathways, including those responsible for circadian rhythm generation, the cAMP signaling pathway, stress response, and neuronal excitability (Hendricks et al., 2000; Hendricks 2001; Shaw et al., 2002; Cirelli et al., 2005a). It is not clear whether these pathways regulate sleep through their effects on

specialized neural circuits in the brain or indirectly through other loci. Moreover, it is unclear if sleep is an actively promoted process in flies, as in mammals.

Material and Methods

Animals. Flies were raised under a 12hr:12hr light:dark schedule at 25°C, and ~50% humidity. Stocks were provided as follows: GAL4 collection was provided by Douglas Armstrong via Greg Suh, 30YGAL4 (Asaf Presente), pars intercerebralis GAL4 lines (G. Korge), UAS shi^{ts1} (T. Kitamoto), and UAS*mc** (A. Sehgal). Other lines were from the Bloomington Stock Centre. Hydroxyurea (HU) ablation was performed as previously described (deBelle and Heisenberg, 1994). MB ablation was verified using MB expression of GFP.

Sleep assays, measures of sleep and activity. One to four day old flies were placed into individual 65mm glass tubes in the Trikinetics Drosophila activity monitoring (DAM) system (Trikinetics, Waltham, MA) under CO2 anaesthesia. A sleep episode was defined as a 5-minute bin of uninterrupted quiescence using the DAM system. Activity counts were summed across all wake bins.

Temperature cycling (TC) assay. Following the end of the 12hr light period of the first day of the experiment, flies were kept for 24hr at 21°C in constant dark (DD). Temperature was then cycled for 14 days (12hr:12hr, 29°C: 21°C in DD). Total sleep and average activity per wake min were averaged during the 29°C or 21°C period of TC across 4 days. In one case, the percentage of total sleep spent at 29°C is reported (Figure 2.10). Days 9-12 were typically used except where indicated to allow complete circadian re-entrainment.

Constant temperature (CT) assay. Flies were maintained for five days under 12:12LD conditions followed by seven days of 24hr DD, at either 21°C or 29°C. Total sleep and average activity per wake min were calculated and averaged across the first three days of LD or DD.

Measures of sleep consolidation. All were averaged across three days of LD or DD under constant conditions (21°C or 29°C). Average sleep bout length was calculated by summing all sleep bouts of all lengths (in minutes) and dividing by the total number of sleep bouts. Consolidation index was calculated as a weighted average of sleep bout length, where each sleep bout was weighted according to its duration in minutes. CI was calculated by summing the square of all sleep bout lengths (min) and dividing by the total amount of sleep. This method reduces the influence of transient awakenings during the sleep phase.

Confocal imaging. Brains of six-day old male flies were dissected and fixed in 3.7% paraformaldehyde for 40min. Brains were washed in phosphate buffered saline (PBS) and PBS containing 0.3% Triton 100-X, incubated and mounted in 80% glycerol and imaged.

Statistical analyses. One-way ANOVA tests were used to compare averages between different genotypes. Paired t-tests were used to compare sleep within a single genotype.
Lifespan curves were generated using Kaplan-Meier Estimates of Survival and were compared using the Logrank Test (NCSS software). Linear regression analysis was performed to determine the relationship between sleep and lifespan (Excel). A p-value of <0.05 was accepted as significant for all analyses.

Sibling Analysis. 30Y, c309, and UAS*shi*^{ts} homozygous males were crossed to virgin 5905 flies, an isogenized w^{-} stock obtained from the DrosDel Collection (Ryder et al., 2004). UAS*shi*^{ts}/+ virgins and 30Y/+ or c309/+ males were crossed to each other, and the sibling progeny from this cross were tested behaviourally in a seven day temperature cycling experiment. Genotyping for the relevant transgenes was performed as follows: at the end of the behavioural experiment, each fly was frozen on dry ice and homogenized in 10mM Tris-HCl, 1mM EDTA, 25mM NaCl, 200g/ml of Proteinase K to extract DNA. Individual PCR reactions were then performed on each DNA sample to identify the presence of UAS*shi*^{ts} (forward primer, 5'-GCAATGCGTTCACATCGCTC-3', reverse primer, 5'-CAAGATTAGTGGTCTCCGAGTTACG-3') or GAL4 (forward primer, 5'-GGCATCATTGAAACAGCAAGGC-3', reverse primer, 5'-GCGGTCTCGTTATTCTCAGCATTC-3'). The MJ Research Peltier Thermo Cycler PTC 200 was programmed to 94°C for 18 seconds, 57°C for 30 seconds, 72°C for 1:30 seconds and cycled 30 times. The product from each PCR reaction was ran on a 1% agarose gel and photographed using a Polaroid camera. PCR genotype results for each fly were matched with sleep data (Total Sleep/Min during 29°C period of temperature cycling, days 3-6), and sleep was averaged for each genotype (GAL4/+, +/UAS*shi*^{ts}, GAL4/UAS*shi*^{ts}).

Lifespan. Recently hatched flies (<3 days old) were collected under CO_2 anaesthesia and placed into vials with a density of <25 males and <25 females (Pittendrigh and Minis, 1972). Flies were maintained at the restrictive temperature (29°C) or permissive temperature (21°C), were transferred to new food vials three or two times a week respectively, and living male flies were counted at transfer (females were not counted, but were transferred along with males). Mean

lifespan was calculated for each genotype, and lifespan survival curves were generated using Kaplan-Meier Survival Curve analyses (NCSS software) with proportion surviving plotted against fly age (in days). Mean lifespan (days) for each genotype was plotted against Total Sleep/24hr (minutes) and linear regression analysis was performed to determine the relationship between sleep and lifespan.

Circadian heat pulse assay. Flies were entrained to a 12:12 LD cycle for 2-4 baseline days at 21°C. A 29°C 6hr heat pulse was administered from ZT6-12, ZT12-18, or ZT18-24. In most cases, following the pulse flies were returned to 21°C. Baseline sleep (Min Sleep/6hr) was averaged over the last baseline day for each independent experiment and was compared to total sleep (Min Sleep/6hr) during the corresponding pulse time ZT6-12, ZT12-18, or ZT18-24. Cumulative sleep loss was calculated by subtracting baseline sleep (min/hr ZT18-24) from sleep during the pulse (min/hr ZT18-24) and summing the differences. Flies were then returned to 21°C to assay rebound sleep. Cumulative sleep rebound was calculated by subtracting baseline sleep (min/hr ZT0-24) from sleep up to 24 hours after the pulse using the corresponding ZT time for subtraction and summing the differences.

Mechanical sleep deprivation assay. Flies were maintained in a 12:12 LD cycle at 29°C for 5 baseline days. Flies were mechanically sleep deprived using a rotating box (Cirelli, 2003; Huber et al., 2004b) for 24hr, starting at lights on (ZT0-24), and rebound sleep was assayed. Minutes of sleep/hr were averaged over the last baseline day for each independent experiment. Cumulative sleep loss was calculated by subtracting baseline sleep (Min/Hr ZT0-24) from sleep during the deprivation (sleep was possible in some flies for brief periods of time) and summing the

differences. Cumulative sleep rebound was calculated by subtracting baseline sleep (Min/Hr ZT0-24) from sleep up to 24 hours after the deprivation and summing the differences. Non-deprived flies were tested in parallel and demonstrated trends upward (29°C) in sleep amount. To remove the upward trend, the average difference in sleep between first day of rebound and last day of baseline in the non-deprived flies was subtracted from the rebound sleep of each individual fly.

Results and Discussion

Mushroom Body Neurons Promote Sleep and Sleep Consolidation

To identify brain regions important for *Drosophila* sleep, we exploited a genetically encoded temperature-sensitive blocker of synaptic transmission, $shibire^{ts1}$ (shi^{ts1}). *shibire* encodes a Drosophila ortholog of dynamin, a GTPase that is essential for synaptic vesicle recycling (Kosaka et al., 1983; Chen et al., 1991; van der Bliek et al., 1991). At elevated temperatures, shi^{ts1} can rapidly block synaptic transmission in neurons (Koenig et al., 1983; Kitamoto, 2000). We targeted shi^{ts1} expression in defined brain regions using the binary GAL4/UAS system (Kitamoto, 2000). We selected GAL4 lines from a collection that previously had been shown to drive expression in the adult brain (Armstrong and Kaiser, 1996), but demonstrating varied expression patterns (Table 2.1). This approach allowed the rapid manipulation of discrete neural circuits and assessment of the consequences on sleep.

Flies were subjected to temperature cycles (TC) of 12 hours 29°C and 12 hours 21°C. Of the 92 GAL4 lines tested, six demonstrated decreases in total sleep at 29°C (Figure 2.1A) but not at 21°C (Figure 2.1B). We also found lines with increased sleep at 29°C (Table 2.1). Given our

interest in sleep promoting circuits, we focused on the short sleep GAL4 lines (SSL). In TC, sleep reductions relative to controls are evident soon after temperature shifts to 29°C ($\leq$1 hour), suggesting direct effects (Figure 2.1C,D). Since reduced sleep may be a consequence of response to temperature shifts, we tested SSL lines maintained at a restrictive (29°C) temperature. Of these, we found three (c253, c747, and c758) with reduced sleep (Figure 2.1E). These phenotypes are temperature dependent as sleep is largely unaffected at 21°C (Figure 2.1F). At 29°C, sleep is reduced during light and dark periods (Figure 2.1G,H). Moreover, these effects are not likely due to differences in genetic background as sibling progeny of heterozygous parents (GAL4/+, UASshi^{ts1}/+) revealed reduced sleep at 29°C in GAL4/UASshi^{ts1} flies (Figure 2.2 A,B).

We assessed GAL4 expression patterns by examining driven patterns of GFP and membrane-linked GFP expression (UAS-mCD8-GFP). Consistent with prior reports (Armstrong and Kaiser, 1996), the most prominent areas of shared expression were the mushroom bodies (MB), neuropil structures central to some forms of long-term memory (Pascual and Preat, 2001) (Figure 2.3 A-H). We then selected other MB expressing GAL4 lines and found that 30Y and c309 GAL4 (Armstrong and Kaiser, 1996) exhibited a robust SSL phenotype in combination with UASshi^{ts1} (Figure 2.1 A,B,E,F). The MB GAL4 line 247 (Zars et al., 2000) in combination with UASshi^{ts1} revealed reduced sleep only during the early morning at 29°C (Figure 2.4). In contrast, 17D (Martin et al., 1998) as well as other MB expressing lines (Table 2.1) exhibit wild-type or even increased sleep at 29°C. These lines may differ in their extent of MB expression. For example, 247, 30Y and 17D are thought to drive expression in only about one-third of MB neurons or less (Schwaerzel et al., 2002; M. Mader and M. Heisenberg, personal communication). The neuroanatomical basis of the long sleepers is unclear as substantial

expression is observed outside the MB (Table 2.1). Although we cannot exclude a role for the MB in inhibiting sleep, these data suggest that the MB play a primary role in promoting sleep.

In addition to reduced sleep, these lines exhibited less consolidated sleep. Reduced sleep was not accompanied by significant decreases in sleep bout number (Figure 2.5C). However, average bout length (ABL; Figure 2.5D) and consolidation index (CI), a weighted ABL (Figure 2.5E), were reduced, suggesting impaired sleep maintenance. While 21°C is nominally permissive, *shi*^{ts1} effects have been observed at 18°C (Sapp et al., 1991), including with 30YGAL4 (data not shown). Nonetheless, consolidation phenotypes were either reduced or absent at 21°C (Figure 2.5 F-H).

The Shibire Induced Short-sleep Phenotype is not Due to Increased Activity or Alteration of Clock Function

Increases in waking locomotor activity are not uniformly evident in SSL flies. Under TC, we found that waking activity at 29°C was not affected in 30Y/UAS*shi*^{ts1} flies (Figure 2.5A). During constant 29°C, waking activity was also unaffected in some SSL lines (Figure 2.5B).

Previous studies in *Drosophila* have implicated clock genes in regulating sleep (Hendricks et al., 2000; Shaw et al., 2002; Hendricks et al., 2003a). We examined two SSL lines during the first four days of TC (days 3-6) rather than days 9-12 (Figure 2.1 A-D). During these days, the circadian contribution to sleep is spread over the day (Figure 2.6A). We also examined sleep in SSL lines in a clockless *per*^{01} background. *shi*^{ts1} effects were evident under both conditions (Figure 2.6 B-E). In addition, we performed temperature shifts from 21°C to 29°C at different times of day and found that sleep was reduced at 29°C in GAL4/UAS*shi*^{ts1} flies at all times (Figure 2.7 A,B). Temperature pulses delivered during a sleep period (ZT18-24), in a

relatively specific MB line (c309/UAS*shi*^{ts1}) lacking a 21°C phenotype, elicits increased sleep loss and subsequent sleep rebound, consistent with a sleep regulatory function (Figure 2.7C).

Mushroom Body Ablation Results in a Short-Sleep Phenotype

In SSL GAL4 lines, expression is observed outside the MB (Figure 2.3 A-H) and some MB expressing lines do not have sleep phenotypes (Table 2.1). To independently assess MB function, we used chemical ablation with hydroxyurea (HU). HU fed to larvae one hour after hatching ablates four neuroblasts that give rise to most MB neurons, and a lateral neuroblast that gives rise to some antennal lobe interneurons (deBelle and Heisenberg, 1994). HU-treated flies demonstrated significant decreases in sleep (Figure 2.8A). Although modest increases in sleep bout number are also observed (Figure 2.8B), sleep maintenance is primarily affected (Figure 2.8 C,D), as in the case of GAL4/UAS*shi*^{ts1} flies (Figure 2.5 A-C). While reduced sleep was accompanied by increased waking activity consistent with prior reports (Martin et al., 1998; Helfrich-Forster, 2002) (Figure 2.8E), activity and sleep phenotypes are not always correlated. For example, HU flies under DD exhibit comparable waking activity to untreated flies in LD, while their sleep levels are significantly reduced (Figure 2.8E). Importantly, these data provide an independent manipulation of MB function in otherwise genetically identical flies to demonstrate a role for the MB in promoting sleep. Notably, we have also observed reduced sleep driving an activated form of protein kinase A (UAS*mc**) using the MB line c309, a manipulation that does not require temperature changes (Figure 2.9) (Li et al., 1995). As this experiment was performed at 25°C, it argues against a role of heat stress as mediating MB effects on sleep.

The 30Y sleep phenotype is not likely due to its effects outside of the mushroom bodies. 30YGAL4 drives expression in the pars intercerebralis (PI), a locus important for sexually

dimorphic aspects of locomotor activity (Belgacem et al., 2002). PI GAL4 lines (mai301 and kurs58) (Siegmund and Korge, 2001) failed to produce sleep phenotypes comparable to 30YGAL4 (Figure 2.10). Additionally, we treated 30YGAL4/*UASshi^{ts1}* flies with HU but did not observe *shi^{ts1}* effects in HU-treated flies (Figure 2.8A). We propose that MB inhibition is largely responsible for the 30YGAL4/UAS*shi^{ts1}* phenotype, thus MB ablation has no further effect on sleep.

Short-Sleep Flies have Reduced Lifespan and Disrupted Sleep Homeostasis

If inhibiting the MB disrupts restorative sleep, then sleep loss may have an adverse consequence on lifespan (Rechtschaffen et al., 1983; Shaw et al., 2002). Testing SSL and other non-SSL MB lines, we observed significant differences in lifespan curves (data not shown) and in most cases, mean lifespan for SSL flies (Figure 2.11A). Although not all SSL lines have reduced mean lifespan, lifespan for several SSL lines (<600 min/24h) at 29°C, is correlated (r^2=0.82, p=0.03) with sleep amount (Figure 2.11A). At 21°C, lifespan effects are reduced or absent with only the SSL line 30Y demonstrating reduced lifespan and sleep (Figure 2.11B). Moreover, we note reductions in lifespan in hydroxyurea-treated flies relative to their untreated controls (Figure 2.11 C,D). These data are suggestive that MB-induced sleep reductions contribute to reduced lifespan.

To determine if sleep homeostasis was altered in 30YGAL4/*UASshi^{ts1}* flies, we mechanically sleep deprived them for 24 hours at 29°C (Cirelli, 2003; Huber et al., 2004b) and assayed rebound sleep. When maintained at 29°C, all flies exhibited a steady increase in sleep (Figure 2.12A). After detrending (see Methods), we found that 30YGAL4/*UASshi^{ts1}* flies lost less sleep than controls, consistent with their reduced 29°C sleep (Figure 2.1C). Nonetheless,

these flies exhibited comparable or increased rebound (Figure 2.12B), suggesting altered sleep homeostasis.

A Learning and Memory Center Promotes Sleep in the Fly

We have uncovered a central role for the mushroom bodies in sleep regulation. We show that transient MB inhibition using temperature cycles rapidly inhibits sleep at the restrictive temperature, indicating an active adult function. Persistent inhibition or ablation also reduces sleep, suggesting that the MB promote sleep. The co-localization of sleep and learning centers in *Drosophila* may reflect shared underlying mechanisms, perhaps a role for synaptic plasticity. In this regard, it is of interest that mutants with altered cAMP signaling disrupt both learning and sleep (Zars et al., 2000; Hendricks et al., 2001). The discovery of a role for the MB should serve to focus genetic studies to elucidate the underlying mechanisms of sleep in this model organism.

Figure 2.1. Sleep in short sleep GAL4 lines. (**A,B**) Average total sleep at 29°C (**A**) and 21°C (**B**) during temperature cycling (TC). (**C,D**) Hourly sleep for c253/+ (**C**) and c253/UAS*shi*^{ts} flies (**D**). Gray bars = 29°C (ZT0-12), black bars = 21°C (ZT12-24). (**E,F**) Average total sleep during constant 29°C (**E**) or 21°C (**F**) conditions. (**G,H**) Hourly sleep for c253/+ (**G**) and c253/UAS*shi*^{ts} flies (**H**). White bars = light (ZT0-12), black bars = dark (ZT12-24). Asterisk (*) = GAL4/UAS*shi*^{ts} combinations significantly different from both GAL4/+ and +/UAS*shi*^{ts} (one-way ANOVA, p<0.05). N=33-124, Number of experiments =3-12 (**A-D**), N=24-170, N experiments = 2-9 (**E-H**). Error bars indicate SEM.

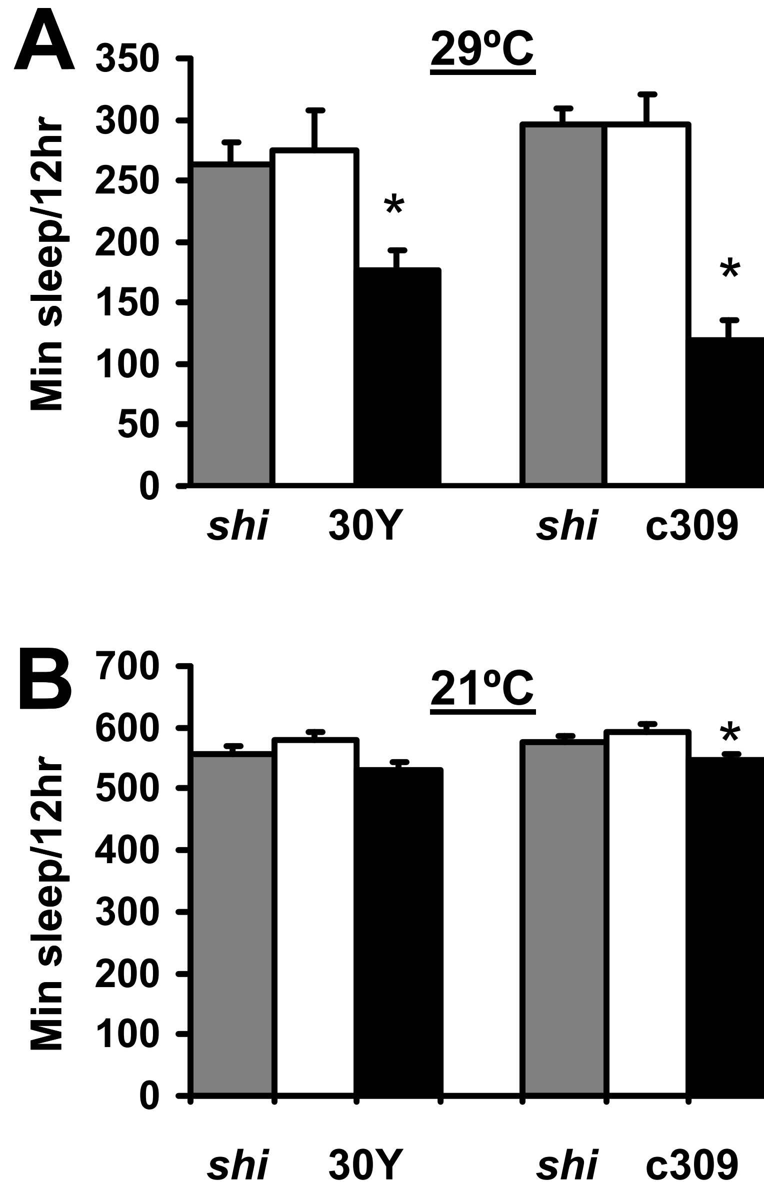


Figure 2.2 The short sleep phenotype cannot be explained by genetic background. Average total sleep occurring during 29°C (**A**) and 21°C (**B**) period of temperature cycling, days 3-6 (Min Sleep/ 12hr). 30Y, c309, and UAS*shi*^{ts} homozygous flies were crossed to an isogenized w^{-} fly stock, siblings (GAL4/+ and +/UAS*shi*^{ts}) were crossed to each other, and the progeny of this cross were tested for behavioural sleep phenotype. Individual flies were genotyped using PCR (GAL4/+, +/UAS*shi*^{ts}, GAL4/UAS*shi*^{ts}), and the sleep phenotype of each genotype was averaged. Asterisk (*) indicates where GAL4/UAS*shi*^{ts} combinations are significantly different from GAL4/+ and +/UAS*shi*^{ts} controls (one-way ANOVA, $p<0.05$). N=17-50, N experiments=2. Error bars indicate SEM.

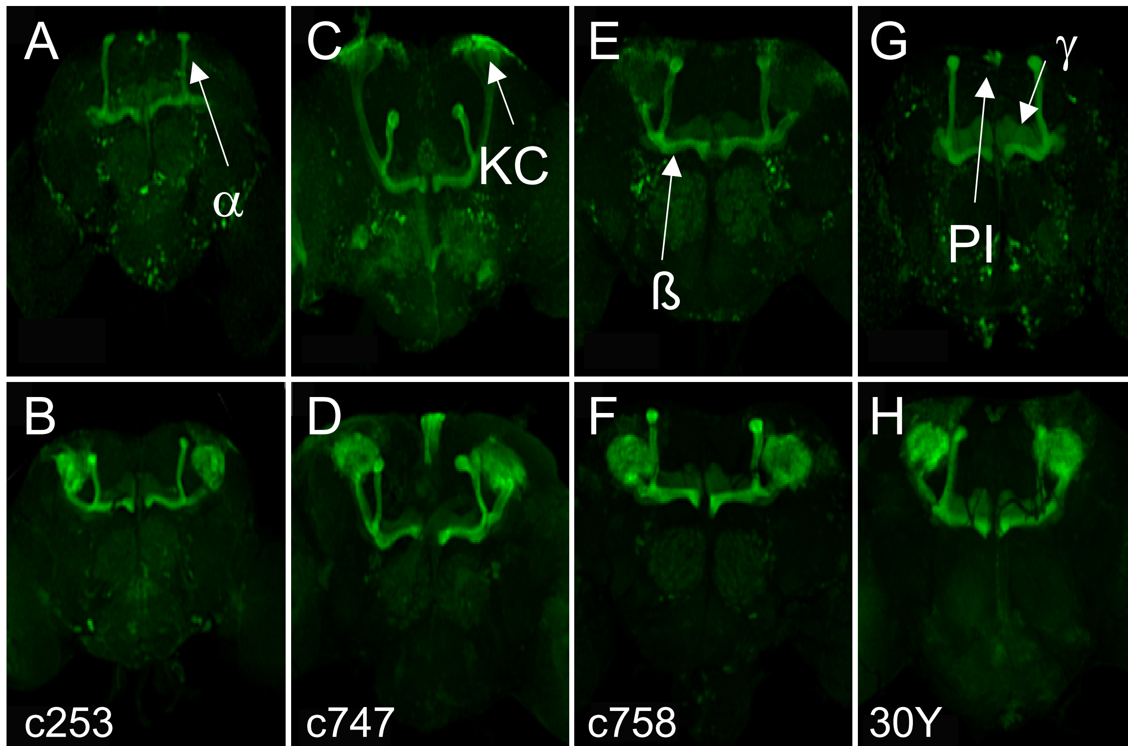


Figure 2.3 Short sleep GAL4 lines share expression within the mushroom bodies. (**A,C,E,G**) UAS*GFP* expression. (**B,D,E,F**) UAS-*mCD8-GFP* expression. Expression patterns of short sleep GAL4 lines c253 (**A,B**), c747 (**C,D**), c758 (**E,F**), 30Y (**G,H**). Alpha, beta, and gamma lobes are labelled. KC= Kenyon cell body layer, PI= pars intercerebralis. Expression patterns were examined in ~10 brains per genotype, in two independent experiments.

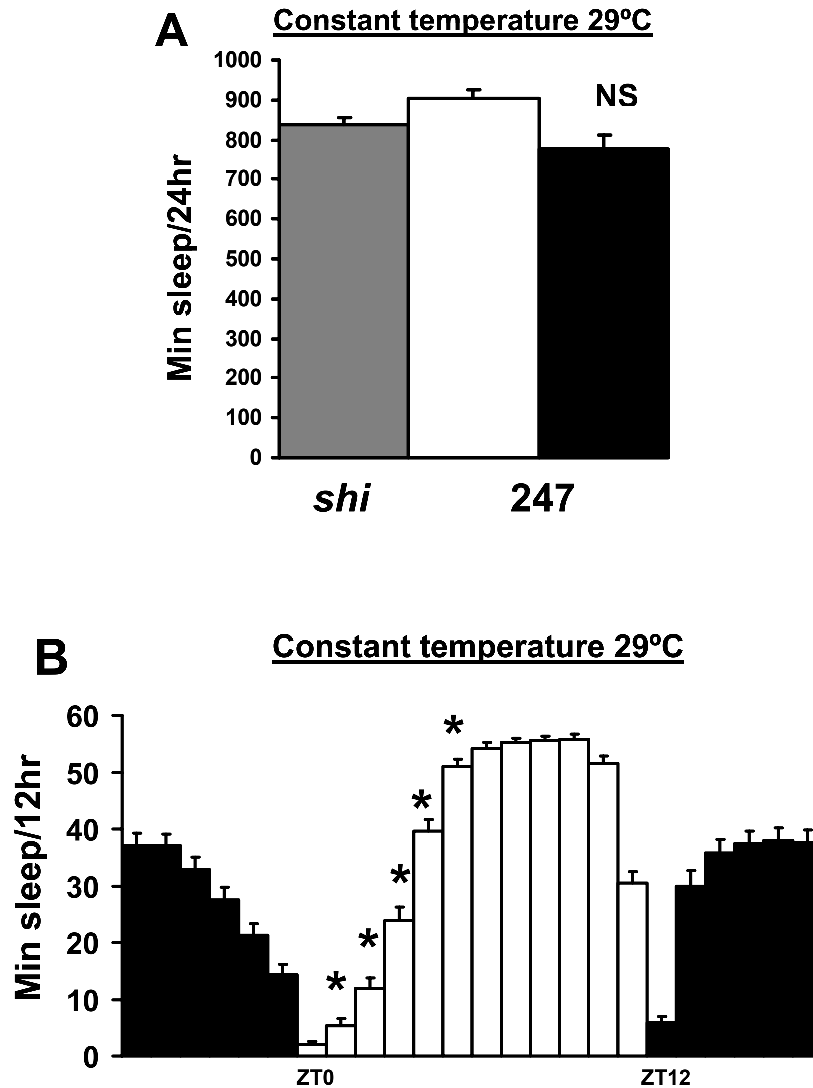


Figure 2.4 Sleep during the light is reduced in the 247 GAL4 line. (**A**) Average total sleep during constant 29°C conditions. (**B**) Hourly sleep for 247/UAS*shi*^{ts} flies. White bars = light (ZT0-12), black bars = dark (ZT12-24). Asterisk (*) = GAL4/UAS*shi*^{ts} hourly sleep significantly different from both GAL4/+ and +/UAS*shi*^{ts} (one-way ANOVA, $p<0.05$). N=52-142, N experiments = 2-9. Error bars indicate SEM.

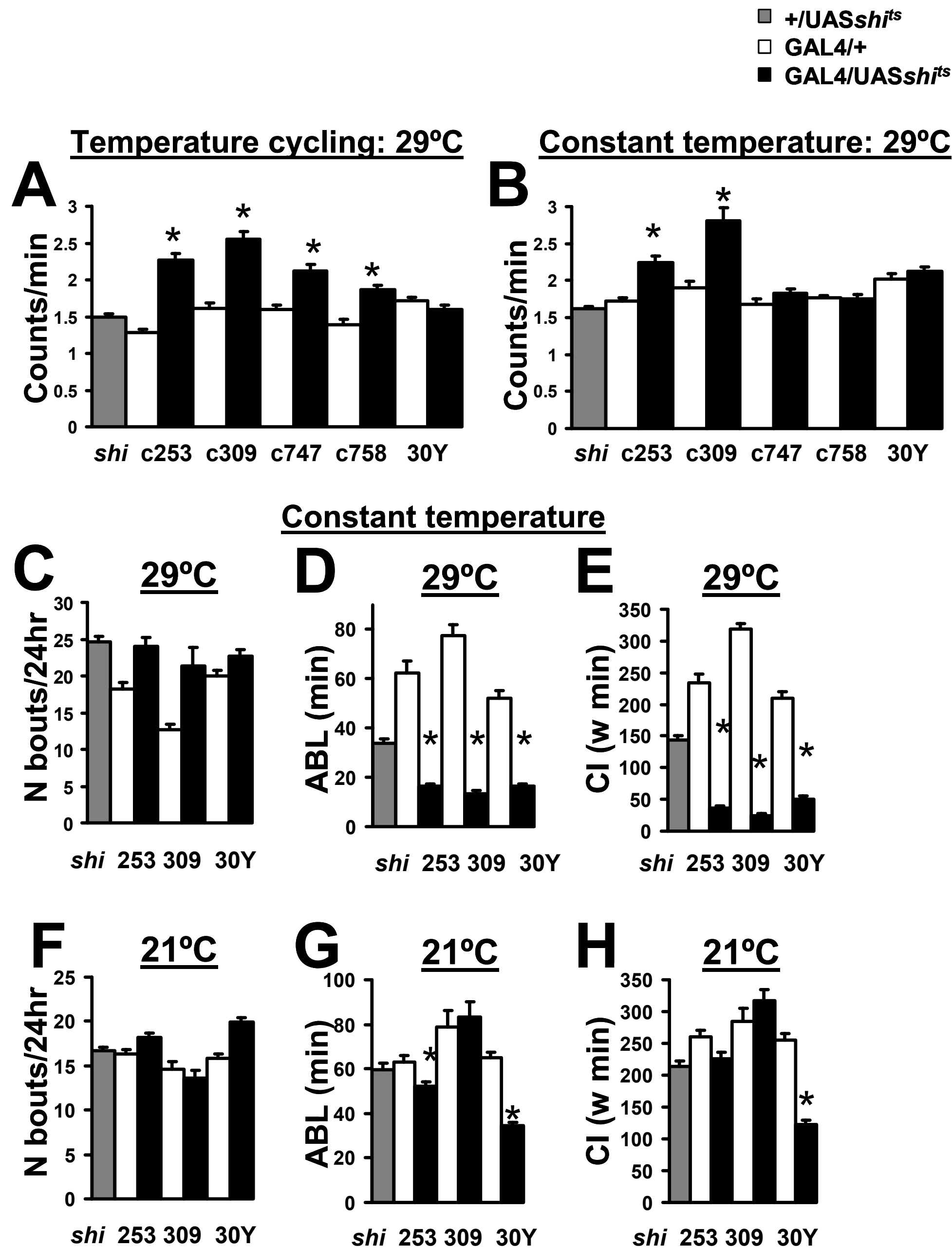


Figure 2.5 Sleep intensity is reduced, and activity is not correlated with the short sleep phenotype. (**A,B**) Average waking activity counts per minute during 29°C (**A**) period of temperature cycling, days 9-12, and constant 29°C (**B**) conditions. (**C-H)**, Sleep intensity – average number of sleep bouts, average sleep bout length, and sleep consolidation index (w= weighted) under constant 29°C (**C-E**) or 21°C (**F-H**) conditions. Asterisk (*) = GAL4/UAS*shi*^{ts} combinations significantly different from both GAL4/+ and +/UAS*shi*^{ts} controls (one-way ANOVA, $p<0.05$). N=33-124, N experiments =3-12 (**A**), N=24-170, N experiments = 2-9 (**B-H**), Error bars indicate SEM.

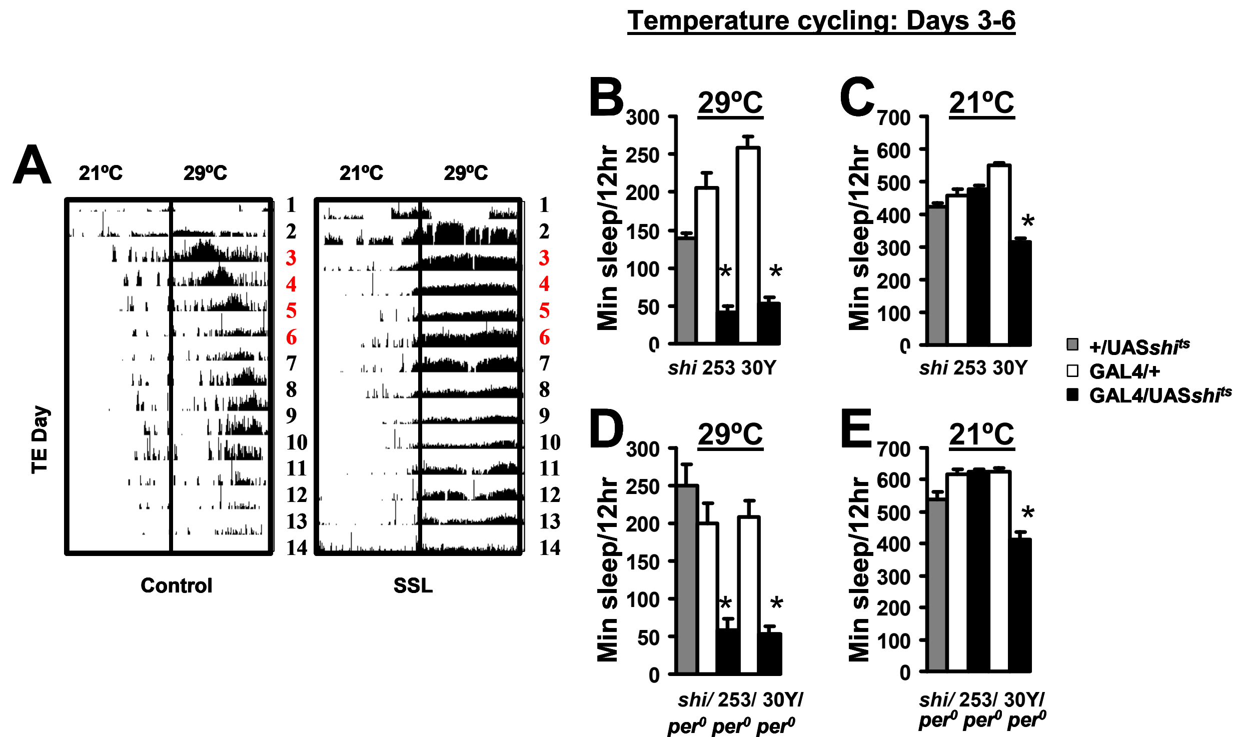


Figure 2.6 The short sleep phenotype is not due to modification of the circadian clock. (**A**) Daily activity during temperature cycling (TC) of control and short-sleep (SSL) fly. (**B,C**) Average total sleep occurring during 29°C (**B**) or 21°C (**C**) period of TC, days 3-6 (Min Sleep/12hr) in short sleep lines c253 and 30Y,and in short sleep lines c253 and 30Y in an arrhythmic *per*^{01} background (also days 3-6) (**D,E**). Asterisks (*) indicate where GAL4/UAS*shi*^{ts} phenotypes are significantly different from both GAL4/+ and +/UAS*shi*^{ts} controls (one-way ANOVA, $p<0.05$). N=36-91, N experiments=4-9 (**B,C**), N=16-42, N experiment=2-3 (**D,E**), Error bars indicate SEM.

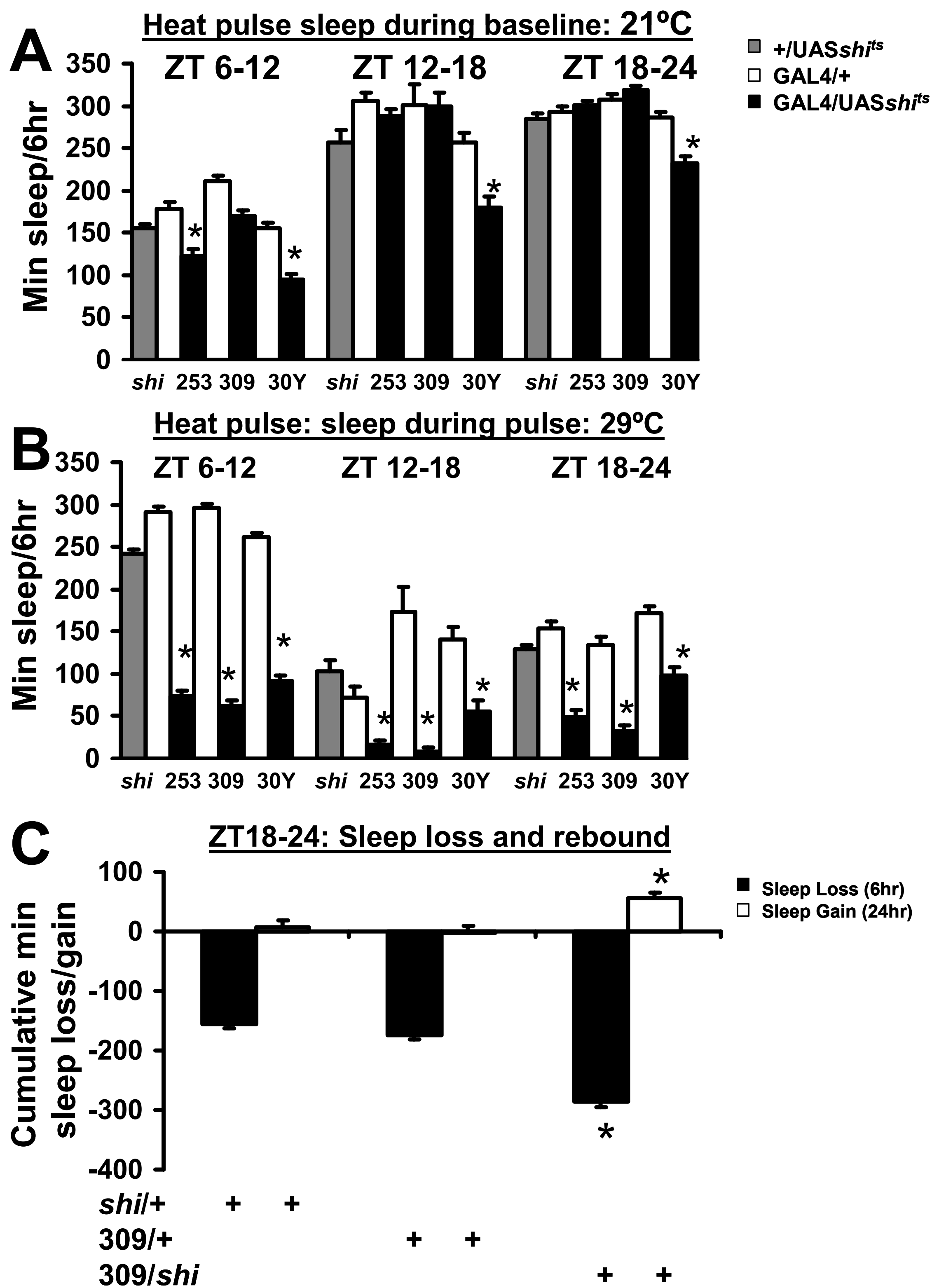

A
Heat pulse sleep during baseline: 21ºC
ZT 6-12
ZT 12-18
ZT 18-24
Min sleep/6hr
350
300
250
200
150
100
50
0
shi 253 309 30Y shi 253 309 30Y shi 253 309 30Y
+/UASshits
GAL4/+
GAL4/UASshits
B
Heat pulse: sleep during pulse: 29ºC
ZT 6-12
ZT 12-18
ZT 18-24
Min sleep/6hr
shi 253 309 30Y shi 253 309 30Y shi 253 309 30Y
C
ZT18-24: Sleep loss and rebound
Cumulative min sleep loss/gain
100
0
-100
-200
-300
-400
Sleep Loss (6hr)
Sleep Gain (24hr)
shi/+
309/+
309/shi

Figure 2.7 29°C temperature pulses elicit reduced sleep at different times of day and can induce sleep rebound. Sleep during 6hr corresponding baseline period (**A**) and sleep during 6hr heat pulse (**B**) for heat pulses administered from ZT6-12, ZT12-18, and ZT18-24, for three short sleep lines, c253, c309, and 30Y. Asterisks (*) indicate where GAL4/UASshi^{ts} phenotypes are significantly different from both GAL4/+ and +/UASshi^{ts} controls (one-way ANOVA, $p<0.05$). N=63-124, N experiments= 2-3 (ZT 6-12), N=11-44, N experiments= 2-3 (ZT 12-18) (except c309/*shi*, c309/+ - one run only), N=57-124, N experiments= 2-5 (ZT 18-24) (**A,B**). Error bars indicate SEM. (**C**) Sleep loss (minutes) during heat pulse and sleep rebound indicated in minutes for first 24 hours.

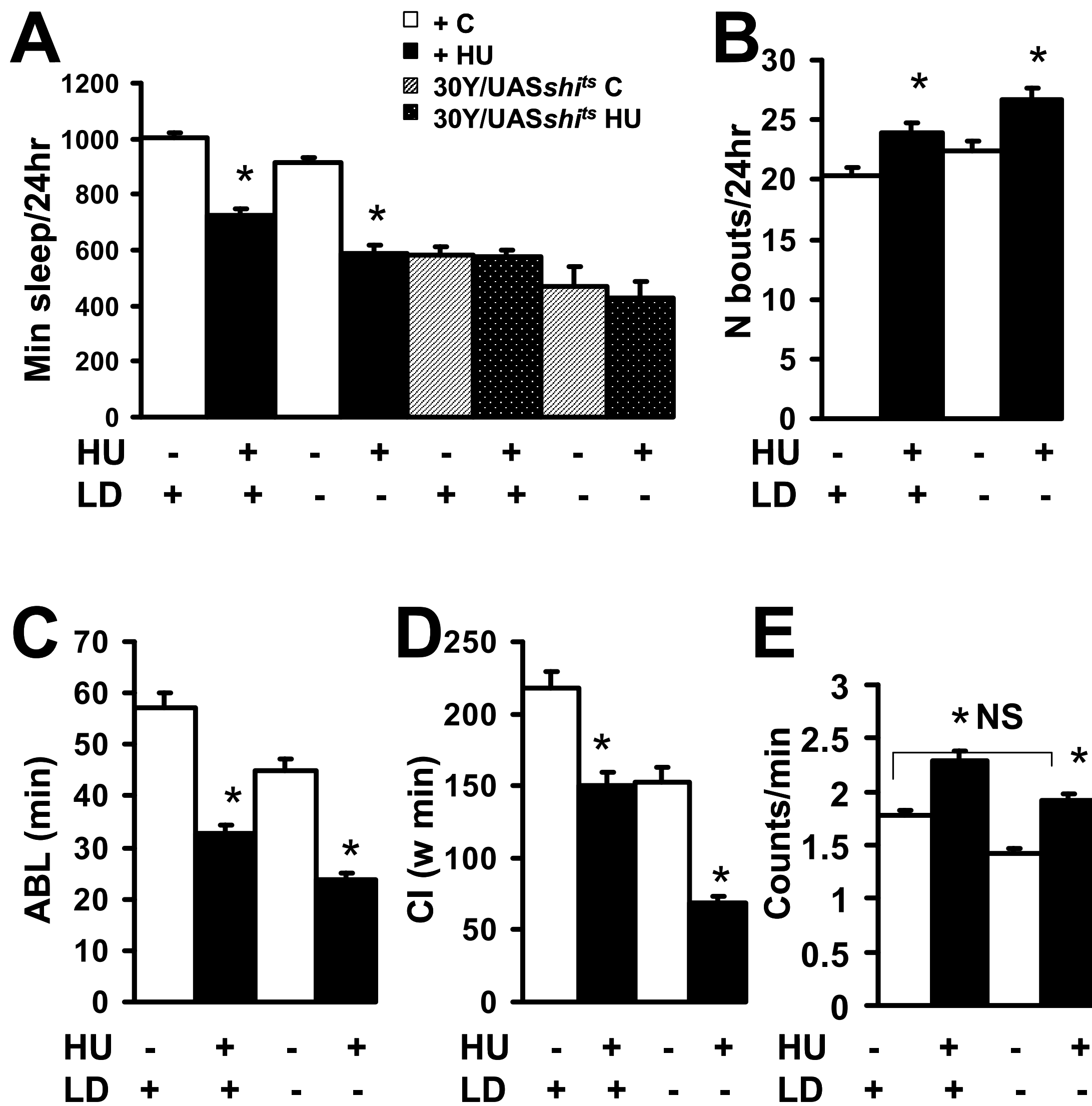


Figure 2.8 Mushroom body ablation reduces sleep. (**A**), Average total sleep in control (**C**) and hydroxyurea (HU) treated wild type (+) and 30Y/UAS*shi*^{ts} flies. (**B-D**), Consolidation – average number of sleep bouts (**B**), average sleep bout length (**C**) consolidation index (w= weighted) (**D**). (**E**), Waking activity. (**B-E**), + flies only. All flies assessed at constant 29°C in light-dark (LD) and constant darkness (DD). Asterisks (*) = significantly different C vs. HU treated comparisons (one-way ANOVA, $p<0.05$). NS = not significant. For LD, N=54-84, N experiments = 3-4. For DD, N=15-62, N experiments = 2-3. Error bars indicate SEM.

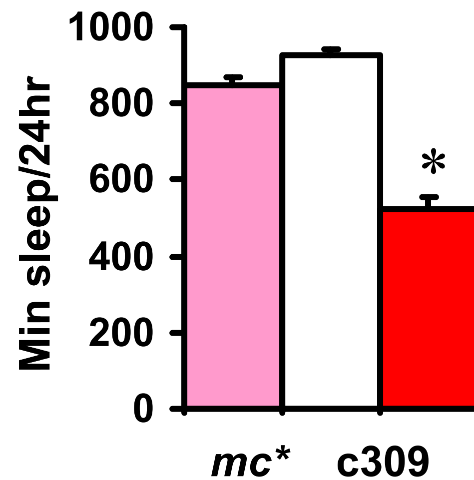


Figure 2.9 Sleep is also reduced in the MB after non-temperature dependent manipulations. Average total sleep (min) during constant 25°C conditions. Asterisk (*) = GAL4/UAS*mc** combinations significantly different from both GAL4/+ and +/UAS*mc** (one-way ANOVA, $p<0.05$). N=42-47, N experiments = 3. Error bars indicate SEM.

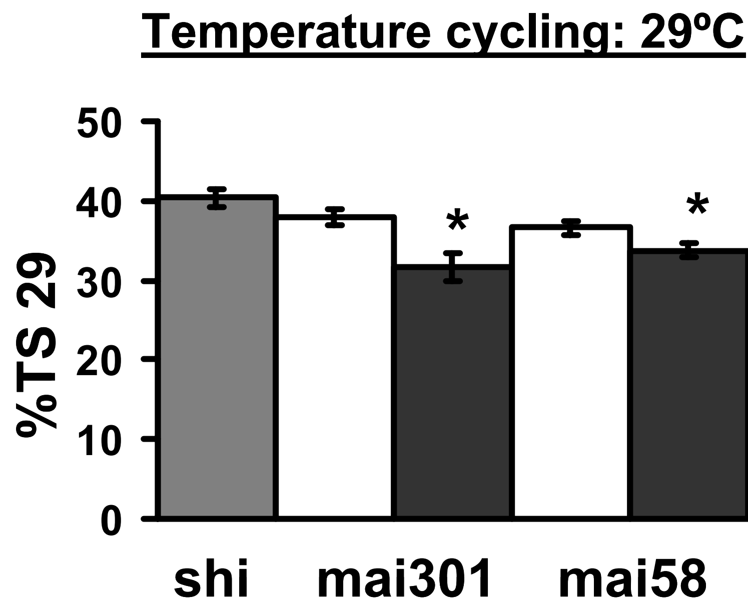
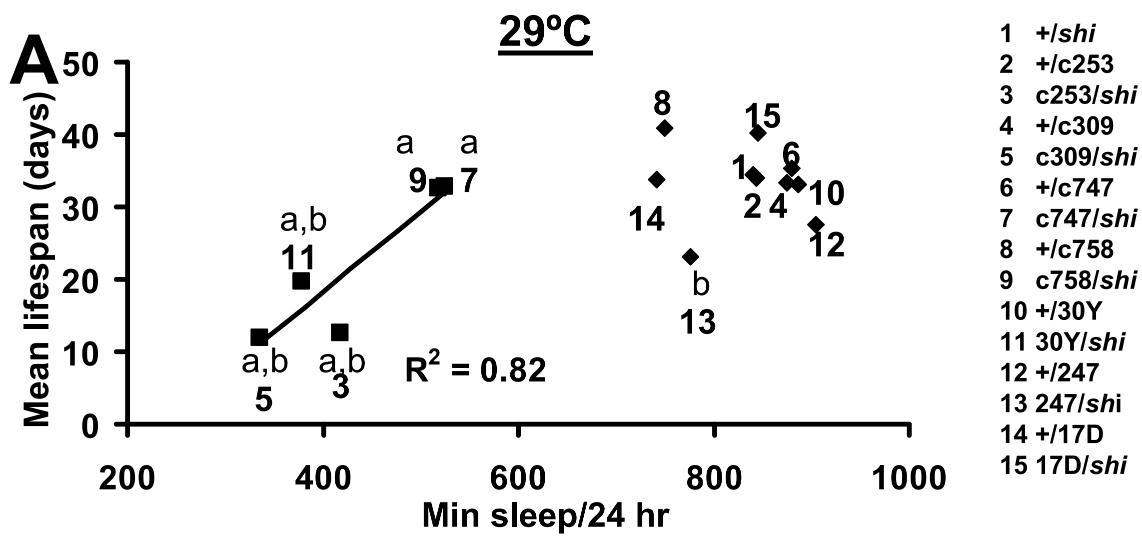
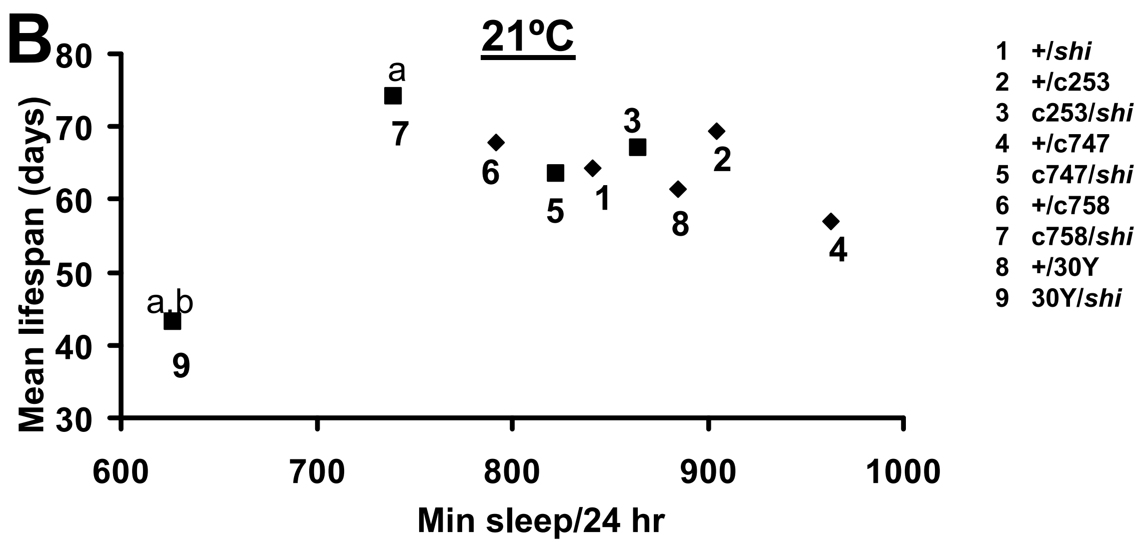
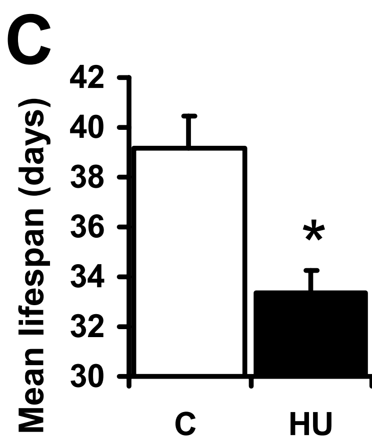


Figure 2.10 Sleep is not dramatically altered by pars intercerebralis inhibition. Average % of total sleep at 29°C during temperature cycling (TC). Asterisk (*) = GAL4/UAS*shi*^{ts} combinations significantly different from both GAL4/+ and +/UAS*shi*^{ts} (one-way ANOVA, $p<0.05$). N=17-29, N experiments = 2. Error bars indicate SEM.


A
29ºC
Mean lifespan (days)
Min sleep/24 hr
R² = 0.82
1 +/shi
2 +/c253
3 c253/shi
4 +/c309
5 c309/shi
6 +/c747
7 c747/shi
8 +/c758
9 c758/shi
10 +/30Y
11 30Y/shi
12 +/247
13 247/shi
14 +/17D
15 17D/shi


B
21ºC
Mean lifespan (days)
Min sleep/24 hr
1 +/shi
2 +/c253
3 c253/shi
4 +/c747
5 c747/shi
6 +/c758
7 c758/shi
8 +/30Y
9 30Y/shi


C
Mean lifespan (days)
C
HU
*

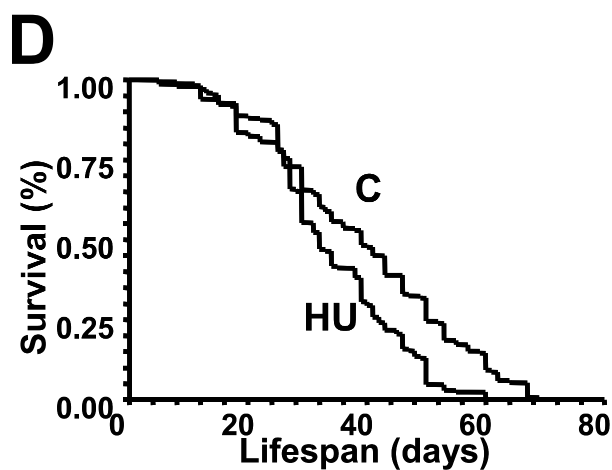

D
Survival (%)
Lifespan (days)
C
HU

Figure 2.11 Lifespan is reduced in short sleep flies. (**A,B**) Relationship between total sleep amount (Min Sleep/24hr) and lifespan (mean lifespan in days) in short sleep and non-short sleep flies maintained under constant 29°C (**A**) or 21°C (**B**) conditions. The relationship between sleep and lifespan is described by a significant linear relationship in short sleep flies at 29°C (p=0.03) (**A**). (**C**), Graph comparing mean lifespan (days) in control (C) and hydroxyurea (HU) treated wild type flies under constant 29°C conditions. Mean lifespan is significantly different between C and HU treated flies (one-way ANOVA, p<0.05). (**D**), Lifespan curves for C and HU treated wild type flies under constant 29°C conditions. Y-axis: proportion of flies surviving, X-axis: age of flies in days after eclosion. Lifespan curves for C and HU treated groups are significantly different from each other (Logrank test, p<0.05). "a" indicates where GAL4/UAS*shi*^{ts} flies sleep significantly less than both GAL4/+ and +/UAS*shi*^{ts} controls (one-way ANOVA, p<0.05), "b" indicates where lifespan in GAL4/UAS*shi*^{ts} flies is significantly less than both GAL4/+ and +/UAS*shi*^{ts} controls (one-way ANOVA, p<0.05), N=100-115 (**A**), N=97-171 (**B**), N=151 (C), 170 (HU) (**C,D**). Error bars indicate SEM.

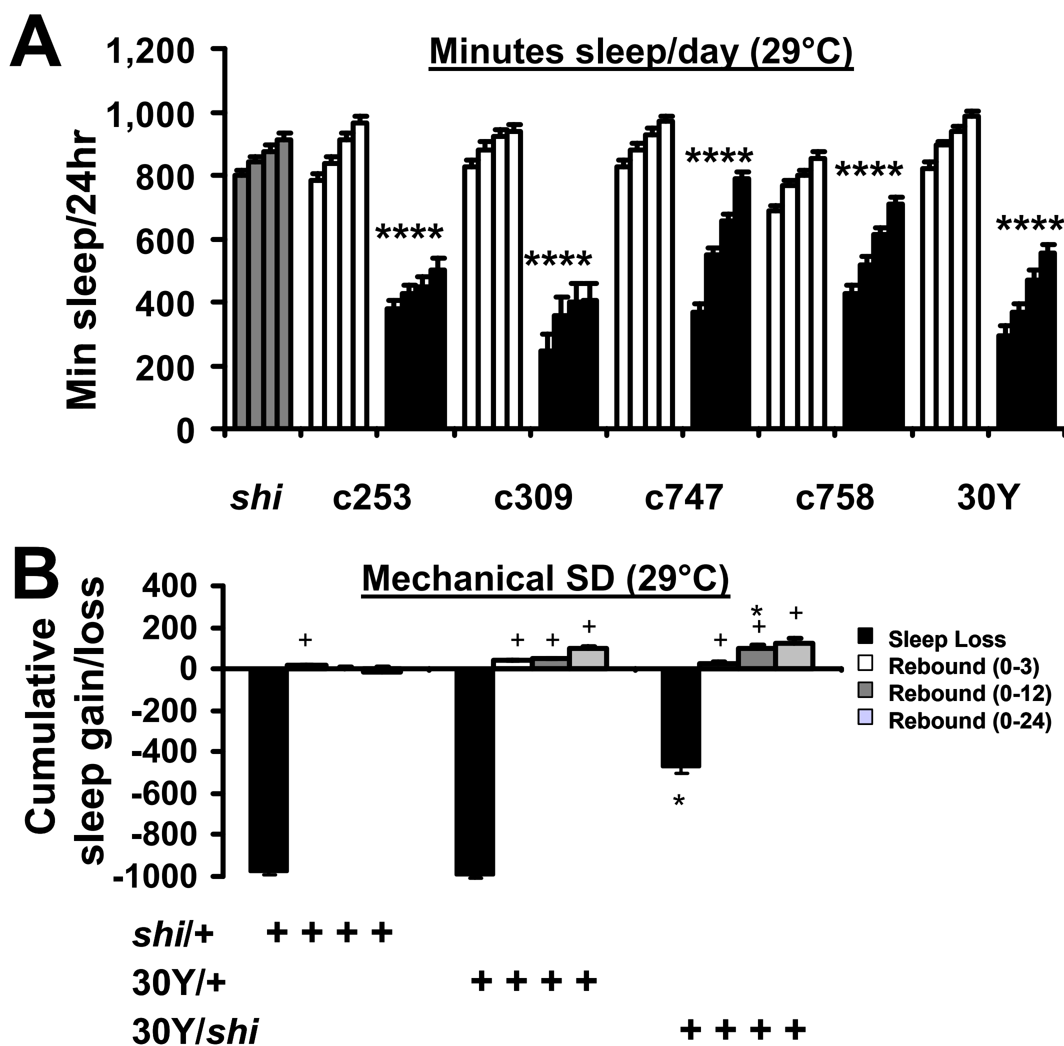


Figure 2.12 Sleep rebound after mechanically induced sleep deprivation. (**A**), Total sleep per day (Min Sleep/24hr) for four days in short sleep and control flies under constant 29°C conditions (normal sleep trend). (**B**) Sleep loss (sleep during deprivation-sleep during corresponding baseline, cumulative minutes) during, and sleep rebound (sleep during rebound-sleep during corresponding baseline, cumulative minutes) after 24hr mechanical sleep deprivation under constant 29°C LD conditions. De-trended sleep rebound indicated in cumulative minutes for three, 12, and 24 hours after the end of the sleep deprivation period. Asterisks (*) indicate where GAL4/UAS*shi*^{ts} phenotypes are significantly different from both GAL4/+ and +/UAS*shi*^{ts} controls (one-way ANOVA, p<0.05) (**A,B**). Plus sign (+) indicates where rebound sleep is significantly different from baseline sleep within a genotype (paired t-test, p<0.05) (**A**). N=24-170, N experiments = 2-9 (**A**), N=55-68, N experiments = 3-4 (**B**). Error bars indicate SEM.

Table 2.1 Table of GAL4 lines screened for sleep phenotypes (crossed to UAS*shi*^{ts1}). Lines were originally screened based on ratio of minutes of sleep at 21°C to minutes of sleep at 29°C (21/29). Expression patterns are summaries of those given on the website, www.fly-trap.org, "NA": GAL4 line on website, but with no description of expression pattern, "not on website": GAL4 line not listed on website. GAL4 Lines that were selected as short sleep GAL4 lines and were further characterized are highlighted in red.

GAL4 line:	Sleep phenotype:			Expression Pattern:	GAL4 line:	Sleep phenotype:			Expression Pattern:
	Min 29	Min 21	21/29			Min 29	Min 21	21/29	
c100	143.8	380.3	2.6	not on website	c546	276.4	589.2	2.1	AL & AGT, CC (EB, FSB - wide field neuron class?)
c199a	145.8	380.8	2.6	NA	c547	374.8	381.1	1.0	EB
c201a	269.7	517.7	1.9	NA	c549	255.5	485.2	1.9	MB, CC, AL
c205	218.3	458.0	2.1	NA	c552	152.1	288.4	1.9	NA
c207	251.3	452.5	1.8	NA	c561a	238.3	507.3	2.1	NA
c228	230.2	530.2	2.3	NA	c577	153.4	389.8	2.6	not on website
c229	251.9	456.4	1.8	NA	c578	180.2	372.0	2.1	OL
c240	288.8	576.8	2.0	NA	c584	293.6	519.7	1.8	NA
c250	223.1	494.4	2.2	NA	c587	284.3	546.8	1.9	NA
c253	68.1	507.3	7.8	NA	c596a	257.1	413.1	1.6	MB, AL, CC, tracts (giant fibre?)
c255	250.7	487.9	1.9	CC (EB), Optic Focii	c597	233.0	408.6	1.8	not on website
c263	308.1	473.6	1.5	AN, AGT, AMMC?	c601	168.5	532.5	3.2	LPC, MPC tracts
c267	132.0	468.0	3.8	NA	c613	215.2	540.2	2.5	Large HT and OL channels
c283	298.4	502.0	1.7	NA	c624	249.5	543.1	2.2	not on website
c284b	195.4	437.5	2.2	NA	c628	205.8	387.7	1.9	NA
c305a	251.6	352.9	1.4	MB (alpha, beta), CC (PB, FSB, N, EB), PI, SOG, OL	c630	355.5	583.6	1.6	NA
c316	166.4	486.3	2.9	DPM cells	c632a	135.1	508.2	4.8	OL, some central tracts, MB (KC)
c318a	187.7	485.6	2.6	NA	c635	211.1	495.2	2.3	Broad: inc. AL, MB, CC (FSB)
c320	312.1	212.3	0.7	NA	c639	275.0	516.3	1.9	Tracheole system?
c335a	102.8	274.4	2.7	NA	c651	209.8	565.5	2.7	DPM cells, AL, OL
c338	256.4	573.4	2.2	NA	c671	145.7	392.7	2.7	Tracts, avoiding main neuropil regions
c355	198.8	452.3	2.3	not on website	c689	236.6	533.4	2.3	Broad, inc. MB (alpha, beta)
c359b	115.3	413.3	3.6	NA	c690	209.4	517.0	2.5	HT
c361a	216.1	470.2	2.2	NA	c693	160.9	400.0	2.5	Tract connecting opposite LPC/lateral horns
c365a	116.3	369.0	3.3	not on website	c694	148.2	501.6	3.4	CC (EB, FSB)
c399	193.8	478.0	2.5	NA	c695	121.4	410.3	3.4	AL, AGT, optic input to LT
c401	259.7	415.0	1.6	NA	c704	153.3	415.6	2.7	MB, CC, OL
c401a	256.6	474.4	1.8	NA	c707	271.1	405.2	1.5	Broad: inc. in OL
c401b	273.9	469.4	1.7	NA	c708a	249.8	544.7	2.2	MB (alpha, beta or alpha prime, beta prime)
c420	192.7	476.1	2.5	NA	c712	172.5	481.7	2.8	not on website
c425a	225.0	451.9	2.0	NA	c726b	165.2	459.8	2.8	Cortex
c435w	238.3	488.1	2.0	not on website	c728	84.5	399.4	4.7	MB, CC, Giant Fibre tract, other tracts
c440	214.1	472.1	2.2	NA	c738	163.8	580.6	3.5	Few large cell bodies/tracts, no obvious structures
c458	80.9	459.1	5.7	not on website	c741	260.5	528.9	2.0	OL (retina?)
c463	221.1	444.2	2.0	NA	c742	195.6	406.7	2.1	not on website
c464	201.4	522.8	2.6	not on website	c743	212.5	452.2	2.1	Tracts; inc. MB extrinsic
c465	299.2	298.9	1.0	NA	c747	98.9	596.1	6.0	Broad: inc. in MB
c469	298.5	469.0	1.6	NA	c758	60.9	508.4	9.7	MB, OL, AL
c470	302.3	617.5	2.0	NA	c767	202.7	303.6	1.5	HT, and a few others
c483	209.1	307.7	1.5	NA	c782	210.9	480.9	2.3	not on website
c492	185.3	564.2	3.0	NA	c800	221.4	441.6	2.0	not on website
c500	360.6	642.8	1.8	NA	c810	193.3	417.8	2.1	MB, CC
c502a	314.8	595.3	1.9	NA	c819	302.3	512.7	1.7	CC (EB, R2 and R4 type Ring Neurons)
c502b	215.0	505.3	2.4	NA	c827	262.2	504.8	1.9	not on website
c538	343.1	558.6	1.6	not on website	189Y	225.9	509.1	2.3	MB, CC (EB), AL
c543	224.5	490.9	2.2	NA	201Y	301.1	437.2	1.5	MB (alpha, beta, gamma)

Abbreviations:

AGT	antennal glomerular tract	LPC	lateral protocerebrum
AL	antennal lobe	LT	lateral triangle
AMMC	antennal mechanosensory center	MB	mushroom body
CC	central complex	MPC	medial protocerebrum
DPM	dorsal paired medial	N	noduli
EB	ellipsoid body	OL	optic lobe
FSB	fan shaped body	PB	protocerebral bridge
HT	horizontal track	PI	pars intercerebralis
KC	kenyon cells	SOG	subesophagael ganglion

CHAPTER 3

Further Characterization of the Role of the Mushroom Bodies in Sleep

We have previously shown that the mushroom bodies (MB) regulate sleep amount (Pitman et al., 2006, Chapter 2). Inhibition of MB synaptic output by expressing a dominant-negative temperature-sensitive *shibire* (*UASshi^{ts}*) transgene (Kitamoto, 2000) caused reductions in sleep amount and sleep consolidation. Ablation of MB neuroblast cells using a cell-division inhibitor, hydroxyurea (HU) (deBelle and Heisenberg, 1994), resulted in a similar phenotype. Importantly, ablation of the MB within *shibire* expressing flies did not enhance the sleep phenotypes, suggesting that MB inhibition was responsible for the sleep reduction, not inhibition of other brain regions. Together, these results indicate a role for the MB in sleep promotion (Pitman et al., 2006).

Work from the Sehgal lab (Joiner et al., 2006) has also implicated the MB in sleep regulation. Similarly, they showed that MB ablation resulted in a reduction of sleep amount, suggesting a role in sleep promotion. However, the majority of their data suggested that the primary function of the MB was to promote wake. Previous data from their lab showed an inverse relationship between cAMP/PKA signaling and sleep amount, such that increased cAMP was correlated with a reduction in sleep. These data suggested that cAMP signaling functioned in part to either activate wake-promoting genes or inhibit sleep-promoting genes (Hendricks et al., 2001). To determine whether specific regions of the brain regulated sleep, they expressed an activated protein kinase A (PKA) catalytic subunit in various brain regions, and assayed the effect on sleep. PKA is a downstream component of the cAMP pathway, and they hypothesized

that over-expressing activated PKA (abbreviated *mc**, Li et al., 1995) would induce the expression of wake-promoting/sleep-inhibiting genes, thereby reducing sleep levels. They found that expression of *mc** in the MB using two different GAL4 drivers, 201Y, and c309, had opposite effects on sleep; with 201Y, sleep was increased, while c309 flies displayed reduced sleep. These data suggested the possibility that some cells in the MB promote sleep, while some promote wake. They then expressed *mc** in the MB using the drug-inducible GAL4 line "MB-Switch" (Mao et al., 2004) and observed reduced sleep, reduced sleep bout length, and accumulation of sleep debt. Using MB-Switch they went on to show that decreasing MB cell activity (by expressing the hyperpolarizing potassium channel *Kir*; Baines et al., 2001), or increasing MB cell activity (by expressing the depolarizing sodium channel, *NaChBac*; Nitabach et al., 2006) increased and decreased sleep amount, respectively. Together, these data suggested that MB cells defined by c309 and MB-Switch GAL4 promote wake (Joiner et al., 2006).

There are a few discrepancies between our work and that of Joiner et al. When we inhibited MB output using the MB GAL4 line c309, we observed a decrease in sleep. While the phenotype is the same as what Joiner et al. showed by over-expressing *mc** using c309, the interpretation of the data is the opposite. We interpret our data to mean that MB output from cells defined by c309 is required for sleep promotion. While this is not inconsistent with a role for PKA in wake-promotion within these cells, it differs from the interpretation that Joiner et al. provide suggesting that PKA accomplishes this reduction in sleep by increasing neuronal cellular activity. The first issue in interpreting these experiments is equating over-expression of a component of a signaling cascade with an increase in cellular activity/excitability. Joiner et al. use the observation that *mc** and *NaChBac* expression in the MB-Switch GAL4 line cause the same phenotype (reduced sleep) to suggest that *mc** expression increases cellular excitability. An

alternative model to explain the discrepancies might be that the MB-Switch GAL4 line consists mostly of wake promoting cells. The c309 GAL4 line on the other hand might contain both PKA sensitive-wake promoting cells, and *shibire* sensitive sleep promoting cells. Alternatively, the same cells may respond differently depending on time of day/behavioral state – for example, c309 cells might promote wake via PKA mechanisms during the day, and promote sleep at night. Within their data, Joiner et al. report that the 30Y GAL4 line, a line which we found to reduce sleep in combination with *shibire* at all times of day, differentially affected sleep in their experiments during the day or night in combination with *mc**. Specifically, sleep was promoted during the day, and reduced at night, suggesting that even within a particular GAL4 line, PKA expression can have opposite effects on sleep.

The discrepancies between our two data sets highlight the importance of understanding that the MB is not a homogenous set of cells. As discussed in the introduction (Chapter 1), different stages of memory formation (acquisition, consolidation, storage) can be localized to particular MB lobes (Review: Keene and Waddell, 2007). It has recently been shown that this difference might be explained in part by the segregation of olfactory inputs onto distinct dendritic regions of the mushroom body calyx, which may be mapped further onto anatomically distinct regions of the MB lobes (Lin et al., 2007). Additionally, it is critical to note that a "mushroom body" GAL4 line does not necessarily imply that this line expresses in all MB cells, or that it doesn't have significant expression outside of the MB. In fact, many "MB" GAL4 lines express in a third of MB cells or fewer, and these expression patterns may not overlap (Schwaerzel et al., 2002; M. Mader and M. Heisenberg, personal communication).

In the following set of experiments we further examined the role of the MB in sleep regulation, in an attempt to resolve some of the discrepancies between the reports on MB sleep

regulation (Joiner et al., 2006; Pitman et al., 2006). We examined sleep in short-sleeper GAL4 lines in which GAL4 function was blocked using tissue-specific GAL80 lines (Lee and Luo, 1999a). We also assayed sleep in MB lobe-specific GAL4 lines, and we discovered and partially characterized novel long-sleeper (wake-promoting) GAL4 lines. Finally, we attempted to define whether intrinsic MB activity and cAMP signaling are sleep or wake promoting by expressing different transgenes expected to excite/inhibit MB activity/ cAMP pathways. The overall conclusion of these experiments is that the MB may contain both sleep promoting and wake promoting cells, although our strongest evidence is still in support of a role for the MB in sleep promotion.

Materials and Methods

Animals. Flies were raised under a 12hr:12hr light:dark schedule at 25°C, and ~50% humidity. Stocks were provided as follows: GAL4 collection was provided by Douglas Armstrong directly or via Greg Suh, 30YGAL4 (Asaf Presente), UAS*shi*^{ts1} (T. Kitamoto), 201Y GAL4 and UAS*mc** (A. Sehgal), MB247GAL80 (M. Heisenberg), *pdf*GAL80/*cry*GAL80 (M. Rosbash), ET21/ET23/ET53/ET88 GAL80 lines (L. Luo), c739GAL4 (R. Davis), gal1471 (T. Preat), 17DGAL4/ H24GAL4/ 247GAL4 (T. Zars), c320 and c305a (S. Waddell), UAS-*Kir*/UAS-DORK C2/UAS DORK NC (M. Nitabach), UAS-PKAc/UAS-PKAm/UAS-PKAmi/UAS-PKAc;UAS-PKAmi (U. Heberlein), UAS-CREB 25.4/UAS-CREB 7.1 (R. Davis), UAS-Gαs/UAS-Gαi (M. Forte), UAS-TNT-IMP/ UAS-TNT-G/ UAS-TNT-E (T. Kitamoto). Other lines were from the Bloomington Stock Center.

Genetics. In the case where >2 transgenes were combined within the same fly (Figures 3.1-3.3, 3.5), transgene insertions were first mapped to a chromosome (if necessary) using standard balancing procedures, and were then double balanced on the opposite chromosome using the CYO/Sc;MRS/TM6B balancer stock. Appropriate double balanced lines were then crossed to each other, and balancers were selected against to obtain progeny containing both transgenes.

Sleep assays, measures of sleep and activity. See Methods section Chapter 2 for details on DAM sleep/activity monitoring system, temperature cycling and constant temperature assays. For temperature cycling, either Min Sleep during 21°C or 29°C phases are reported, or % of total sleep spent during 29°C phase is reported. Days 9-12 were typically used except where indicated (days 3-6) to allow complete circadian re-entrainment. Sleep/activity/sleep consolidation was measured for four days under constant conditions (29°C or 25°C). Daily sleep profiles were created by graphing the average %sleep/hr, averaged over four days.

Measures of sleep consolidation. See Methods section Chapter 2 for details. ABL = average sleep bout length, CI = sleep consolidation index.

Gene switch protocol. RU-486 (mifipristone, Sigma) was dissolved in 100% ethanol and diluted into agar/sucrose behavioral tube food to a final concentration of 500µM. Flies used for behavioral analysis were loaded into drug containing tubes and fed for two days prior to the start of the experiment.

Confocal imaging. See Methods section Chapter 2 for details.

Statistical analyses. See Methods section Chapter 2 for details.

Results and Discussion

Suppression of GAL4/UASshi^{ts} phenotypes using MB and circadian GAL80

We examined suppression of the *UASshi^{ts}* induced short-sleep phenotype in the short-sleep GAL4 lines 30Y and c309 using a GAL4 inhibitor, GAL80 (Lee and Luo, 1999a). We used seven different GAL80 lines; these included MB247GAL80 (MBGAL80), which suppresses GAL4 expression in cells in all five MB lobes (Krashes et al., 2007), as well as four unpublished GAL80 lines generated by enhancer trap insertion method (L. Luo, Stanford). The four unpublished lines have not been fully characterized for MB suppression. However, the insertion sites of the GAL80 elements are known, and we have estimated the relative amounts of suppression by co-expression with a broad GAL4 line, OK107 (expressed in all MB lobes, pars intercerebralis, optic lobe, and subesophageal ganglion) and UAS-GFP (Table 3.1). Among these lines, we estimate that ET53 (inserted near *headcase*), displays the most MB suppression, while the ET21 (*PNGase*), ET88 (*CG7097*), and ET23 (*EIF2*β) lines show progressively less MB suppression. We also used two GAL80 lines that suppress GAL4 expression specifically in circadian cells, *pdf*GAL80 (large and small LNvs) and *cry*GAL80 (LNvs and LNds) (Stoleru et al., 2004).

We found that short-sleep phenotypes of 30Y under temperature cycling (TC) and constant 29°C temperature (LD) (CT) were suppressed by MBGAL80 (Figure 3.1 A-E), suggesting that *UASshi*^{ts} expression within the MB is required for the observed reduction in sleep (Chapter 2). Surprisingly, the short-sleep phenotype of c309 was only partially suppressed by MBGAL80 under both TC and CT conditions (Figure 3.1 A-D, F). Specifically, MBGAL80 suppressed the short-sleep phenotype during the light phase, but not the dark phase (Figure 3.1F). The sleep phenotype during the light phase in 30Y and c309 was partially blocked by *pdf*GAL80 (30Y), and the sleep phenotype during the light phase in c309 was partially blocked by *cry*GAL80 (Stoleru et al., 2004), suggesting that normal activity of the LNvs and/or LNds may also promote sleep at this time (Figure 3.1 A-F). Since *pdf*GAL80 did not block the c309 sleep phenotype during light, this may suggest a role for the LNd specifically in sleep promotion in this GAL4 line. There is some evidence that 30Y and c309 GAL4 contain circadian cell expression (Bridget Lear, personal communication, Chapter 4), although the extent of circadian expression has not been fully quantified. Preliminary experiments in which we suppressed GAL4 with the enhancer trap GAL80 lines ET53, ET21, ET23, and ET88 produced varying effects on the short-sleep phenotypes of 30Y and c309. The short-sleep phenotypes of 30Y were mostly blocked by ET88, whereas ET21 blocked the sleep phenotype specifically during the light phase (Figure 3.2 A-C). The short sleep phenotypes of c309 were also blocked completely by ET88, and partially by ET23. All four GAL80 lines blocked the c309 sleep phenotype in the light phase, while the dark phase sleep phenotype was blocked to increasing degrees by ET53 and ET21 (Figure 3.2 A, B, D). Notably, short-sleep controls (30Y/*UASshi*^{ts} and c309/*UASshi*^{ts}) were not included in these experiments, thus it is difficult to make firm conclusions at this time.

Nonetheless, these experiments yielded interesting preliminary data, which will be repeated with appropriate controls.

Taken together, this data suggests that the short-sleep phenotypes of 30Y and c309 GAL4 are not produced by the same sub-groups of cells, since MBGAL80 blocks the 30Y phenotype in both light and dark, but only blocks the c309 phenotype in the light. Another possibility is that GAL80 blocks 30Y and c309 GAL4 with different efficiency in different cell groups. This should be verified by examining GAL80 suppression of GFP in all flies. Also, because the circadian GAL80 lines block components of the 30Y and c309 short-sleep phenotypes, this suggests involvement of the circadian system in sleep promotion (Figure 3.1). Finally, while strong conclusions cannot be made, it appears that there are additional as yet uncharacterized cells that promote sleep during the dark phase in both 30Y GAL4 (ET88), and c309 (ET88, ET23, ~ET21) (Figure 3.2). ET88 appears to only suppress ~50% of the MB cells labeled by GFP in OK107, but has the greatest effect on suppressing the short-sleep phenotype in both 30Y and c309 GAL4 lines. Also, it is important to note that the GAL80 line ET53 appears to suppress all but a small amount of α/β lobe MB expression in OK107, yet it promotes only minimal suppression of the c309 short-sleep phenotype. This suggests that a population of α/β lobe cells may be responsible for this phenotype. Together, these data suggest that the MB may contain both light and dark phase specific sleep promoting cells, and also perhaps wake promoting cells (ET88 data), which may work to regulate sleep by exciting/inhibiting each other. To determine the identity of these cells, GAL4 suppression in 30Y and c309 by MBGAL80 and the ET GAL80 lines should be fully characterized, and these experiments will need to be repeated with the appropriate controls. As a starting point, we should compare GAL4 expression patterns of c309, 30Y, and 247GAL4. We previously found that while 247GAL4 in combination with *shibire* did

not alter overall levels of sleep, sleep was reduced during the first half of the light phase (Figure 2.4). It is possible that these cells represent the same light specific sleep-promoting cells within 30Y and c309 that were blocked by MBGAL80.

c632a may Identify a MB Neuron Subset Important for Sleep Promotion

As part of our original GAL4/*UASshi*^{ts} screen, we discovered a short-sleep GAL4 line, c632a, with an interesting expression pattern including both circadian neurons, and a very small number of MB α/β lobe cells. The insertion position of this enhancer trap GAL4 line has subsequently been mapped to the newly characterized circadian gene, *clockwork orange* (*cwo*) (Lim et al., 2007). The circadian expression of c632a has been localized to at least LNv cells (Lim et al., 2007), and based on the native protein expression of CWO, expression may include all other circadian cells (Matsumoto et al., 2007). In combination with *UASshi*^{ts}, c632a flies exhibit all of the behavioral characteristics of a short-sleeper fly, including reduced sleep and sleep consolidation during constant 29°C conditions (Figure 3.3 A-D), reduced sleep during the 29°C phase of TC during days 9-12 (Figure 3.3E) and days 3-6 (Figure 3.3F), and reduced sleep in a *per*^{01} mutant background (Figure 3.3 G-H).

Given the significant amount of circadian cell expression, and the observation that circadian cells may also promote wake (Figure 3.1) we used the MBGAL80 line to suppress MB GAL4 expression in c632a. We found that MBGAL80 suppressed MB GAL4 expression, but not circadian cell expression in c632a (compare Figure 3.3.4Ai-Aiii). MBGAL80 only partially suppressed the TC 29°C phenotype (Figure 3.4B), and in fact appeared to enhance the slight decrease in sleep seen at 21°C (Figure 3.4C). During CT, MBGAL80 did not suppress the overall sleep phenotype (Figure 3.4D), but when examined more closely, blocked the short-sleep

phenotype during the light phase (Figure 3.4F), and also blocked the consolidation phenotype (Figure 3.4E). Unfortunately, it was not possible to examine the contribution of circadian cells to the short-sleep phenotype of c632a in this experiment, since the *pdf*GAL80/*UASshi^{ts}*/c632a cross resulted in lethality. The crosses were raised at 25°C, a temperature that occasionally results in developmental lethality in 632a/*UASshi^{ts}* flies, suggesting that the cross should be repeated at a lower temperature (18°C).

In general, the MBGAL80 suppression of 632a resembles MBGAL80 suppression of c309, suggesting perhaps that the set of α/β lobe cells in c632a GAL4 are responsible for a portion of the short-sleep phenotypes of both lines (specifically, some sleep promotion during the light, and sleep consolidation). Our data also suggest that circadian cell expression in this line might be involved in other aspects of the short-sleep phenotype, such as sleep during temperature cycling, and/or sleep promotion during the dark phase.

Negative Mushroom Body Lines

We next examined sleep in MB “lobe-specific” GAL4 lines as an independent method to examine the contribution of different MB cell groups to the observed *UASshi^{ts}* induced short-sleep phenotype (Chapter 2). We used two α/β lobe specific GAL4 lines, c739 (McGuire et al., 2001; Krashes et al., 2007), and 17D (Martin et al., 1998; Zars et al., 2000), two γ lobe specific GAL4 lines, H24 (Martin et al., 1998; Zars et al., 2000) and gal1471 (Isabel et al., 2004), and a line expressed in γ lobe, and a few α/β lobe cells, 201Y (Connelly et al., 1996; Martin et al., 1998; Zars et al., 2000). We were surprised to see that under TC, there was little to no effect on sleep of these more specific GAL4 lines. Both c739 and H24 significantly reduced sleep, but not to the same magnitude as other short-sleeper GAL4 lines (Figure 2.5A). We considered the

possibility that these GAL4 lines might express at a lower level than other short-sleep GAL4 lines, and therefore, not activate *UASshi*^{ts} induction at a high enough level to observe a sleep phenotype. We doubled the GAL4 dosage in two MB specific GAL4 lines, 17D and gal1471, but even doubling the GAL4 dosage did not result in a TC sleep phenotype (Figure 3.5B, x2). We also combined specific MB lobe drivers together (Figure 3.5B, αβ/αβ, γ/γ) to examine whether they consisted of two subpopulations of cells which would together express at a high enough level to produce a short-sleep phenotype, however this was not the case. Additionally, we considered that perhaps we would only observe a short-sleep phenotype if more MB lobes were represented (α/β and γ), based in part on the observation that ablation of most MB cells using hydroxyurea results in a short-sleep phenotype, and that our short-sleep GAL4 lines were broadly expressing (Figure 3.3; Figure 2.8). To address this possibility, we combined α/β and γ lobe specific GAL4 drivers together in the same fly (Figure 3.5B, αβ/γ), and examined sleep. Again, this manipulation did not result in a short-sleep phenotype. Finally, we combined α/β and γ lobe specific GAL4 drivers together with the GAL4 line 247, which was shown to have little affect on sleep, but expresses in the α/β and γ lobes, and a short-sleep GAL4 line, c253 (Figure 3.5B, αβ/αβγ, γ/αβγ). While this manipulation did not enhance the slight short-sleep phenotype when c739 was combined with 247, when the GAL4 lines 17D, c739, and gal1471 were combined with c253, the c253 short-sleep phenotype appeared to be enhanced (<10% TS 29°C compared to 12% in c253/*UASshi*^{ts} alone, Figure 2.1), although this control was not included in the run, so this cannot be said with certainty. It should also be noted that the slight short-sleep phenotype of c739 (Figure 3.5A) was suppressed when combined with other negative GAL4 lines (Figure 3.5B). These two results are difficult to interpret, but suggest that "sleep neutral" circuitry might not be neutral when placed into a different anatomical context.

Taken together, these results suggest a potential role for a population of α/β lobe cells defined by c739 GAL4 in sleep promotion, but more importantly, indicates that there are regions of the MB which do not appear to regulate sleep at all. As additional evidence for this, a number of GAL4 lines labeled as expressing in the MB our original GAL4/*UASshi*^{ts} screen (Table 2.1), and other GAL4 lines selected from GAL4 collections and/or published papers based MB expression do not reduce sleep in combination with *UASshi*^{ts} (data not shown). Again, this highlights the importance of not considering the MB or even MB lobes to be a homogeneous set of cells, and indicates that sleep-promoting MB cells defined by short-sleep GAL4 lines are likely not the same α/β or γ cells labeled by these MB lobe specific GAL4 lines. Alternatively, "sleep-neutral" GAL4 lines may contain cells that both promote sleep AND promote sleep, resulting in a net effect of zero on sleep amount.

Many Mushroom Body GAL4/UAS Combinations do not Alter Sleep Amount

As a final experiment designed to examine the nature of MB function in sleep regulation we performed a screen with a variety of different UAS transgenes expected to affect MB function, and seven different MB GAL4 lines. We chose two short-sleep GAL4 lines, 30Y and c309, one α/β lobe specific GAL4 line, 17D, one γ lobe specific GAL4 line, 201Y, one GAL4 line thought to express in all five MB lobes, but with no effect on sleep when expressing *UASshi*^{ts}, OK107 (data not shown), and one line thought to express in the α/β and γ lobes, 247, but with no overall effect on sleep when expressing *UASshi*^{ts} (slight reduction in sleep during the light phase) (Figure 2.4), and the MB-Switch line, predicted by Joiner et al. (2006) to contain wake-promoting cells. We crossed these 7 GAL4 lines to 18 different UAS lines (not all GAL4/UAS combinations are represented) (See Table 3.2 for description of UAS lines). We

chose UAS lines that would both excite and inhibit cellular function, as well as UAS lines that would both activate and inhibit cAMP signaling. Briefly, the cAMP pathway follows a cascade of events beginning with G-protein coupled receptor activation > Gαs subunit activation of adenylyl cyclase > cAMP activation > activation of protein kinase A (PKA) catalytic subunit > phosphorylation of cAMP responsive binding protein (CREB), and finally, activation of gene transcription (via the CREBa subunit) (Review: Davis, 2005).

We examined sleep under CT, 25°C (Table 3.3). To determine whether or not a particular GAL4/UAS combination affected sleep, %sleep in the GAL/UAS combination was subtracted from %sleep in the GAL4 and UAS control flies, and only GAL4/UAS combinations which resulted in a 10% or greater change in sleep compared to both controls were considered to have a sleep phenotype (red filled cells, Table 3.3 – Note: combinations close to reaching this threshold are filled pink). We were able to repeat the published decrease in sleep seen with MB-Switch/UAS-*mc**, c309/UAS-*mc**, and potentially the light/dark differences in 30Y/UAS-*mc**, although this data was compiled from only 2 surviving flies (data not shown) (Joiner et. al., 2006). Of the four GAL4 lines shown not to affect sleep amount when crossed to *UASshi*^{ts} (17D, 201Y, 247, and OK107), only OK107/UAS-Kir (increase), 17D/UAS-PKAc (increase), and 247/UAS-TNT-E (decrease) affected sleep amount, and in the case of OK107/UAS-Kir, the flies that hatched were very small, and unhealthy. In 30Y and c309 GAL4, and the MB-Switch line, the only manipulation that changed sleep amount (with the possible exception of an increase in sleep in c309/UAS-EAG) was alteration of cAMP signaling (as published, Joiner et. al, 2006). It should be noted that manipulating cAMP signaling often resulted in developmental lethality, adult lethality, and/or wing expansion phenotypes (notes to Table 3.3), indicating developmental effects of transgene expression. The changes in sleep that we observed are rather difficult to

interpret. In 30Y GAL4, overall sleep was increased in combination with UAS-*mc**, UAS-PKAc, UAS-PKAi, and UAS-PKAc;UAS-PKAi. UAS-PKAc and UAS-PKAi are predicted to have opposite effects on cAMP signaling, and these are predicted to cancel each other in the UAS-PKAc;UAS-PKAi fly (Rodan et al., 2002). To complicate matters further, in 30Y GAL4, sleep was reduced in combination with UAS-Gαs, a manipulation which would be predicted to increase sleep, based on the above data. In c309 GAL4, sleep was decreased in combination with UAS-*mc**, however was increased in combination with UAS-PKAc, UAS-PKAm, UAS-PKAmi, UAS-PKAc;UAS-PKAi, and UAS-CREB-25.4. These results are contradictory, and are difficult to explain based on predicted phenotype alone. While its possible that these transgenes behave in unpredictable ways in these tissues, which appear to be highly sensitive to cAMP signaling, it is also likely that these are the effects of experimental variability, and may fail to repeat upon further experimentation. Unfortunately, it is not possible from this data to make any strong conclusion on whether MB activity is intrinsically sleep promoting or wake promoting.

While the results of the GAL/UAS screen were rather negative in nature, they verified that GAL4 lines that do not alter sleep in combination with *UASshi*^{ts} do not generally alter sleep in combination with other UAS lines, suggesting that these GAL4 lines encompass non-sleep regulatory MB tissues. Unfortunately, the observation that many UAS lines did not affect sleep in combination with sleep regulatory GAL4 lines may be a limitation of the GAL4/UAS system itself. Thum et al. (2006) examined the effects of *UASshi*^{ts}, UAS-TNT and UAS-Kir among others on development, adult paralysis, and olfactory learning, and found that the selected transgene could have dramatically different effects on behavior depending on the time the transgene was active during development, and the properties of the targeted cells. For example, adult induced *UASshi*^{ts} expression impaired olfactory memory as published (McGuire et al.,

2001), whereas adult induced expression of UAS-TNT, a manipulation expected to produce a similar phenotype, had no effect on memory formation (Thum et al., 2006).

Long-Sleep GAL4 Lines Identify Potential Novel Sleep Circuits

As part of our GAL4/*UASshi*^{ts} sleep screen (Table 2.1) we found a few GAL4 lines that increased sleep in combination with *shibire* (long-sleeper lines), suggesting that the brain also contained wake-promoting areas. Upon retesting, many of these lines failed to repeat (data not shown), however, four GAL4 lines show promise as long-sleeper GAL4 lines (Figure 3.6 A-C), although even the data for these three lines tends to be inconsistent, possibly due to heterozygosity (c320), increased developmental and adult lethality (c320, c305a), and overall difficulty in observing an increase in sleep due to ceiling effects. While a short-sleep phenotype is more difficult to attribute to lethality, an increase in sleep might be an indirect result of sickness, and so, these data should not be over interpreted until experimental conditions are improved in which to better examine the role of these GAL4 lines in sleep.

We examined sleep during TC in the heterozygous c320 GAL4 line, sleep was increased in those flies thought to contain the GAL4, labeled "wings", since these flies also had a wing expansion phenotype (Figure 3.6A). A homozygous c320 GAL4 line (obtained from S. Waddell) was then used to examine sleep during CT, and while overall baseline sleep amount was not affected (data not shown), sleep bout length was reduced (Figure 3.6C). Progeny of this cross all exhibited a wing-expansion phenotype, suggesting that the sleep result from the single fly in Figure 3.6A (wings) represented a real effect. c596a and c547 GAL4 both increase sleep during TC, and c547 increases sleep during CT (Figure 3.6B), whereas the CT long-sleep phenotype of the c596a line is rather inconsistent (data not shown).

Of the three long-sleep GAL4 lines, two (c320 and c596a) have significant MB expression, the third is specific to the ellipsoid bodies of the central complex (c547) (Armstrong and Kaiser, 1996). c320 GAL4 expression was characterized in detail by Krashes et al. (2007), and found to contain significant α'/β' lobe expression, two lobes which we do not believe to be represented by our short-sleep GAL4 lines based on analysis of GFP expression patterns, although this remains to be fully characterized. Another potential long-sleeper GAL4 line found in our screen was c305a, which also contains MB α'/β' lobe expression (Krashes et al., 2007). This line was pursued, however it produced inconsistent data, and ultimately does not appear to increase sleep significantly above the *UASshi*^{ts}/+ control (data not shown). A verified homozygous c305a line obtained recently from S. Waddell resulted in lethality when crossed to *UASshi*^{ts}, which could explain the previous inconsistent data, for example, if our copy of this line was also heterozygous. It is a general problem with the GAL4 collection that we screened that these lines tend to lose the GAL4 insertion, which is difficult to see since the GAL4 is labeled with w+.

In addition to expressing in MB cells, c320 and c596a both express in cells of the central complex (c320: fan shaped body; c596a: fan shaped body; Armstrong and Kaiser, 1996). Since c547 GAL4 is extremely specific to the ellipsoid body, perhaps the long-sleep phenotype observed in these lines is due to inhibition of central complex (CC) function. Of note, the c739 GAL4 line has also been shown to exhibit significant ellipsoid body and fan shaped body expression (Rodan et al., 2002), which may explain its relatively minor effects on sleep, despite apparently strong α/β lobe expression. A role for the fan shaped body of the CC in visual learning has recently been shown (Liu et al., 2006), and the CC has been linked to activity regulation (Martin et al., 1999), behavioral characteristics shared with the mushroom bodies. It is

possible the MB and CC form a learning/activity/sleep anatomical circuit. These data are very preliminary, and much remains to be investigated in the role for the α'/β' lobes/central complex in sleep regulation, including examining sleep consolidation, assaying the lines under experimental conditions less likely to result in lethality (for instance, raising crosses at 18°C), and also, examining the contribution of both tissues (MB/CC) to the observed increase in sleep using GAL80.

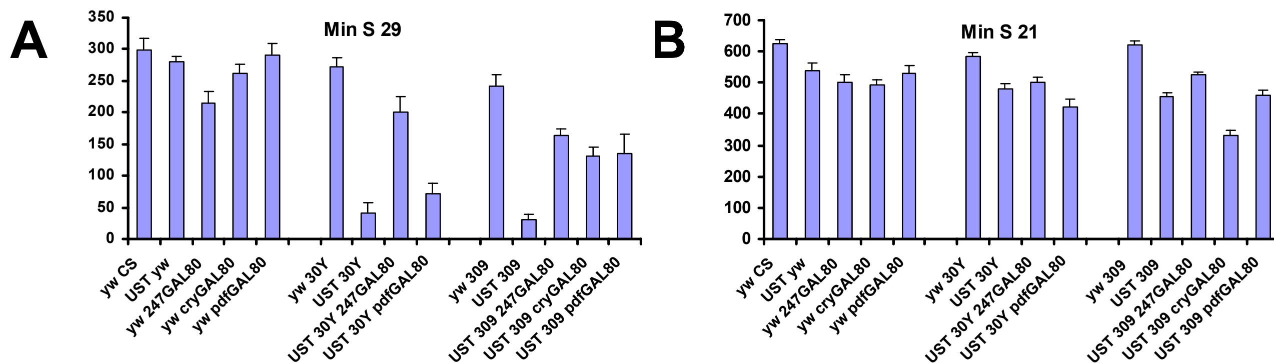
The Mushroom Bodies May Promote both Sleep and Wake

Although the results of the preceding work are preliminary, they advance the finding that the MB regulate sleep in several different ways. Results from GAL80 experiments suggest that the MB promote sleep during both the light and dark phase, and suggest that different cells might be responsible for sleep promotion at different times of day. It seems likely based on four separate pieces of data presented here (light phase suppression of 632a/c309/30Y short-sleep phenotype, lack of suppression with c309/ET53, specific expression pattern of c632a, c739/*shibire* phenotype), that at least some cells within the α/β lobes may promote sleep during the wake phase.

Additionally, for the first time, this data strongly implicates a role of the circadian system in sleep promotion. The extent of circadian cell expression in 30Y and c309 GAL4 lines needs to be quantified to determine which particular cells groups are responsible. These data also suggest that the MB may be divided into "wake-promoting" and "sleep promoting" cell groups, although a strong conclusion cannot be made without further anatomical characterization. Results from the c632a and c739 GAL4 lines, and also ET53 GAL80 suggest that the α/β lobes may contain sleep-promoting cells, and results from long-sleeper GAL4 lines suggest that the α'/β' lobes

could potentially contain wake-promoting cells. It will be important to characterize short-sleep and long-sleep GAL4 lines for lobe-specific expression using lobe-specific antibody staining (Krashes et al., 2007), since it is possible that this could explain differences seen between the 30Y and c309 GAL4 short-sleep phenotypes, and may also explain why some broadly expressing MB GAL4 lines have no apparent effect on sleep regulation. Alternatively, the data presented in this chapter also suggest a potential role of the central complex in wake-promotion, a result which could be of critical importance in determining the anatomical circuitry responsible for sleep/wake regulation.

Temperature Cycling



Constant Temperature

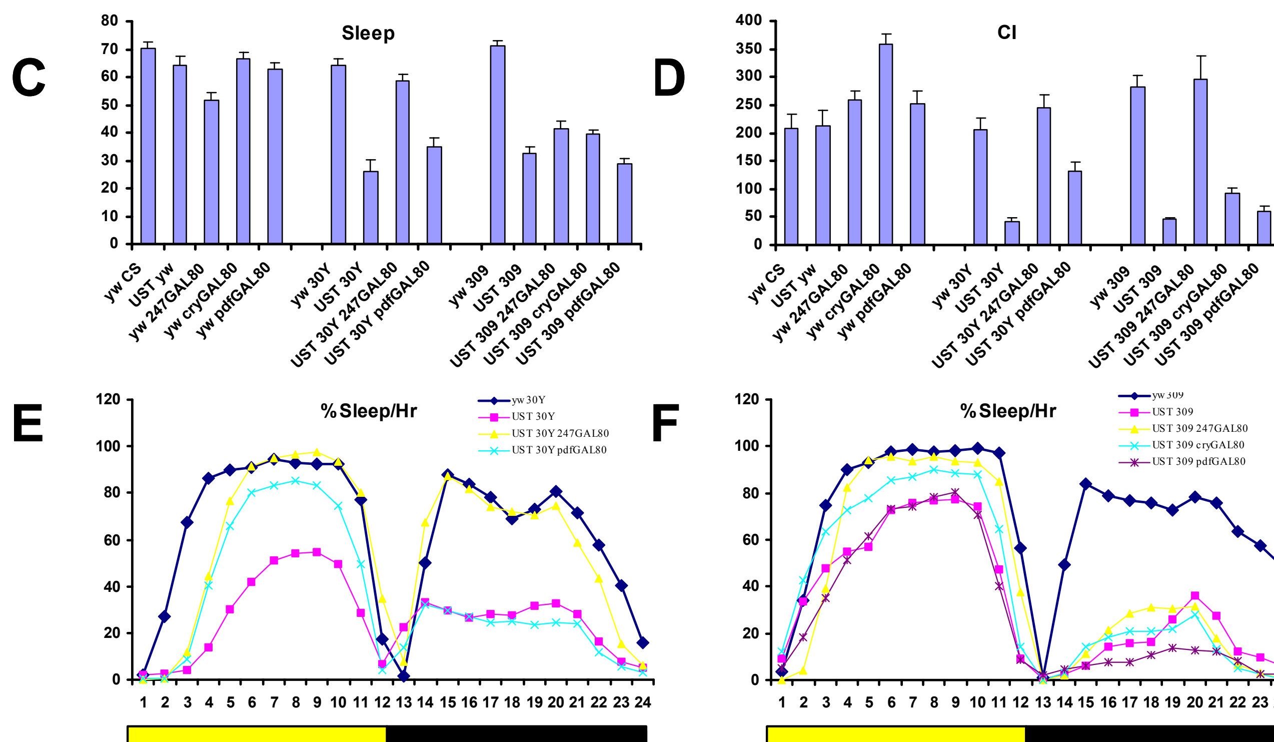


Figure 3.1 GAL80 suppression of 30Y and c309 GAL4 reveals a role for both circadian and mushroom body cells in sleep promotion. (**A,B**) Average total sleep (Min) at 29°C (**A**) and 21°C (**B**) during temperature cycling. (**C-F**) Average %Sleep/24hr (**C**) average sleep consolidation (CI) (**D**), and average %Sleep/Hr (**E,F**) during constant 29°C temperature. Yellow bar represents light phase (ZT1-12), black bar represents dark phase (CT13-24). Abbreviations: *yw* = yellow white, CS = Canton S, UST = UAS*shi*^{ts}, MB = mushroom body, *cry* = *cryptochrome*, *pdf* = *pigment dispersing factor*. The first 6 bars represent control genotypes, including wild type (*yw* CS), UAS*shi*^{ts} heterozygous outcross (UST *yw*), GAL80 heterozygous outcross (*yw* GAL80). The following bars represent data from outcrossed heterozygous GAL4 controls (*yw* GAL4), GAL4/UAS*shi*^{ts} controls (UST GAL4), and GAL4/UAS*shi*^{ts} /GAL80 suppression flies (UST GAL4 GAL80). N = 14-23, N expt = 1 (**A,B**). N = 7-26, N expt = 1 (**C-F**). Error bars indicate SEM.

Constant Temperature

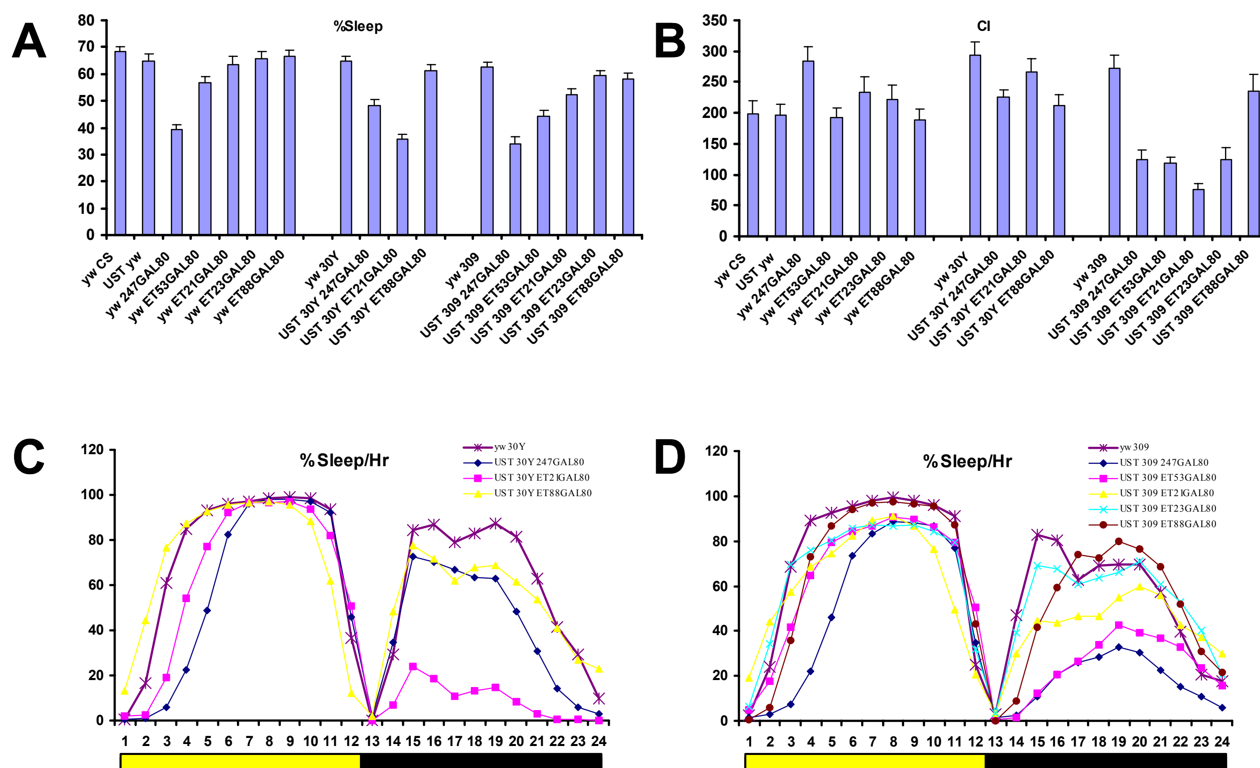
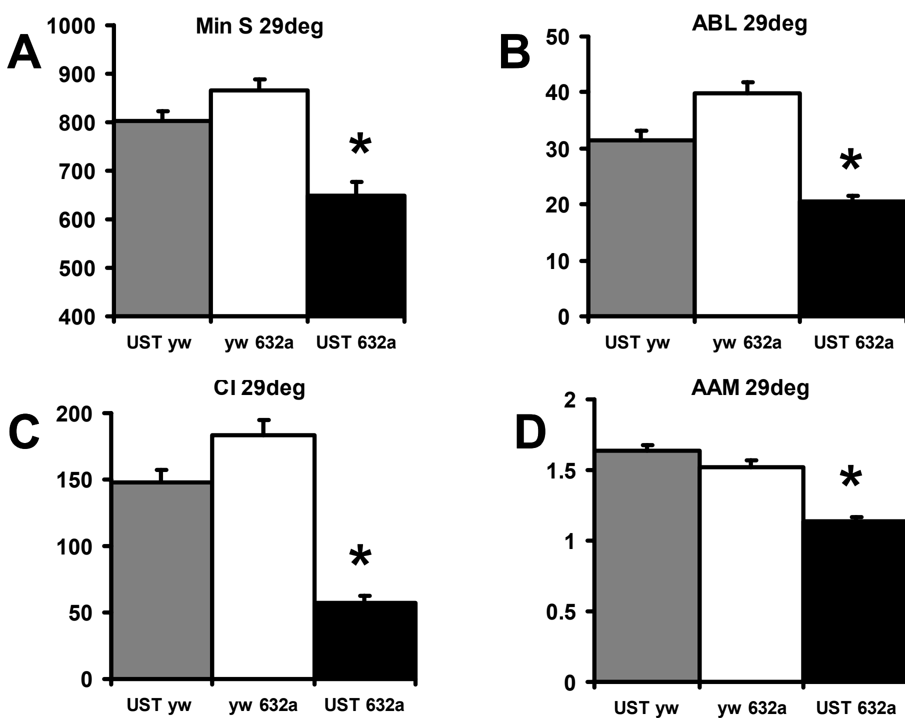


Figure 3.2 GAL80 suppression of 30Y and c309 sleep GAL4 reveals a role for uncharacterized cells in sleep promotion. (**A-D**) Average %Sleep/24hr (**A**) average sleep consolidation (CI) (**B**), and average %Sleep/Hr (**C,D**) during constant 29°C temperature. Yellow bar represents light phase (ZT1-12), black bar represents dark phase (CT13-24). Abbreviations: *yw* = yellow white, CS = Canton S, UST = UAS*shi*^{ts}, MB = mushroom body, ET = enhancer trap. The first 7 bars represent control genotypes, including a wild type control (*yw* CS), UAS*shi*^{ts} heterozygous outcross (UST *yw*), and GAL80 heterozygous outcross (*yw* GAL80). The following bars represent data from outcrossed heterozygous GAL4 controls (*yw* GAL4) and GAL4/UAS*shi*^{ts} /GAL80 suppression flies (UST GAL4 GAL80). N = 12-23, N expt = 1. Error bars indicate SEM.

Constant Temperature



Temperature Cycling

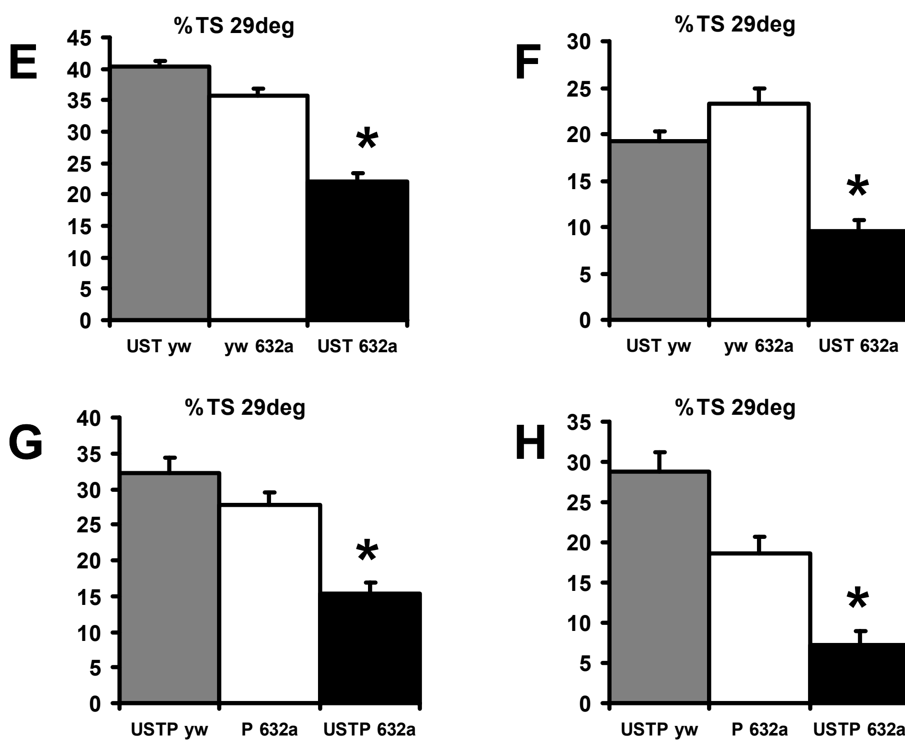
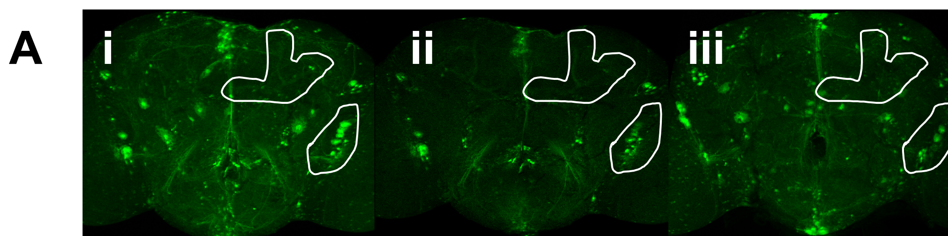
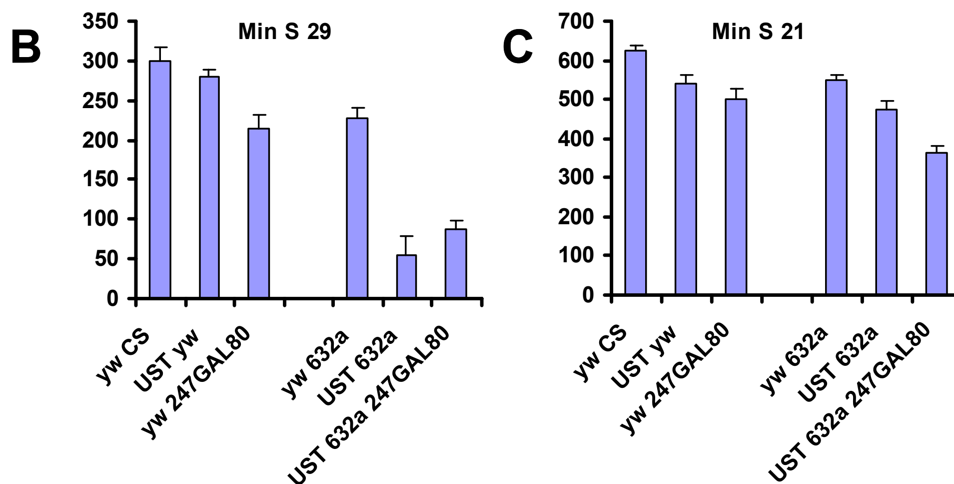


Figure 3.3 c632a GAL4 is a short-sleep GAL4. (**A-D**) Average Min Sleep/24hr (**A**), average sleep bout length (**B**), average sleep consolidation (CI) (**C**) and average waking activity/min (AAM) (**D**) during constant 29°C temperature. (**E-H**) Average % of total sleep occurring during 29°C period of TC, days 9-12 (**E**) and days 3-6 (**F**) and in an arrhythmic per^{01} background (**G**: days 9-12, **H**: days 3-6). Abbreviations: Abbreviations: *yw* = yellow white, UST = UASshi^{ts}, USTP = UASshi^{ts} ;per^{01}, P = per^{01}. Asterisks (*) indicate where GAL4/UASshi^{ts} phenotype is significantly different from both GAL4/+ and +/UASshi^{ts} controls (one-way ANOVA, $p<0.05$). N = 24-105, N experiments = 3-7 (**A,D**), N = 24-88, N experiments = 3-7 (**B,C**), N = 42-91, N experiments = 3-8 (**E,F**), N = 26-27, N experiments = 2 (**G,H**). Error bars indicate SEM.



Temperature Cycling



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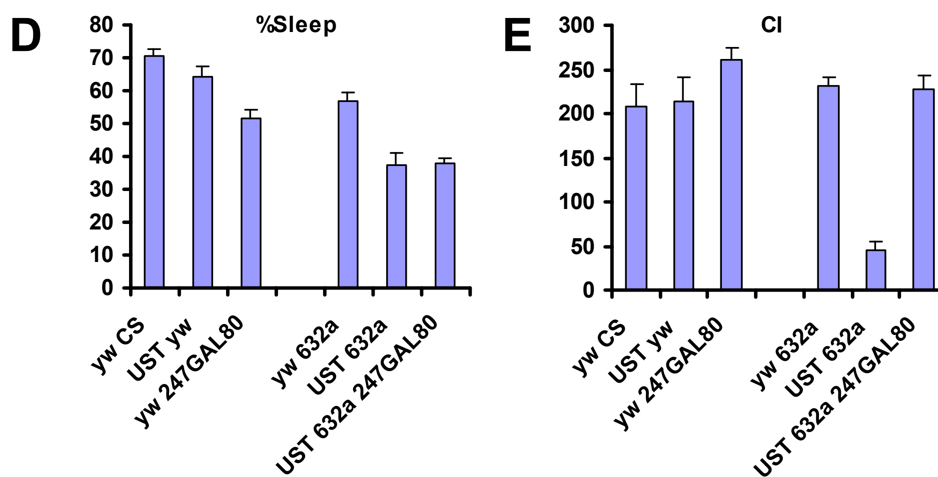


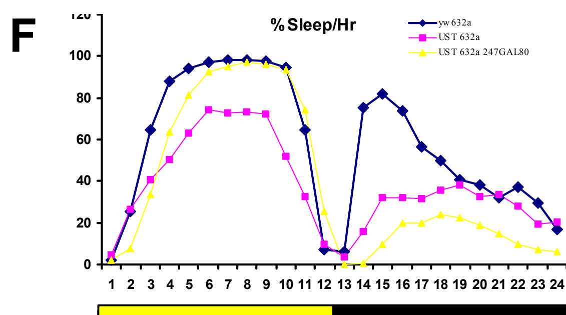


Figure 3.4 GAL80 suppression of c632a GAL4 reveals a role for a discrete sub-set of mushroom body cells in sleep promotion. (**A**) c632a/UAS-GFP, whole brain reconstruction (**i**), c632a/UAS-GFP, single slice from reconstruction in (i) highlighting mushroom body expression (**ii**), c632a/UAS-GFP, whole brain reconstruction, with MB247GAL80 blocking mushroom body expression (**iii**). Mushroom body cells and circadian lateral neurons are circled. (**B,C**) Average total sleep (Min) at 29°C (**A**) and 21°C (**B**) during temperature cycling. (**D-F**) Average %Sleep/24hr (**D**) average sleep consolidation (CI) (**E**), and average %Sleep/Hr (**F**) during constant 29°C temperature. Yellow bar represents light phase (ZT1-12), black bar represents dark phase (CT13-24). Abbreviations: *yw* = yellow white, CS = Canton S, UST = UAS*shi*^{ts}, MB = mushroom body. The first 3 bars represent control genotypes, including a wild type control (*yw* CS), UAS*shi*^{ts} heterozygous outcross (UST *yw*), and GAL80 heterozygous outcross (*yw* 247GAL80). The following bars represent data from outcrossed heterozygous GAL4 controls (*yw* 632a), GAL4/UAS*shi*^{ts} controls (UST 632a), and GAL4/UAS*shi*^{ts}/GAL80 suppression flies (UST 632a 247GAL80). N = 10-22, N expt = 1 (**B,C**). N = 9-25, N expt = 1 (**D-F**). Error bars indicate SEM.

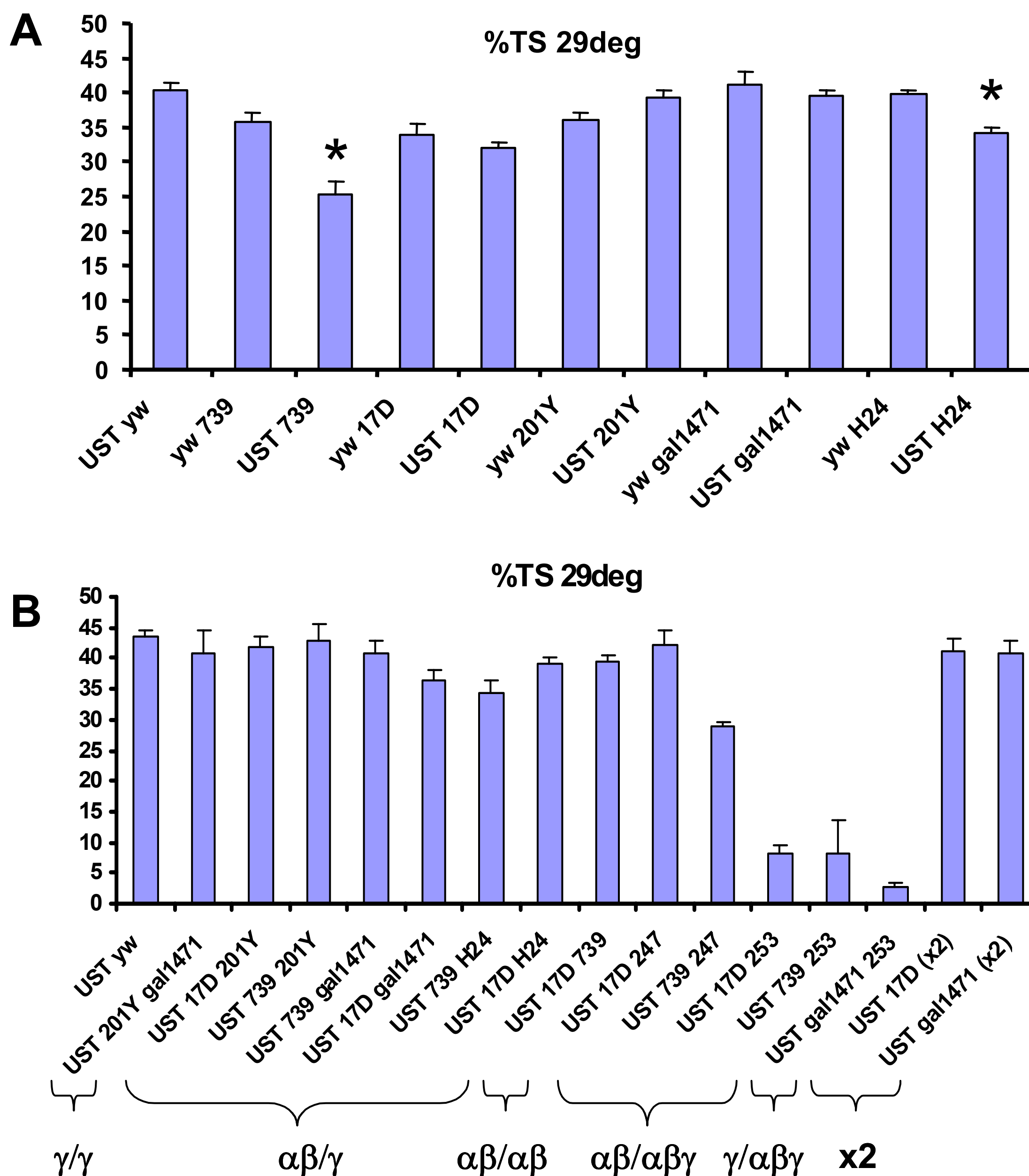
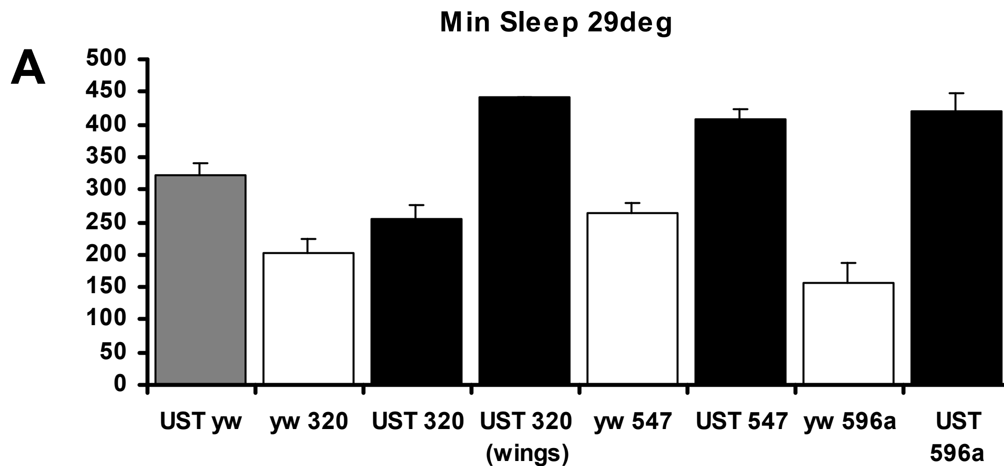


Figure 3.5 Mushroom body lobe specific GAL4 lines do not dramatically alter sleep amount. (A,B) Average % of total sleep occurring during 29°C period of TC, days 9-12. Abbreviations: *yw* = yellow white, UST = UAS*shi*^{ts}. Asterisks (*) indicate where GAL4/UAS*shi*^{ts} phenotype is significantly different from both GAL4/+ and +/UAS*shi*^{ts} controls (one-way ANOVA, p<0.05). N = 19-91, N experiments = 2-8 (**A**), N = 3-16, N experiments = 1 (**B**). Error bars indicate SEM.



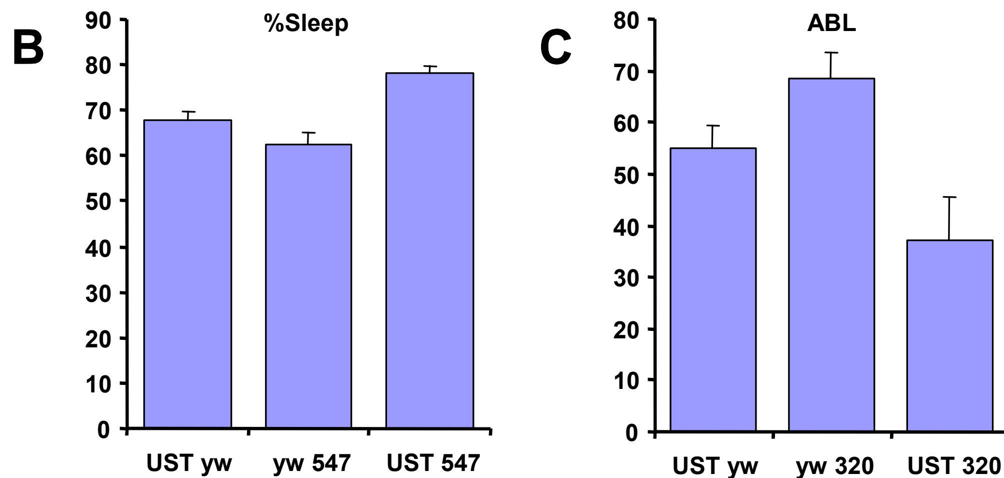


Figure 3.6 Three GAL4 lines may contain wake promoting cells. Average total sleep (Min) at 29°C (**A**) during temperature cycling. (**B,C**) Average %Sleep/24hr (**B**) average sleep bout length (Min) (**C**) during constant 29°C temperature. Abbreviations: *yw* = yellow white, UST = UAS*shi*^{ts}. Note that "wings" refers to the fact that this fly had an unexpanded wing phenotype. N = 1-19, N experiments = 1 (**A**), N = 10-16, N experiments = 1 (**B**), N = 5-16, N experiments = 1 (**C**).

Table 3.1 Estimation of GAL80 suppression of OK107/UAS-GFP by four enhancer trap GAL80 lines. GAL80 line name, genomic (gene) insertion location, and estimated % suppression is noted for the following anatomical regions: optic lobe (OL), subesophageal ganglion (SOG), pars intercerebralis (PI), and mushroom bodies (MB). Also indicated is an estimation of which regions on the MB are NOT suppressed by GAL80.

GAL80 Line	Insertion	OL	SOG	PI	MB	MB Detail (cells NOT suppressed)
ET53	headcase	100	95	0	95	alpha/beta lobe core remains?
ET21	PNGase	100	100	95	75	alpha/beta lobe, some gamma lobe?
ET88	CG7097	95	95	50	50	alpha/beta/alpha prime/beta prime/gamma
ET23	EIF2beta	100	95	90	25	alpha/beta/alpha prime/beta prime/gamma

Table 3.2 Description of UAS lines used in mushroom body GAL4/UAS screen. UAS line, description of the transgene and proposed function on cellular activity or cAMP signaling, and primary reference for the UAS line.

UAS LINE	DESCRIPTION/ FUNCTION	EXCITABILITY	/cAMP ACTIVITY	REFERENCE
UAS-na	*narrow abdomen* , sodium leak channel	increase		Lear et al., 2004
UAS-EAG	*ether a go-go* , voltage-gated potassium channel	decrease		Broughton et al., 2004
UAS-Kir	human inward rectifying potassium channel	decrease		Baines et al., 2001
UAS-DORK NC	*Drosophila* open rectifying potassium channel, non conducting	no effect		Nitabach et al., 2002
UAS-DORK C2	*Drosophila* open rectifying potassium channel, conducting	decrease		Nitabach et al., 2002
UAS-mc*	activated human PKA catalytic subunit		increase	Li et al., 1995
UAS-PKAc	*Drosophila* PKA catalytic subunit		increase	Kiger et al., 1999
UAS-PKAi	*Drosophila* PKA catalytic subunit, inactivated		decrease	Li et al., 1995
UAS-PKAmi	*Drosophila* PKA catalytic subunit, inert		no effect	Kiger and O'Shea, 2001
UAS-PKAi;UAS-PKAc	*Drosophila* PKA catalytic/ inactivated catalytic subunit		no effect	Rodan et al., 2002
UAS-CREB 25.4	*Drosophila* CREB2a transcriptional activator subunit		increase	Perazonna et al., 2004
UAS-CREB 7.1	*Drosophila* CREB2a transcriptional activator subunit		increase	Perazonna et al., 2004
UAS-CBZ	dominant negative dCREB2a		decrease	Eresh et al., 1997
UAS-gas	G-protein coupled receptor adenylyl cyclase activating subunit		increase	M Forte
UAS-gai	G-protein coupled receptor adenylyl cyclase inhibiting subunit		decrease	M Forte
UAS-TNT-IMP	tetanus toxin light chain - inactivated	no effect		Sweeney et al., 1995
UAS-TNT-G	tetanus toxin light chain - cleaves synaptobrevin (inhibit synaptic transmission)	decrease		Sweeney et al., 1995
UAS-TNT-E	tetanus toxin light chain - cleaves synaptobrevin (inhibit synaptic transmission)	decrease		Sweeney et al., 1995

Table 3.3 Results of a mushroom body GAL4/UAS screen designed to examine the effect of different transgenes on sleep amount. NR = number of runs (experiments), AVE = average %sleep (4 days), SE = standard error. Average %sleep and SE were averaged together where >1 run is indicated. MB-S(+) indicates MB-Switch activated by RU-486, MB-S(-) indicates MB-Switch without RU-486 added (non-activated control) – See Methods for details. Refer to Table 2 for descriptions of UAS transgenes and text for description of MB GAL4 lines. Note that some crosses resulted in complete developmental lethality (LETHAL), adult lethality during experiment (DEAD), or male specific developmental lethality (no m). X indicates that this cross was not done. See Table notes for explanation of text colors. Red filled cells indicates that %sleep in the GAL4/UAS combination was >10% greater/less than both the UAS and GAL4 controls. Pink filled cells indicates that %sleep in one of the controls was >10% greater/less than the GAL4/UAS combination, and the other control was >8% different. In the case of MB-Switch, induced (+) is subtracted from the non-induced (-) control.

	yw			GAL4	17D			201Y			30y			c309			247			OK107			MB-S (+)			MB-S (-)		
%SLEEP LD	NR	AVE	SE		NR	AVE	SE	NR	AVE	SE	NR	AVE	SE	NR	AVE	SE	NR	AVE	SE	NR	AVE	SE	NR	AVE	SE	NR	AVE	SE
yw	x	x	x		5	62.5	2.2	3	65.8	2.5	6	54.2	2.7	6	61.3	2.5	3	47.9	2.9	2	72.7	2.0	x	x	x	x	x	x
UAS TRANSGENE																												
UAS-na	4	55.4	2.8		1	67.4	1.9	1	64.2	2.6	1	52.5	3.2		LETHAL		1	58.2	2.3	1	73.4	2.2	x	x	x	x	x	x
UAS-EAG	3	52.5	3.1		1	64.8	3.0	1	70.0	2.6	1	55.6	3.8	1	68.8	2.9	1	49.4	9.0	1	69.9	2.8	x	x	x	x	x	x
UAS-Kir	2	54.6	3.2		1	57.2	5.2	x	x	x		LETHAL			LETHAL			LETHAL		1	89.4	2.7	1	64.8	1.5	1	60.6	3.5
UAS-DORK NC	2	55.8	2.0		1	58.1	2.5	x	x	x	x	x	x	2	61.9	2.1	x	x	x		x	x	1	63.2	1.9	1	69.0	2.1
UAS-DORK C2	2	60.5	2.7		1	56.4	2.0	x	x	x	1	50.2	2.7	2	69.0	3.2	x	x	x	1	71.6	1.8	1	61.8	2.1	1	57.6	1.8
UAS-mc*	4	56.8	2.4		3	57.8	3.1	3	62.1	4.2	1	64.9	5.6	3	30.7	4.5	1	45.9	1.8	x	x	x	1	42.8	2.7	1	66.7	2.0
UAS-PKAc	1	60.1	3.0		1	74.4	1.9	x	x	x	2	69.4	2.3	1	71.1	1.6	1	58.1	2.7	x	x	x	x	x	x	x	x	x
UAS-PKAi	1	58.8	3.4		1	56.8	4.0	x	x	x	2	72.4	1.5	1	81.5	1.3	1	53.0	3.4	x	x	x	x	x	x	x	x	x
UAS-PKAmi	1	64.2	1.5		1	66.8	2.5	x	x	x	2	63.9	1.5	1	75.5	1.0	1	57.2	2.8	x	x	x	x	x	x	x	x	x
UAS-PKAi;UAS-PKAc	1	63.0	3.2		1	64.9	2.2	x	x	x	2	72.6	1.8	1	71.8	1.5	1	55.1	3.8	x	x	x	x	x	x	x	x	x
UAS-CREB 25.4	2	61.9	2.1		2	69.6	2.8	x	x	x	2	59.2	3.5	1	74.8	1.9	x	x	x	x	x	x	x	x	x	x	x	x
UAS-CREB 7.1	2	65.7	2.7		1	70.1	2.1	x	x	x	2	no m	x	2	66.1	2.7	x	x	x	x	x	x	x	x	x	x	x	x
UAS-CBZ	3	58.3	2.5		1	59.3	3.2	1	69.9	1.8	1	57.6	1.0	1	64.0	1.3	1	56.2	2.0	x	x	x	x	x	x	x	x	x
UAS-gαs	3	57.1	2.3		x	x	x	1	56.3	3.3	1	44.7	2.2	1	60.0	2.2	1	56.4	2.1	1	65.5	1.7	x	x	x	x	x	x
UAS-gαi	3	62.8	2.0		x	x	x	x	x	x	1	62.6	2.0	1	58.3	2.0	1	58.7	2.2	1	77.7	0.6	x	x	x	x	x	x
UAS-TNT-IMP	4	67.4	2.7		x	x	x	1	66.0	1.6	1	49.6	3.1	1	65.6	4.8	x	x	x	2	71.1	2.0	1	69.4	2.2	1	64.5	2.8
UAS-TNT-G	3	60.4	2.0		1	67.0	2.9	1	64.2	1.8		LETHAL		1	64.2	1.4	1	46.2	3.1	2	76.7	2.3	1	70.8	4.4	1	72.4	2.0
UAS-TNT-E	4	63.5	3.1		1	64.9	2.0	3	61.9	3.8		LETHAL			LETHAL		1	39.3	3.6	2	DEAD	x	1	74.5	1.2	1	66.3	2.4

NOTES:
mini flies
most flies were developmental lethal
wing expansion phenotype

CHAPTER 4

Clk Promotes Consolidated Sleep in Circadian and/or Mushroom Body Neurons

If the function of sleep is to be elucidated, the genes and anatomical regions involved in its regulation must first be determined. Sleep is regulated homeostatically, and is also regulated by a daily circadian rhythm, which dictates the proper timing of sleep, and consolidates sleep into a continuous bout. One attractive hypothesis for how these processes interact is that molecular machinery within a cell or within an interconnected neural circuit may be capable of both sensing sleep need (homeostatic sleep drive) and regulating sleep timing. We have chosen to examine sleep in the *Drosophila Clock (Clk)* mutant as a means to examine this hypothesis.

Clock is a bHLH transcriptional activator central to the molecular feedback loop required for generation of circadian rhythms in flies (Allada et al., 1998) and mice (King et al., 1997). Additionally, mutations in *Clock* result in a reduction in total sleep and sleep bout length in flies (Hendricks et al., 2003a) and mice (Naylor et al., 2000), and a disrupted homeostatic response to sleep deprivation in mice (Naylor et al., 2000) and flies (Shaw et al., 2002; Hendricks et al., 2003a). It is possible that the effect of *Clock* on sleep might be via expression in non-pacemaker tissues, since *Clock* is expressed widely throughout the body and brain of mice (King et al., 1997) and flies (Houl et al., 2006). In flies, *Clk* expression includes the mushroom bodies, a non-oscillator tissue recently shown to regulate sleep (Pitman et al., 2006; Joiner et al., 2006, Chapter 3, this thesis). In agreement with its role as a transcription factor, and the observation that *Clock* is broadly expressed, mutations in *Clock* result in the alteration of transcript levels of ~270 non-cycling genes (McDonald and Rosbash, 2001). Transcripts increased in a *Clock* mutant

background include those involved in the immune response, which were also shown to up-regulated by wake in the fly (McDonald and Rosbash, 2001; Cirelli et al., 2004). Levels of cycling transcripts are also affected by mutations in *Clock* (McDonald and Rosbash, 2001; Ceriani et al., 2002). Of these, it is interesting to note that genes involved in cellular detoxification are generally down regulated in *Clock* mutants (Ceriani et al., 2002), but are up-regulated by wake in the fly (Cirelli et al., 2004). These data suggest that *Clock* may function within, and/or outside of the pacemaker to regulate transcription of many genes, including those shown to vary by sleep/wake state.

Other circadian genes have been shown to affect sleep amount and homeostasis in both mice and flies, although none to the extent of *Clock*. Mutations in *Drosophila cycle* or its mouse ortholog BMAL1 disrupt sleep amount, sleep fragmentation, and sleep rebound (Shaw et al., 2002; Hendricks et al., 2003a; Laposky et al., 2005). Mutations in *Drosophila period* and *timeless,* and their mouse orthologs *period* and *cryptochrome* affect sleep/consolidation/rebound, although much more subtly than either *Clock* or *cycle* (Kopp et al., 2002; Shaw et al., 2002; Wisor et al., 2002; Hendricks et al, 2003a; Shiromani et al., 2004). In humans, it was recently shown that a polymorphism in the *period3* gene affects sleep, but not circadian rhythms (Viola et al., 2007). Subjects homozygous for the *period3*^{5} allele exhibit increased slow wave sleep, increased slow wave activity during NREM sleep, and reduced cognitive impairment following sleep deprivation, but no alterations in circadian rhythms (Viola et al., 2007). Together, these data suggest a role of circadian genes in sleep regulation, beyond simply imparting sleep timing information.

In addition to the proposed genetic link between sleep and circadian rhythms, there is anatomical evidence linking sleep and rhythms in mammals. Lesion experiments in mice (Easton

et al., 2004) and monkeys (Edgar et al., 1993) suggest that the circadian pacemaker of the mammalian brain, the suprachiasmatic nucleus (SCN) may be actively involved in wake-promotion, since SCN lesions result in increased sleep amount. Although the SCN does not synapse directly onto sleep regulatory regions, SCN activity could promote wake through either multi-synaptic pathways (Review: Fuller et al., 2006), or via release of an as yet undiscovered "alertness factor". Physiological data suggests that sleep directly influences SCN activity, since, superimposed onto spontaneous SCN activity is a separate electrical firing pattern that is correlated with sleep/wake state. SCN neurons fire rapidly during waking, REM sleep, and NREM sleep deprivation, and less during NREM sleep and REM sleep deprivation (Deboer et al., 2003). Taken together, these data suggest that changes in SCN firing activity are regulated by sleep/wake state, and that the SCN may respond to homeostatic sleep need.

The role of circadian pacemaker neurons in sleep regulation in the fly has not been described, however preliminary data from this thesis (Figure 3.1 and Figure 3.4) suggests that circadian neurons might promote sleep in *Drosophila*. While the role of circadian neurons in sleep has not been fully investigated, the contribution of different classes of circadian neurons to circadian behavior has now been well described. Briefly, circadian locomotor behavior under light-dark entrained conditions can be grouped into "morning" and "evening" behavior, where flies anticipate both the Dark>Light transition (morning) and Light>Dark transition (evening). The circadian system in *Drosophila* is a collection of six main cell groups, totaling about 150 neurons in number (Figure 1.4). Circadian neurons in the fly are defined by whether on not they express the PER protein (Ewer et al., 1992), and are named by their positions in the brain. There are three groups of lateral neurons, which line the border between the optic lobes and central brain, the ventral lateral (small and large – sLNv, lLNv), and dorsal lateral (LNd) neurons. The

sLNv and lLNv groups are also defined by their expression of the circadian output neuropeptide, pigment dispersing factor (PDF). There are also three groups of dorsal neurons, within the dorsal protocerebrum of the brain, these are named dorsal neuron groups (DN1 – divided into anterior and posterior subsets, DN2, DN3). An seventh group consists of a single neuron that is considered an LNv, but does not express the PDF neuropeptide. The sLNv appear to drive morning behaviors, while the LNd, some DN1 cells, and the PDF(-) LNv are believed to drive evening behaviors (Grima et al., 2004; Stoleru et al., 2004).

In the following experiments, we have further examined the role of *Clk* in *Drosophila* sleep. Published data on *Clk* in *Drosophila* sleep (Shaw et al., 2002; Hendricks et al., 2003a) reported data on only one allele of *Clk*, the semi-dominant Clk^{Jrk} allele (Allada et al., 1998). Similarly to mice, Clk^{Jrk} mutants exhibit reduced sleep amount and sleep consolidation (Hendricks et al., 2003a), and exhibit an exaggerated sleep rebound following sleep deprivation (Shaw et al., 2002). The role of *Clk* in regulating sleep in the adult was examined by over-expressing the Clk^{Jrk} mutation under the control of a heat-shock promoter (Hendricks et al., 2003a). The results of these experiments were questionable, given the low number of flies used in these experiments, but suggest that adult expression of hs-Clk^{Jrk} results in reduced sleep amount (Hendricks et al., 2003a). Since three additional *Drosophila Clock* alleles have now been described, and given the arguable nature of some of the existing data, we decided to further examine the role of *Clock* in sleep in *Drosophila*. We were also interested in whether genetic background might contribute to the Clk^{Jrk} sleep phenotype, since genetic background has been found to strongly influence other mutant phenotypes, including the severity of anatomical defects in mutations that disrupt mushroom body development (deBelle and Heisenberg, 1996), and mutations of genes shown to influence sleep amount (*shaker*, Cirelli et al., 2005a). Additionally,

we have examined the role of circadian pacemaker neurons in sleep regulation. The results of these experiments suggest that mutations in *Clk* reduce sleep amount in LD and DD, but that this phenotype may be modified by genetic background, particularly in DD. We show that *Clk* mutations robustly reduce consolidated sleep, and while this phenotype is also modified by genetic background, it can be rescued using a GAL4 driver expressing in both circadian neurons, and sleep-promoting mushroom body neurons. Since control flies do not exhibit increased consolidation themselves we do not think this is a result of genetic background, however this remains to be fully characterized. Finally, we have found that circadian neurons, perhaps the sLNv and lLNv in particular, may promote sleep. Together, these data suggest that *Clk* may promote consolidated sleep via a circuit comprising circadian pacemaker and mushroom body neurons.

Materials and Methods

Animals. Flies were raised under a 12hr:12hr light:dark (LD) schedule at either 18°C or 25°C, and ~50% humidity. Stocks were provided as follows: c309GAL4 was provided by Douglas Armstrong via Greg Suh, 30YGAL4 (Asaf Presente), UASshi^{ts1} (T. Kitamoto), Clk^{Jrk}, Clk^{ar}, *pdf*GAL4, *cry16*GAL4, *tim*GAL4, *cry13*GAL4 (M. Rosbash). Other lines were from the Bloomington Stock Center.

Clk^{p} Allele Cleanup. Clk^{p} mutants from the Bloomington Stock Center contain an unrelated mutation in the background resulting in homozygous lethality. To remove the lethal mutation we crossed balanced Clk^{p} flies to *yw*, and allowed recombination to occur in heterozygous female

progeny, and crossed these to 3rd chromosome double balanced males. We then used the male progeny of this cross to set up 61 single male lines, keeping only those that were homozygous viable (1/61 lines).

Genetics. In the case where >2 transgenes were combined within the same fly, transgene insertions were first mapped to a chromosome (if necessary) using standard balancing procedures, and were then double balanced on the opposite chromosome using the CYO/Sc;MRS/TM6B balancer stock. Appropriate double balanced lines were then crossed to each other, and balancers were selected against to obtain progeny containing both transgenes. Standard recombination schemes were used in the case where mutations/transgenes were combined on the same chromosome.

Sleep assays, measures of sleep and activity. See Methods section Chapter 2 for details on DAM sleep/activity monitoring system, temperature cycling and constant temperature assays. Sleep/activity/sleep consolidation in light:dark was measured for four days, and in constant darkness for either 4 or 7 days, at 29°C or 25°C, as noted. Daily sleep profiles were created by graphing the average %sleep/hr, averaged over four days.

Circadian behavioral analyses. Locomotor activity of individual male flies was measured using Drosophila Activity Monitors (Trikinetics). Monitoring conditions included LD cycles for 5 days, followed by DD cycles for 7 days. Data were analyzed using ClockLab analysis software (Actimetrics) with the significance level of the $\chi 2$ periodogram set to $\alpha = 0.01$.

Flies with a $\chi2$ statistic ≥10 over the significance line were scored as rhythmic, commonly referred to as "Power-Significance", abbreviated "P-S" in text and on figures. Average period (tau), P-S, and %Rhythmic (%R) are reported for individual genotypes. Normalized activity plots (eductions) for LD were generated by normalizing the average activity of each individual fly to 1, and averaging genotype data. Flies with little or no activity over the final day of the analysis, or throughout the entire analysis, were considered potentially sick and removed.

Measures of sleep consolidation. See Methods section Chapter 2 for details. ABL = average sleep bout length, CI = sleep consolidation index, weighted average sleep bout length.

Genetic Background Analysis. *Clk*^{Jrk} homozygous males were crossed to isogenic *w*^{1118} virgin females (referred to as Bloomington stock number 5905, obtained from DrosDel Collection, Ryder et al., 2004). *Clk*^{Jrk} /+ virgins and *Clk*^{Jrk} /+ males were crossed to each other, and the sibling progeny from this cross were tested behaviorally in a 5LD 7DD experiment, at 25°C. Genotyping for the relevant transgenes was performed as follows: at the end of the behavioral experiment, each fly was frozen on dry ice and homogenized in 10mM Tris-HCl, 1mM EDTA, 25mM NaCl, 200g/ml of Proteinase K to extract DNA. Individual PCR reactions were then performed on each DNA sample to amplify the region of *Clk* containing the *Clk*^{Jrk} mutation (forward primer, 5'-CCTCCAGCAACAGAATGAGC-3', reverse primer, 5'-CTGCTGATGTTGCTGCTG-3'). This yields a single DNA band, when visualized on an agarose gel. Following amplification, the PCR product was purified using a PCR purification kit (Qiagen), and the product was sequenced using the Big Dye Terminator Cycle Sequencing Kit (Applied Biosystems). The sequencing product was purified using CentriSep gel columns and

was sent to the Northwestern University Center for Genetic Medicine Genomics Core Facility to be run on a gel. Sequencing data was then analyzed, and *Clk*^{Jrk} mutants were identified by a single base pair change (C to T). Sequenced genotype results for each fly were matched with sleep and circadian data, which was averaged for each genotype (+/+, *Clk*^{Jrk}/+, *Clk*^{Jrk}/ *Clk*^{Jrk}).

RT-PCR. Flies were collected at approximately CT2, and fly heads were separated on dry ice. Total RNA was extracted from flies using TRIzol reagent (Invitrogen). Following extraction, RNA was treated with DNase I to remove genomic DNA contamination. RNA levels were measured using the QuantiTect SYBR green RT-PCR kit (QIAGEN), using primers spanning intron 1. Semi-quantitative PCR was performed under non-saturating conditions. The relative amount of *Clk* transcript was quantified as described previously (Livak and Schmittgen, 2001). Samples were run in triplicate.

Statistical analyses. Two tailed t-tests were used for all statistical comparisons. Comparisons were considered significant with a p value <0.05.

Results and Discussion

Clk Promotes Consolidated Sleep

We examined sleep, consolidated sleep, activity, and sleep/activity distribution in four alleles of *Clk*, including the semi-dominant *Clk*^{Jrk} (*Jrk*) allele (Allada et al., 1998), the recessive *Clk*^{ar} (*ar*) allele (Allada et al., 2003), a homozygous lethal deletion line which deletes the entire

Clk gene and several other genes, D1 (Allada et al., 1998), and a newly generated allele from the Exelixis gene disruption collection, containing a piggybac transposon insertion located within the 5' UTR of *Clk, Clk*^{p} (Figure 4.1A). Since the *Clk*^{p} allele had not been described, we first compared *Clk* mRNA levels in wild type and *Clk*^{p} flies, and found that *Clk* mRNA levels were reduced to ~40% of wild type (Figure 4.1B). Based on behavioral data, we predict that *Clk*^{p} is a relatively weak recessive allele, since it retains some rhythmicity both as a homozygote, and heterozygous with *ar* (Table 4.1). *Clk*^{p} also exhibits weak phenotypes compared to other *Clk* alleles in sleep and LD circadian behavior (data to follow).

%Sleep was significantly reduced in both light:dark (LD) and constant darkness (DD) in all *Clk* alleles and *Clk* allele complementation crosses, with the exception of *Clk*^{p} homozygotes (Figure 4.2 A,B). Importantly, this decrease in sleep was not necessarily due to an increase in activity, since many *Clk* mutant flies did not show increased activity levels (Figure 4.2 C,D), and sleep was not correlated with activity levels (Figure 4.2 E,F). Sleep was decreased in *ar*, D1, and *Clk*^{p} heterozygotes, suggesting that these alleles may dominantly affect sleep amount. The observation that *Clk*^{p} homozygotes do not have a sleep phenotype, but *Clk*^{p}/+ heterozygotes do suggests that genetic modifiers may be present in the *Clk*^{p} stock that act to reduce the *Clk*^{p} phenotype, which is revealed following outcross to a wild type stock.

Rhythmicity is reduced in D1/+, and *ar*/+, and it is dramatically reduced in *Jrk*/+ (Table 4.1) suggesting the possibility that all three alleles may have semi-dominant (*ar*, D1), or dominant (*Jrk*) effects on rhythmicity, in addition to sleep. This differs from previously published results (Allada et al., 1998, Table 1; Allada et al., 2003, Table 1), which imply that the *ar* and D1 alleles are recessive, and *Jrk* is semi-dominant, when examining circadian behavior. It is possible that genetic modifiers may have accumulated in the stocks in the intervening years

since these reports were published. Alternatively, it is possible that the choice of wild type strain to outcross *Clk* mutants to (*yellow white* in our studies, Canton S in Allada et al., 1998, unclear in Allada et al., 2003) may have influenced the penetrance of *Clk* mutant phenotypes. To examine this possibility, *Clk* mutant alleles should be outcrossed to different wild type strains and phenotypes re-examined, or, should be isogenized into the same genetic background.

We noticed that the magnitude of the sleep phenotype was reduced in DD in some alleles (eg. *ar*), an observation that was also made in *Clock* mutant mice (Naylor et al., 2000). When we examined daily sleep amount we noticed that these genotypes exhibit a “trend” towards increased sleep/time (Figure 4.2G), which produced a sleep difference of up to ~25% in some flies from the start of the experiment to the end (DD day 7 – LD day 1; Figure 4.2H). Age-related increases in sleep in flies have been reported (Koh et al., 2006), so this might reflect an accelerated age response, or other possibilities such as a differential response to the tube food, isolation, sickness, or accumulated sleep debt in *Clk* mutant flies.

In addition to assaying total sleep amount, we also examined the extent of sleep consolidation, or “fragmentation” in *Clk* mutants. The amount of consolidated sleep was quantified using a weighed average sleep bout (CI) calculation, which takes into account both the number of sleep bouts, and length of sleep bouts. It “weights” longer sleep bouts more heavily in the calculation, by squaring bouts of all lengths. Therefore, a consolidated fly would be one in which sleep occurred in a few uninterrupted bouts. Consolidation does not refer to amount of sleep occurring during the light or dark phase, terminology which has been used in mammalian sleep to describe this feature. While %sleep was unaffected in *Clk^p^* homozygous mutants, sleep consolidation was decreased in all homozygous *Clk* alleles, including *Clk^p^*, and *Clk* heterozygotes, with the exception of *Clk^p^*/+, in both LD and DD (Figure 4.2 I,J). As with sleep,

consolidation increased with time in *Clk* mutant flies, and actually decreased with time (flies became less consolidated) in *Clk* heterozygotes and *Clk*^{p} homozygotes (Figure 4.2 K,L). The effect of *Clk* on consolidated sleep is robust, and supports the idea that one of the main functions of *Clk* may be to consolidate sleep into a single bout, or, to maintain sleep once initiated. This supports published data on *Clk* mutant flies and mice, which have reported a reduction in sleep bout length (Naylor et al., 2000; Hendricks et al., 2003a).

Clk mutant flies (*Jrk, ar*) exhibit a striking alteration of circadian behavior in LD, and arrhythmicity in DD (Figure 4.2 M-P, Table 4.1). Wild type flies are diurnal, and show anticipation to both lights on and lights off events by an increase in activity preceding these transitions (Figure 4.2M). Sleep is primarily confined to the dark phase, although in males, a significant portion of sleep occurs during mid-day (Figure 4.2P). *Clk* mutants fail to anticipate L>D and D>L transitions (Figure 4.2N), and switch to sleeping primarily during the light phase, although sleep is distributed throughout both L and D (Figure 4.2P). In *Clk*^{p} homozygotes, some of these typical *Clk* phenotypes are retained, including reduced anticipation to D>L and L>D transitions (Figure 4.2O) and a reduced amplitude of sleep distribution (Figure 4.2P). We considered the hypothesis that sleep may be reduced in *Clk* mutants due to a masking phenomenon, whereby light inhibits activity, forcing sleep to occur during the light phase. This may be part of the phenotype, however, the fact that sleep is reduced in *Jrk* flies and other *Clk* mutants (data not shown) during the light as well supports the idea that sleep amount itself is affected by *Clk* mutations, and not simply sleep timing.

We were concerned that genetic modifiers might have enhanced the *Clk* phenotype in some alleles (ie. *Jrk*) and reduced the phenotype in others (ie. *Clk*^{p}). To test this hypothesis we crossed *Jrk* flies to an isogenic strain, intercrossed siblings from this cross, and then tested

progeny of this intercross for behavior (Figure 4.3). This effectively exchanges a large amount of genetic background from the original *Jrk* stock with the isogenic stock, creating *Jrk/Jrk, Jrk*/+, and +/+ siblings with ~50% genetic background in common. In a typical experiment, WT shares 50% of its genetic background with *Jrk*/+, and *Jrk*/+ shares 50% of its genetic background with *Jrk/Jrk*, but *Jrk/Jrk* and WT share no common genetic background (Figure 4.3A). Following backcrossing, we found that as before, sleep in LD was significantly reduced in both *Jrk*/+ and *Jrk/Jrk* flies as compared to +/+ flies (Figure 4.3B), but not during DD in backcrossed *Jrk*/+ and *Jrk/Jrk* flies (Figure 4.3C). Rhythmicity was increased (Table 4.2) and the magnitude of *Jrk* consolidation phenotypes were reduced in both LD (Figure 4.3D) and DD (Figure 4.3E), although there was a trend towards a significant *Jrk* effect in both LD and DD. While non-backcrossed *Jrk* flies showed a significant increase in activity in both LD and DD (Figure 4.2 C,D), this phenotype was abolished in backcrossed *Jrk* flies (Figure 4.3 F,G). Additionally, while non-backcrossed *Jrk* flies do not experience an age-related increase in sleep or consolidation (Figure 4.2 G,K), backcrossed *Jrk* flies do (Figure 4.3 H,I), similar to other *Clk* flies. It is important to note that the wild type phenotype is not typical in these flies. Compared to other wild type flies, backcrossed wild type flies have dramatically reduced sleep in DD (~60% in Figure 4.2B compared to ~45% in Figure 4.3C) reduced sleep consolidation (~175 in Figure 4.2J compared to ~80 in Figure 4.3E), and reduced rhythmicity in DD (100% in Table 4.1, compared to 76% rhythmic in Table 4.2). When examined in greater detail, 3 of the 5 arrhythmic "WT" flies have extremely reduced sleep (22%, 23%, and 36%), suggesting that these may represent PCR/sequencing errors which mis-genotyped these flies. Alternatively, there may be a second mutation in the *Jrk* stock that reduces sleep and rhythmicity, which is tightly linked with the *Jrk* mutation, but occasionally separates through recombination. The low number of flies in

the *Jrk* group should also be taken into consideration, since only eight *Jrk/Jrk* flies were generated from these crosses. We have noted in other instances that the *Jrk* mutation increases developmental lethality, which may explain the small N. Together, this data suggests that genetic modifiers may enhance *Jrk* phenotypes in the *Jrk* stock, however, a greater number of *Jrk/Jrk* flies should be examined before this can be claimed conclusively. Given the potential influence of genetic modifiers in the *Jrk* stock, we should perform similar backcrossing experiments to determine if genetic modifiers are present in other *Clk* alleles, including *ar* and Clk^{p}.

Arrhythmicity is Not Highly Correlated with Sleep Consolidation or Sleep Amount

Based on the observation that both consolidation and rhythmicity are reduced in *Clk* mutants, we were interested in whether this represented a general principle, that decreases in DD consolidation are an indirect result of reduced circadian rhythmicity. We were also interested in whether mutations of other genes comprising the circadian molecular feedback loop would duplicate the range of *Clk* phenotypes. We examined sleep, consolidation, and rhythmicity in three wild type genotypes and 8 arrhythmic mutant genotypes, including *ar*, *Jrk*, cyc^{01}, per^{01}, and tim^{01}, all null mutations of pacemaker genes, and null mutations in the clock output peptide, pigment dispersing factor (pdf^{01}) in two different genetic backgrounds, as well as its recently identified receptor, *gop* (*groom of pdf*), also known as *pdfr* (Hyun et al., 2005; Lear et al., 2005; Mertens et al., 2005). We found that among arrhythmic mutants, sleep was reduced only in cyc^{01} flies in LD and DD (Figure 4.4A), consistent with published reports, although using different experimental methods (Hendricks et al., 2003a). Consolidation was reduced in cyc^{01}, per^{01}, and pdf^{01}w- flies in both LD and DD, and in *pdfr* in DD, although the magnitude of the effect was comparable to *Clk* only in cyc^{01} (Figure 4.4B). An effect of cyc^{01} on sleep bout length has been

published (Hendricks et al., 2003a), however these authors did not observe a consolidation phenotype for *per^{01}*. The fact that only one mutation (*cyc^{01}*) resulted in a comparable sleep and consolidation phenotype to *Clk,* despite the fact that all genotypes were arrhythmic (Figure 4.4C) suggests that the *Clk* phenotype is relatively unique. It is important to note that CYC is the binding partner of CLK, which may explain the phenotypic similarity, since CLK dimerizes with CYC to initiate gene transcription (Darlington et al., 1998; Lee et al., 1998; Rutila et al., 1998; Bae et al., 1998, 2000). To further examine the possibility that rhythmicity and sleep phenotypes are correlated, we tested an additional 18 circadian mutants that result in either reduced rhythmicity or shortened/lengthened periods. We found that %sleep in DD, and consolidation in LD were poorly correlated with rhythmicity, and consolidation in DD was only weakly correlated with rhythmicity (Figure 4.4 D-F). We conclude that not only are the range of *Clk* phenotypes relatively unique, but that in general, mutations in many circadian genes do not affect sleep. Since it does not appear to be a general principle that circadian mutants exhibit sleep phenotypes, this suggests that the role of *Clk* in sleep may potentially represent a non-pacemaker function for this gene. It should be mentioned once again however that genetic background may modify not only *Jrk* sleep phenotypes as shown (Figure 4.3), but also sleep phenotypes of other circadian mutants. Before completely ruling out the role of any gene on sleep regulation, the contribution of genetic background should be assessed. Again, the best way to do this would be through a backcrossing/genotyping scheme, or backcrossing all circadian mutations into a similar genetic background.

Clk Phenotypes can be Rescued by Clk Expression in Cells Defined by 30Y GAL4

Given that *Clk* is expressed in both circadian and mushroom body (MB) neurons in the fly (Houl et al., 2006), *Clk* promotes sleep (Naylor et al., 2000; Hendricks et al., 2003a; this thesis), MB neurons promote sleep (Pitman et al., 2006; Joiner et al., 2006; Chapter 3), and circadian neurons may promote sleep (Figure 3.1, Figure 3.4), we attempted to rescue the *Clk* sleep phenotype by expressing wild type *Clk* within MB and circadian neurons. We were primarily interested in the adult function of *Clk* in sleep, especially since *Clk* over-expression throughout development results in lethality in combination with many GAL4 drivers. We took advantage of the GAL80^{ts} system (McGuire et al., 2003), whereby we were able to temporally control GAL4 expression using a temperature sensitive GAL80 expressed under the ubiquitous *tubulin1*α promoter (*tubulin*GAL80^{ts}). Flies were raised at 18°C, when the GAL80 is active, and were tested at 29°C, a temperature where GAL80 becomes inactive, relieving repression on GAL4. We focused rescue experiments on the recessive *Clk*^{ar} allele, since the sleep-dominant *Clk*^{Jrk} allele was less likely to rescue based on previously published rhythmicity data (Allada et al., 2003), and our own preliminary data (data not shown). This may be due to developmental axonal defects in pacemaker neurons caused by the *Jrk* mutation (Park et. al., 2000). Unfortunately, placing the *ar* mutation into the *tubulin*GAL80^{ts}; UAS*Clk* (TGUCar) genetic background, and/or testing flies at 29°C resulted in a reduction of the *ar* %sleep phenotype in controls during both LD and DD, making an assessment of total %sleep rescue impossible (Figure 4.5 A,B). However, control flies still exhibited sleep consolidation phenotypes, and exhibited all circadian features of *Clk* mutants in LD and DD.

We attempted to rescue *Clk* consolidation phenotypes using the MB/circadian GAL4 lines 30Y and c309, both of which result in reduced sleep and consolidation in combination with

UAS*shi*^{ts} (Chapter 2), and the pan-neuronal GAL4 line, *elav* (combination of *elav* with UAS*shi*^{ts} results in lethality, precluding testing of this line in those experiments). Of the three drivers, only 30Y rescued the *Clk* sleep consolidation phenotype (Figure 4.5 C,D). Since we could not assess total sleep rescue, we examined sleep in greater detail (LD %sleep/hr) in rescue and control flies. The *tubulin*GAL80^{ts}; UAS*Clk-ar* control sleeps equivalently to wild type flies during the light, but much less than wild type during the dark (yellow line, Figure 4.5 E-G), making it possible to compare rescue flies (dark blue line, Figure 4.5 E-G) to wild type (purple line, Figure 4.5 E-G) and *ar* (pink and light blue lines, Figure 4.5 E-G) controls during the dark phase. When we examined rescue during the dark only, we observed that 30Y (Figure 4.5E) and *elav* (Figure 4.5G), but not c309 (Figure 4.5F) rescued sleep amount. This observation was confirmed by examining total %sleep during the dark (Figure 4.5H), which also uncovered a partial rescue of consolidation during the dark with *elav* (Figure 4.5I). It is not clear why *elav* rescues sleep and consolidation (partially) during the dark, but not the light. It is possible that *Clk* works in some cell groups labeled by *elav* to promote sleep and consolidation, others to reduce sleep and consolidation, canceling out any overall effect.

All three drivers rescued components of circadian behavior (controls: Figure 4.5 J-O, experimental groups: Figure 4.5 P-R) including morning (30Y, Figure 4.5P; *elav*, Figure 4.5R) and evening (30Y, Figure 4.5P; 309, Figure 4.5Q; *elav*, Figure 4.5R) anticipation, and more weakly, period and rhythmicity (Table 4.3). There was no correlation in 30Y rescue flies between consolidation in DD and rhythmicity, suggesting that these two behaviors are separable (r = -0.11, data not shown). These results were relatively surprising, since we expected that 30Y and c309 would either both rescue or not rescue *Clk*, as they both contain sleep promoting cells, and that *elav*, since it is expressed in all neurons, would rescue all *Clk* phenotypes. It was also

surprising that both 30Y and c309 rescued circadian phenotypes of *Clk*. There was some previous evidence that 30Y and c309 contained circadian cell expression (Chapter 3, Figure 3.1), and these data further support this possibility. Since period and rhythmicity are weakly rescued in both 30Y and c309, and evening behavior is rescued in both lines, this suggests that both 30Y and c309 are expressed in at least a subset of the morning cells, sLNv, and evening cells, DN1, LNd, and PDF(-) sLNv (Grima et al., 2004; Stoleru et al., 2004). The exact circadian expression of these lines should be confirmed by co-labeling GAL4/UASGFP expression with PER protein expression.

Clk Over-expression Results in Reduced Consolidation in c309 and elav GAL4

To examine the possibility that *Clk* over-expression itself caused a sleep/consolidation phenotype we then examined the results of *Clk* over-expression in 30Y, c309, and *elav* GAL4 (Figure 4.6). While %sleep was unaffected by *Clk* over-expression in all three lines (Figure 4.6 A,B), consolidation was reduced in both c309 and *elav*, but not 30Y, in LD and DD (Figure 4.5 C,D). In addition, *Clk* over-expression resulted in an advanced evening peak of sleep (Figure 4.6 E-G) and activity in all three lines (controls: Figure 4.6 H-L, over-expression: Figure 4.6 M-O), and significantly shortened circadian period, especially in c309 and *elav* (Table 4.4). This provides another example of the separation between sleep and circadian phenotypes, since the evening peaks of activity and sleep were shifted by *Clk* over-expression in all three GAL4 lines, but only c309 and *elav* exhibited consolidation phenotypes.

Although over-expression data can be difficult to interpret, one possibility is that *Clk* over-expression does not cause a consolidation phenotype in 30Y because *Clk* is already present in these cells, where it works to promote consolidation, as supported by rescue data (Figure 4.4).

In c309 and *elav*, *Clk* may either not be present normally, or function in sub-sets of cells to both promote and inhibit consolidated sleep. Another possibility is that *Clk* levels are different between 30Y and c309/*elav* GAL4 lines. A moderate level of *Clk* expression in 30Y may rescue the *Clk* consolidation phenotype, but a higher level of *Clk* expression in c309/*elav* may result in an ectopic consolidation phenotype. Increasing 30Y gene dosage, and/or measuring *Clk* levels in 30Y/UAS*Clk* and c309,*elav*/UAS*Clk* over-expression flies should help to resolve these possibilities. The observation that *Clk* over-expression in circadian cells in 30Y, c309, and *elav* results in an alteration of circadian behavior also supports the idea that ectopic expression of *Clk* results in disrupted normal behavior, when expressed at the wrong time, and/or in the wrong place. Interestingly, these data resemble those of PKA over-expression in Joiner et al. (2006). c309 was sensitive to PKA over-expression, resulting in reduced overall sleep, but PKA promoted sleep during the day and inhibited sleep at night with the 30Y driver, resulting in little overall effect on sleep. Perhaps the 30Y sleep-promoting cells and the c309 sleep promoting cells employ different molecular mechanisms within the neurons defined by these drivers to promote sleep. For example, c309 cells may use a PKA dependent pathway, and 30Y cells may use a *Clk* dependent pathway.

Circadian Cells May Promote Sleep

While it has been shown that mushroom body neurons promote both sleep and consolidated sleep (Pitman et al., 2006; Joiner et al., 2006), there is also evidence that circadian neurons may also promote sleep (Chapter 3; and Figure 4.4). To further investigate this possibility, we examined sleep in flies in which circadian neurons were either inhibited via UASshi^{ts} expression (Figure 4.7), or, genetically ablated (Figure 4.8). We chose to examine sleep

in four circadian GAL4 lines that have been shown to alter period length to various degrees when crossed to UAS*shi*^{ts}, as an independent means to confirm that they were functioning to affect behavior (V. Kilman, submitted). We focused on *pdf*GAL4, which is expressed in only the sLNv and lLNv (Stoleru et al., 2004), *cry*13GAL4, which is expressed in the sLNv, lLNv, LNd, 2-4 DN1 cells and 2 DN3 cells (Stoleru et al., 2004; Shafer et al., 2006), *cry*16GAL4, which is expressed in the sLNv, lLNv, LNd, some ellipsoid body neurons (Zhao et al., 2003) and probably some DN cells (V. Kilman, personal communication), and *tim*GAL4, which is expressed in all circadian cells, as well as many non-circadian cells (Zhao et al., 2003; Stoleru et al., 2004).

Sleep was examined in circadian GAL4/UAS*shi*^{ts} flies under both temperature cycling (Figure 4.7 A-F) and constant 29°C conditions (Figure 4.7 G-L). Most drivers had small effects on sleep at the restrictive temperature (*pdf*, *cry*13, *cry*16, Figure 4.7 A,D,E,G,H,I,K), however, only *tim*GAL4 consistently reduced sleep in both sleep assays (Figure 4.7 A,F,G,H,L). This could reflect the fact that it is the broadest expressing of all the circadian lines, but might also be due to the significant amount of expression in uncharacterized, non-circadian, cells. Blocking circadian cell expression using *pdf*GAL80 or *cry*GAL80 in these flies might offer some explanation as to whether the phenotype is due to circadian cell inhibition, or inhibition of these additional areas. A homozygous *pdf*GAL4;UAS*shi*^{ts} stock, which presumably expresses *shibire* to a higher level in *pdf* cells, showed a reduction in sleep during the permissive temperature during temperature cycling (Figure 4.7 B,C). This may be the result of genetic background, or may represent a “leakiness” of the UAS*shi*^{ts} transgene, where the function of sleep promoting circuitry (as defined by *pdf*GAL4) at 21°C is inhibited. It will be necessary to test this line under constant 29°C and 21°C temperatures to assess these possibilities.

Genetic ablation of circadian cells, by expressing a pro-apoptotic (cell death) gene, *hid* (Zhou et al., 1997), using the *pdf* and *cry*13 GAL4 drivers resulted in an overall reduction in sleep and consolidation with *pdf* (Figure 4.8 A,B), due primarily to reduced sleep during the light phase (Figure 4.8 C,D), and a reduction of sleep with *cry*13 in the dark phase (Figure 4.8 E,F). A shift in sleep timing would not be predicted to alter overall sleep levels, but the *pdf* results may be explained by a masking effect of light on sleep. This could be tested by performing *pdf*/UAS*hid* ablation in a per^{01} mutant background. *Pdf shibire* and *hid* data consistently suggest a role for *pdf*GAL4 neurons in sleep promotion, however we observed only a slight sleep reduction in *cry*13/UASshi^{ts} flies. It is possible that *shibire* was not expressed to high enough levels by *cry*13 in adult flies to influence sleep amount, but at high enough levels during development, when *hid* is required to ablate cells. In fact, we are confident that cells were ablated in both *cry*13 and *pdf*, since the evening peak of circadian anticipation was advanced in *pdf* cell ablated flies (controls: Figure 4.8 G-I, Figure 4.8K), and reduced in *cry*13 cell ablated flies (controls: Figure 4.8 G,H,J; Figure 4.8L), and morning anticipation was reduced in both, in agreement with published reports (Stoleru et al., 2004).

The observation that inhibition and/or ablation of morning cells results in a reduction of sleep (in *pdf*), and both morning and evening cells results in a slight reduction during the dark phase (in *cry*13) suggests that these cells groups may promote sleep at different times of day. Since the non-overlapping cells between *pdf* and *cry*13 are the evening cells (in *cry*13), this suggests that morning cells may promote sleep during the light phase, whereas evening cells may promote sleep during the dark phase.

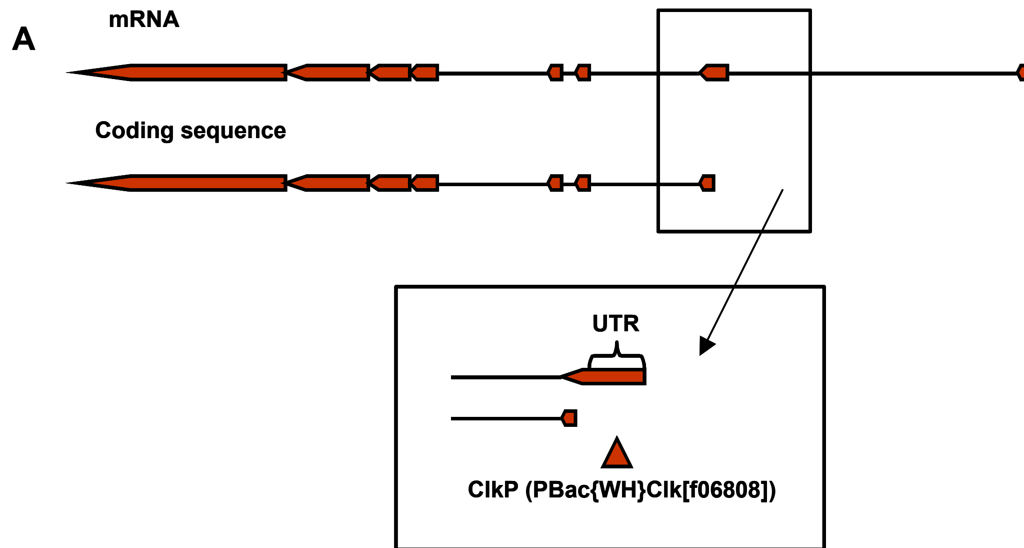
Circadian and Sleep Regulatory Regions May Overlap Both Anatomically and Genetically

The preceding data has advanced the finding that *Clk* is involved in sleep, particularly sleep consolidation, and suggests that the *Clk* consolidation phenotype may be due to loss of *Clk* from a circuit comprising circadian and/or mushroom body neurons. In addition, this data is the first to describe a role for circadian neurons in *Drosophila* in sleep. The majority of the data presented here supports a role for circadian neurons in sleep promotion, rather than wake, which has been the case in mammals (Edgar et al., 1993; Easton et al., 2004). The reason for this difference in insects is unclear. It is possible that circadian neurons promote both sleep and wake in *Drosophila*, but that the circadian GAL lines used in these experiments either represent mainly sleep promoting cells, or both groups equally, which may explain why some of them (*cry*16, for example) did not consistently alter sleep. Another possibility is that there are regions within the SCN in mammals that promote sleep, but that lesion studies have spared these cells. Electrophysiological data showing that SCN firing rates are increased during REM sleep can be interpreted to suggest that the SCN may actively promote sleep during this stage, however the authors interpret this to mean that downstream sleep regions influence SCN activity, and not the reverse (Deboer et al., 2003).

The idea that *Clk* promotes sleep is not new, however, the data reported here provide a more comprehensive assessment of the *Drosophila Clk* phenotype, including data from four mutant alleles, and rescue of the consolidation phenotype. Rescue of consolidation occurred exclusively with the 30Y GAL4 driver, offering a starting place to determine where and how *Clk* is working. It will be important to determine whether the *Clk* consolidation phenotype is rescued via circadian cell, or mushroom body cell expression in this line. We can use the MBGAL80 to assay whether consolidation is rescued in 30Y following MB expression block. We can also

block circadian cell expression in 30Y using *pdf*GAL80 and *cry*GAL80. If MB expression is responsible for *Clk* rescue, we can then attempt to determine the further narrow down the identity of these cells by using more restricted MB drivers, which do not express in circadian cells, or by using other MBGAL80 lines. If circadian cell expression is responsible for *Clk* rescue, we can easily determine which cells are labeled in 30Y via PER/GFP double label. We can independently assess the role of *Clk* on sleep in circadian and MB cells by knocking down *Clk* function using RNAi, or by over-expressing a UASClk^{Jrk} construct, in hopes of replicating the sleep and consolidation phenotypes. Finally, it may be possible that *Clk* works in both circadian and mushroom body cells together to promote sleep, since the work presented in this thesis supports a role for the activity of both types of neurons in sleep promotion. Many circadian neurons, including sLNvs, LNds, and most of the DNs, send axonal projections within the region of mushroom body Kenyon cell body layer, or the calyx dendritic region (Kaneko et al., 2000), where they could synapse, forming an anatomical circuit. In this proposed circuit, *Clk* may relay signals regarding sleep timing to the mushroom bodies via promoting circadian cell electrical activity at specific times of day. Within MB neurons, *Clk* may sense an accumulation of sleep need, and activate the transcription of genes required to activate sleep-promoting MB circuitry, with the end result of reducing sleep need.

Once it has been established which regions of the brain *Clk* is working to promote sleep, we can then begin to ask how *Clk* accomplishes this, by assaying the role of *Clk* target genes in sleep, and placing them into anatomical and molecular pathways. If we consider the success that the *Drosophila* circadian field has experienced recently in dissecting the complex genetic and anatomical circuit regulating circadian behavior, this goal seems within reach.



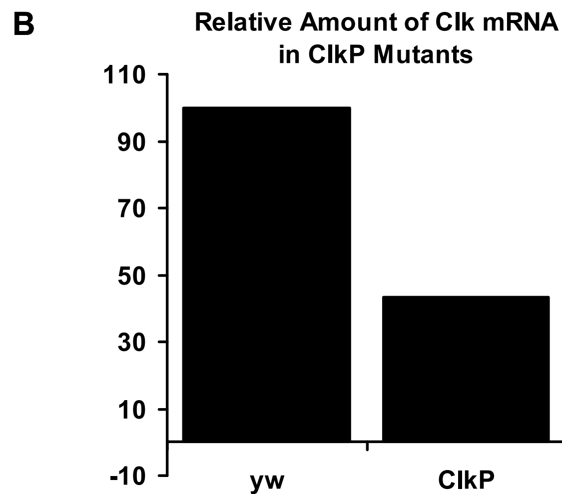
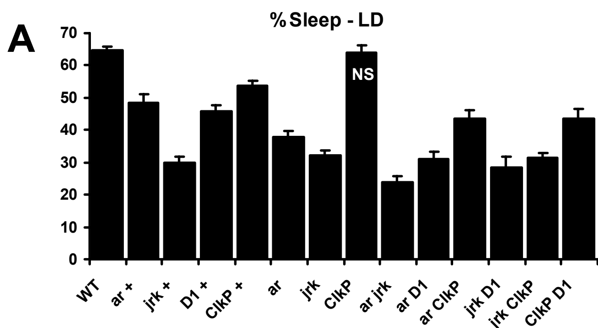


Figure 4.1 ***Clk^p* is a new allele of *dClk.*** (**A**) *Clk^p* (PBac{WH}*Clk*[f06808]) is a piggbac p-element inserted within the untranslated region (5'UTR) of the first coding exon (second exon) of *Clock* (diagram reproduced based on information from "FlyBase" *Drosophila* database). (**B**) Relative *Clk* mRNA transcript level in *Clk^p* mutant fly heads compared to *yw* (*yellow white*) controls. N expt = 1.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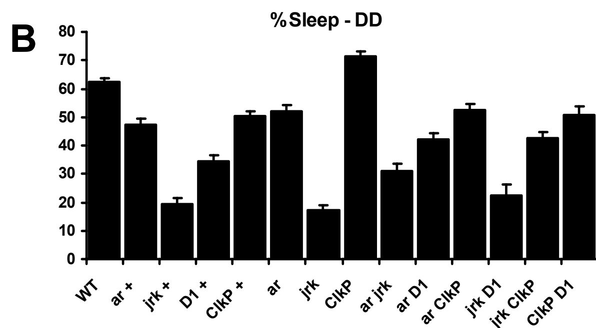
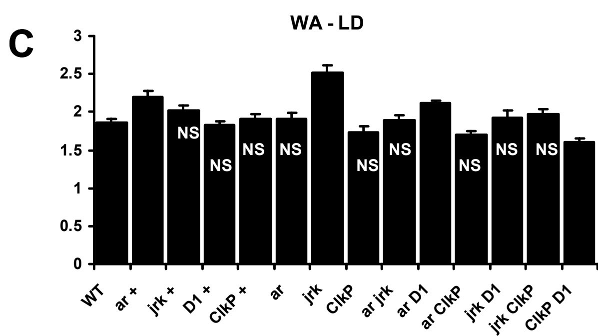
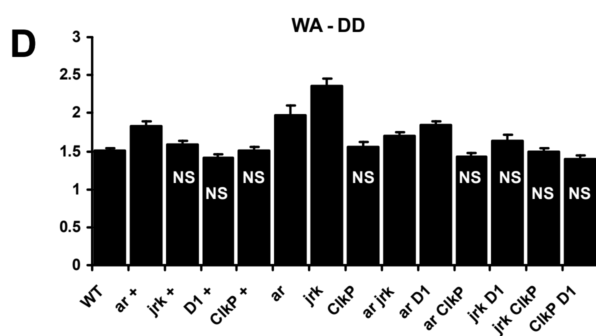
% Sleep - LD
NS
WT
ar +
jrk +
D1 +
ClkP +
ar
jrk
ClkP
ar jrk
ar D1
ar ClkP
jrk D1
jrk ClkP
ClkP D1


B
% Sleep - DD
WT
ar +
jrk +
D1 +
ClkP +
ar
jrk
ClkP
ar jrk
ar D1
ar ClkP
jrk D1
jrk ClkP
ClkP D1


C
WA - LD
NS
WT
ar +
jrk +
D1 +
ClkP +
ar
jrk
ClkP
ar jrk
ar D1
ar ClkP
jrk D1
jrk ClkP
ClkP D1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WT
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ar
jrk
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ar jrk
ar D1
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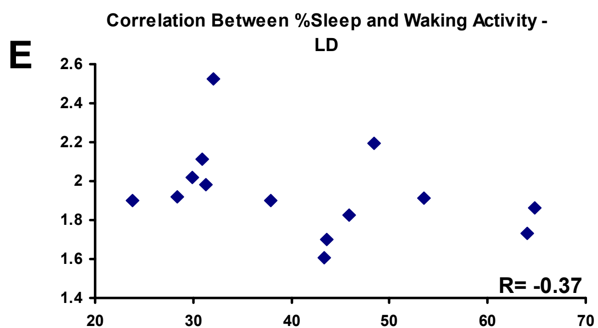
jrk ClkP
ClkP D1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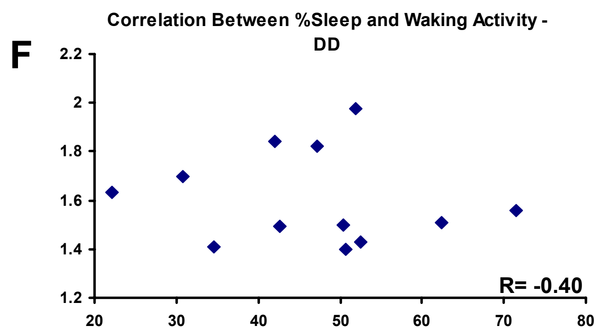
Correlation Between %Sleep and Waking Activity - LD
R= -0.37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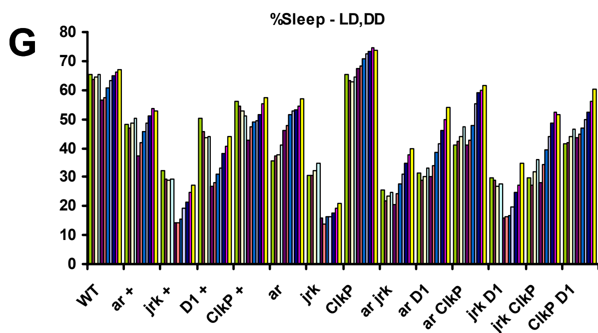
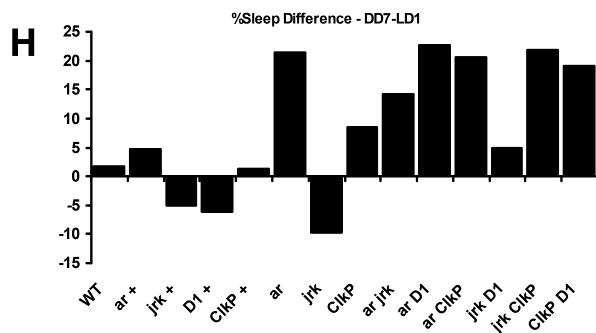
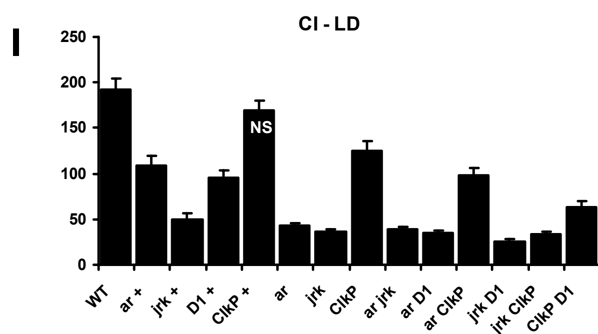

G
%Sleep - LD,DD
WT
ar +
jrk +
D1 +
ClkP +
ar
jrk
ClkP
ar jrk
ar D1
ar ClkP
jrk D1
jrk ClkP
ClkP D1


H
%Sleep Difference - DD7-LD1
WT
ar +
jrk +
D1 +
ClkP +
ar
jrk
ClkP
ar jrk
ar D1
ar ClkP
jrk D1
jrk ClkP
ClkP D1

I



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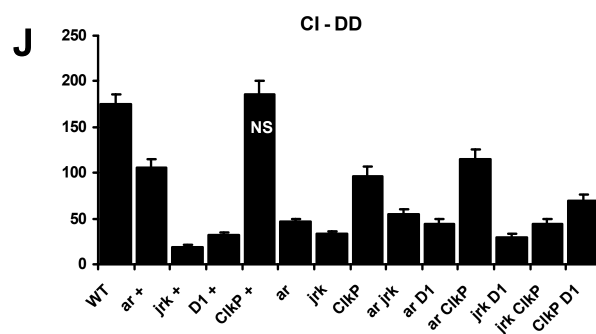

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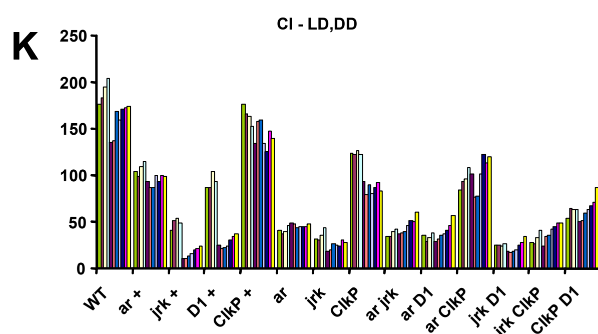

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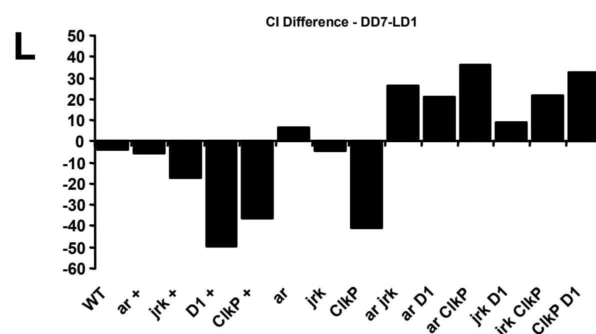

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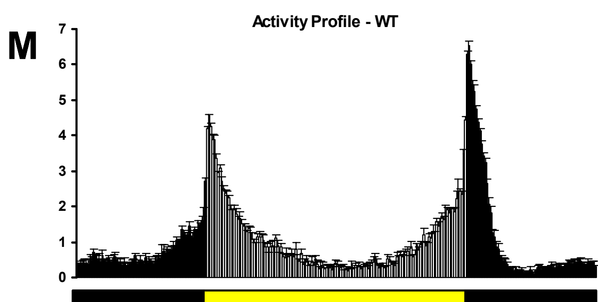

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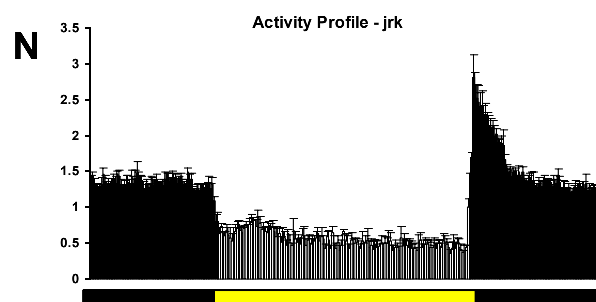
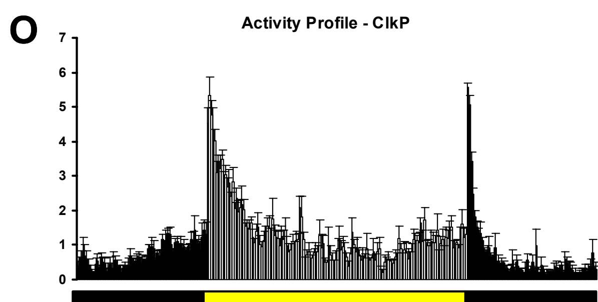


O



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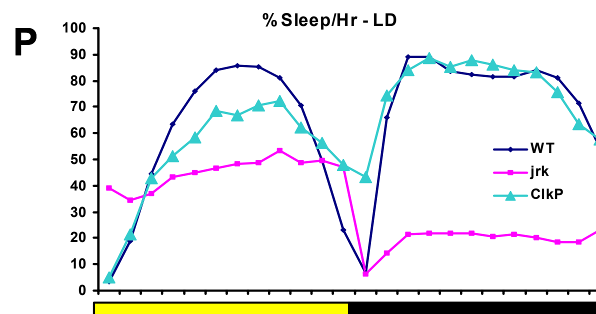
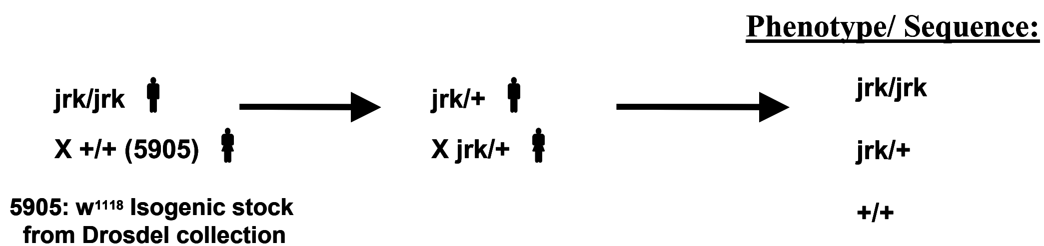
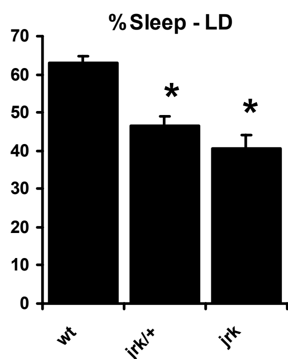
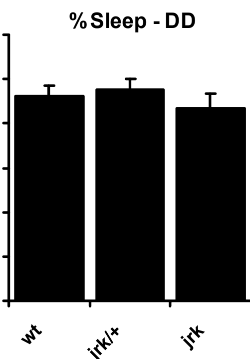


Figure 4.2 Sleep and sleep consolidation are reduced in *Clk* mutants. *Clk* mutant phenotypes in entrained, 25°C, 12hr light:12hr dark (LD, 4 days) conditions (**A,C,E,I,M-P**), and non-entrained, 25°C constant darkness (DD, 7 days) conditions (**B,D,F,J**), or both (**G,H,K,L**) including; (**A,B**) average %sleep/24hr, (**C,D**) activity (WA: waking activity, average activity counts/waking minute), (**E,F**) correlation between %sleep and WA, (**G**) %sleep/day/11 day LD:DD experiment, (**H**) sleep difference across 11 day LD:DD experiment (%sleep DD day 7 - %sleep LD day 1), (**I,J**) sleep consolidation (CI: weighted average sleep bout length (min)), (**K**) CI/day/11 day LD:DD experiment, (**L**) sleep consolidation difference across 11 day LD:DD experiment CI DD day 7 - CI LD day 1), (**M-O**), activity profiles (24hr profile of 4 day average normalized activity/5min) for wild type (WT: *yellow white*/ Canton S outcross) (**M**) Clk^{Jrk} (*Jrk*) mutants (**N**), and Clk^{p} mutants (**O**), (**P**) sleep profiles (24hr profile of 4 day average %Sleep/Hr) for WT, *Jrk,* and Clk^{p} mutants. Yellow bars = 12 hr light phase, black bars = 12 hr dark phase (**M-P**) "**+**" = wild type allele – homozygous genotype was crossed to *yw* control, heterozygous progeny represented. All genotypes were compared to WT using 2-tailed t-test, if comparison is not significantly different (P<0.05), the bar is labeled "NS". N = 16-79 (LD), N = 16-75 (DD), N expt = 2-6. Error bars indicate SEM.


A
Phenotype/ Sequence:
jrk/jrk
X +/+ (5905)
5905: w1118 Isogenic stock
from Drosdel collection
jrk/+
X jrk/+
jrk/jrk
jrk/+
+/+


B
%Sleep - LD
wt
jrk/+
jrk


C
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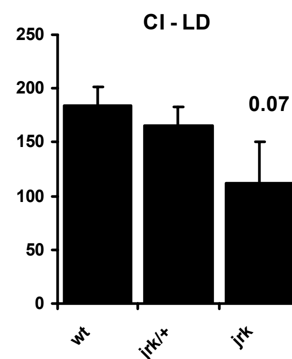
wt
jrk/+
jrk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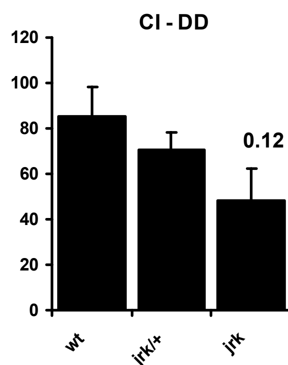
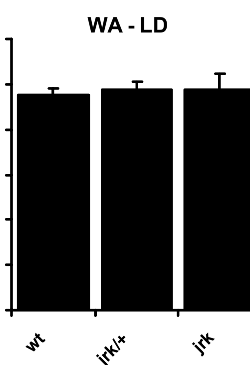
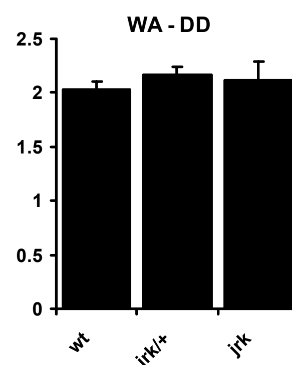
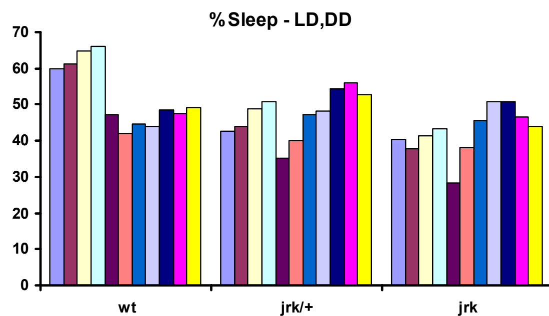
CI - LD
0.07
wt
jrk/+
jrk


E
CI - DD
0.12
wt
jrk/+
jrk


F
WA - LD
wt
jrk/+
jrk


G
WA - DD
wt
jrk/+
jrk


H
%Sleep - LD,DD
wt
jrk/+
jrk

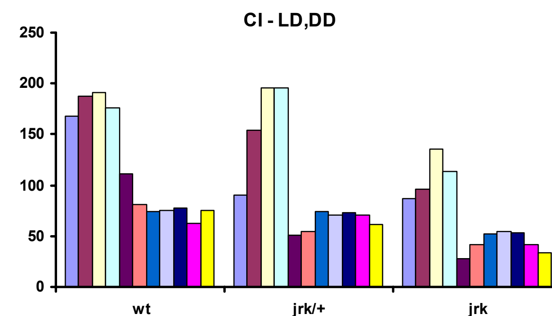

I
CI - LD,DD
wt
jrk/+
jrk

Figure 4.3 Sleep and activity phenotypes may be influenced by genetic background in *Jrk* mutants. (**A**) *Jrk* backcrossing genetic scheme *Jrk* = *Jrk* in scarlet genetic background. (**B-I**) Backcrossed homozygous *Jrk (Jrk/Jrk)*, heterozygous *Jrk (Jrk/+)*, and wild type (+/+, WT) sleep phenotypes in entrained, 25°C, 12hr light:12hr dark (LD, 4 days) conditions (**B,D,F**), non-entrained, 25°C constant darkness (DD, 7 days) conditions (**C,E,G**), or both (**H,I**) including; average %sleep/24hr (**B,C**), sleep consolidation (CI: weighted average sleep bout length (min)) (**D,E**), activity (WA: waking activity, average activity counts/waking minute) (**F,G**), %sleep/day/11 day LD:DD experiment (**H**), CI/day/11 day LD:DD experiment (**I**). *Jrk* and *Jrk*/+ flies were compared to WT using 2-tailed t-test, significant ($P<0.05$) comparisons are indicated with asterisk (*). N = 8-37 (LD), N = 8-36 (DD), N expt = 1. Error bars indicate SEM.

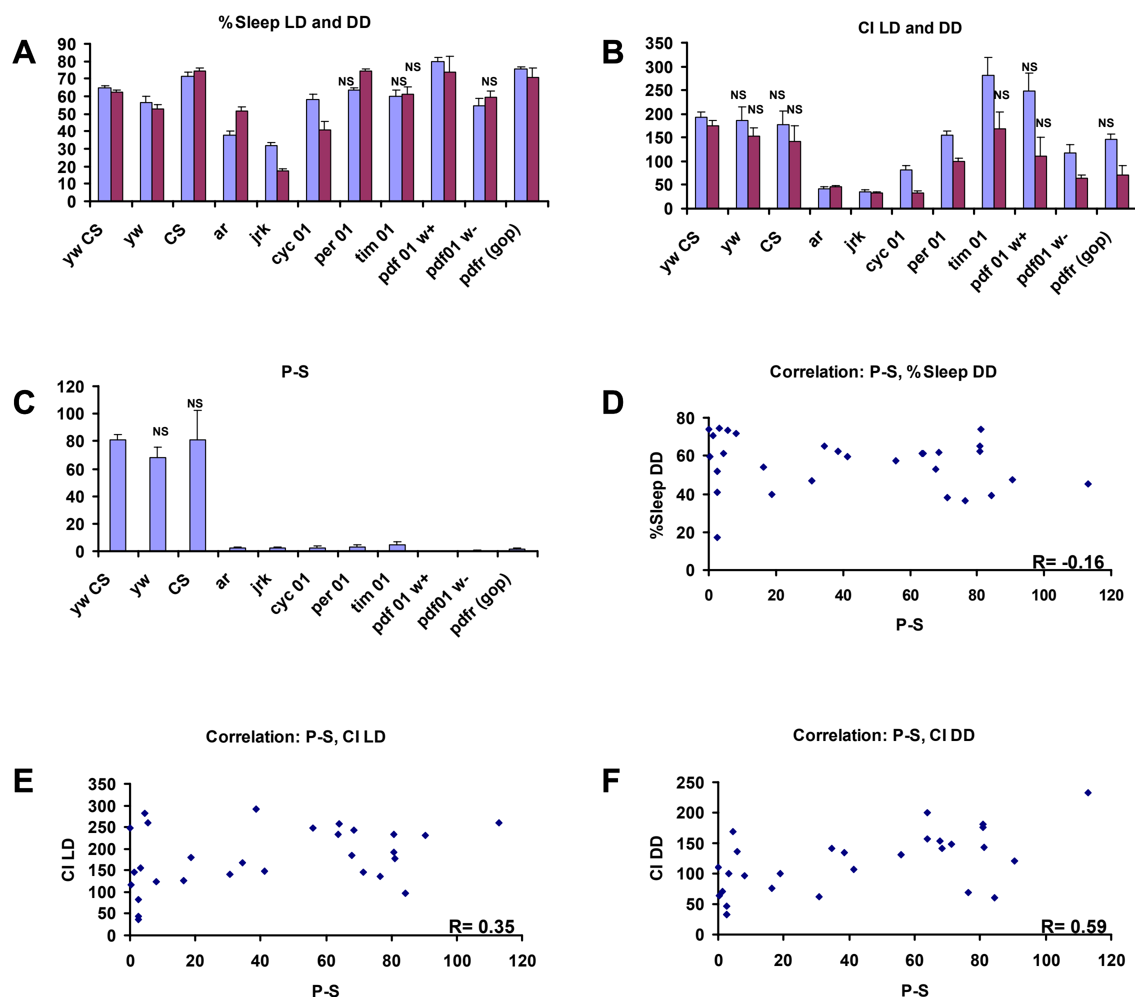


Figure 4.4 Arrhythmicity is not highly correlated with sleep/consolidation phenotypes. (**A**,**B**) Sleep and sleep consolidation phenotypes in wild type strains (*yw*CS outcross, *yw*, CS homozygous stocks) and arrhythmic circadian mutant strains in entrained, 25°C, 12hr light:12hr dark (LD, 4 days), and non-entrained constant darkness (DD, 4 days) conditions. Sleep (%sleep/24hr) (**A**) and sleep consolidation (CI: weighted average sleep bout length (min)) (**B**). (**C**) Rhythmicity (P-S: power-strength) values for wild type strains and arrhythmic mutant strains in DD. (**D-F**) Correlation of rhythmicity (P-S) with %sleep/24hr DD (**D**), CI (weighted average sleep bout length, min) LD (**E**), and CI DD (**F**). "w+" indicates that allele is in a red eyed genetic background, "w-" indicates that allele is in a white eyed genetic background, "gop" = groom of pdf. All genotypes were compared to *yw* CS using 2-tailed t-test, if comparison is not significantly different (P<0.05), the bar is labeled "NS". N = 4-79 (LD), N = 4-75 (DD), N expt = 1-6 (**A-F**). Error bars indicate SEM.

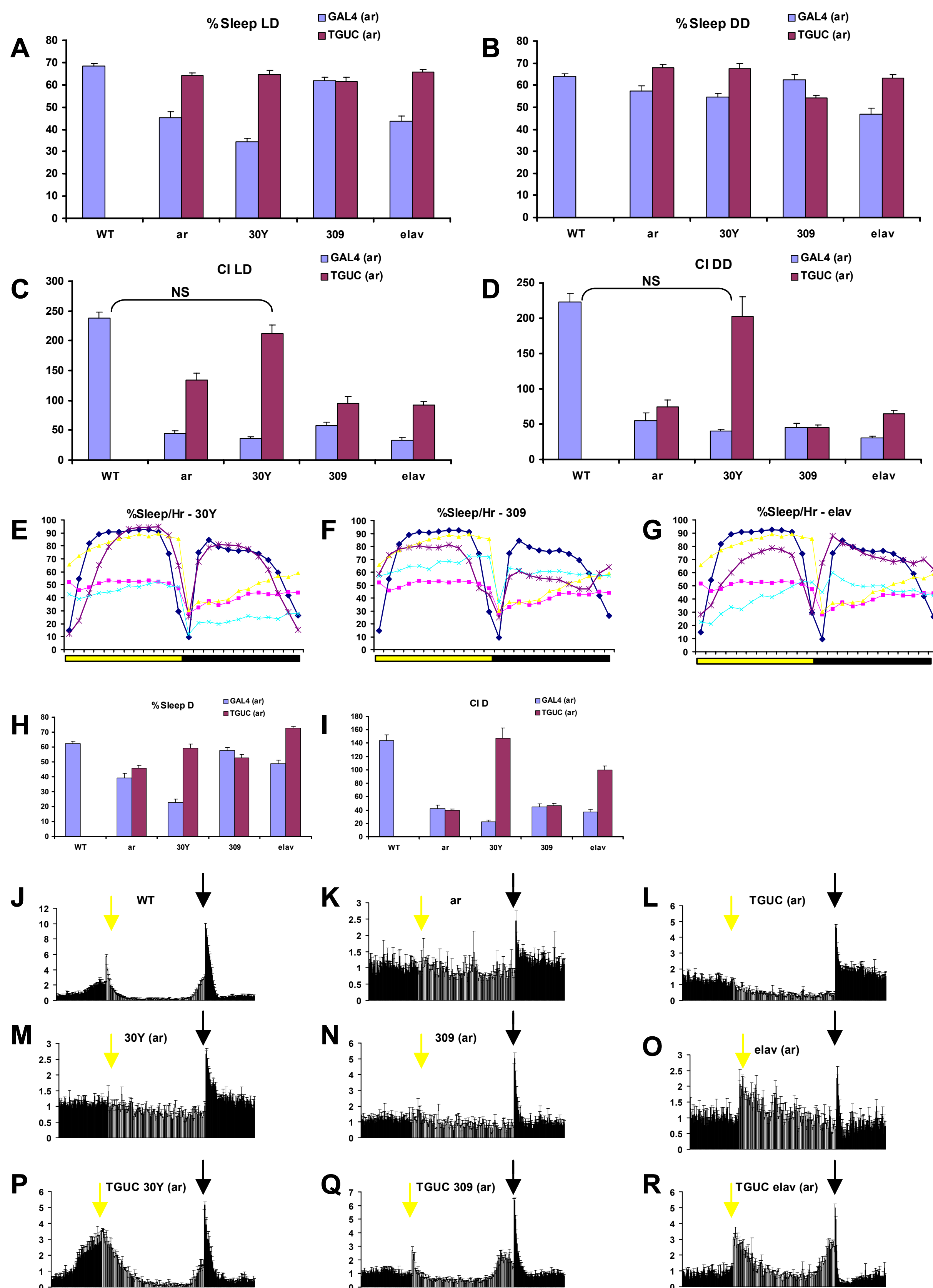
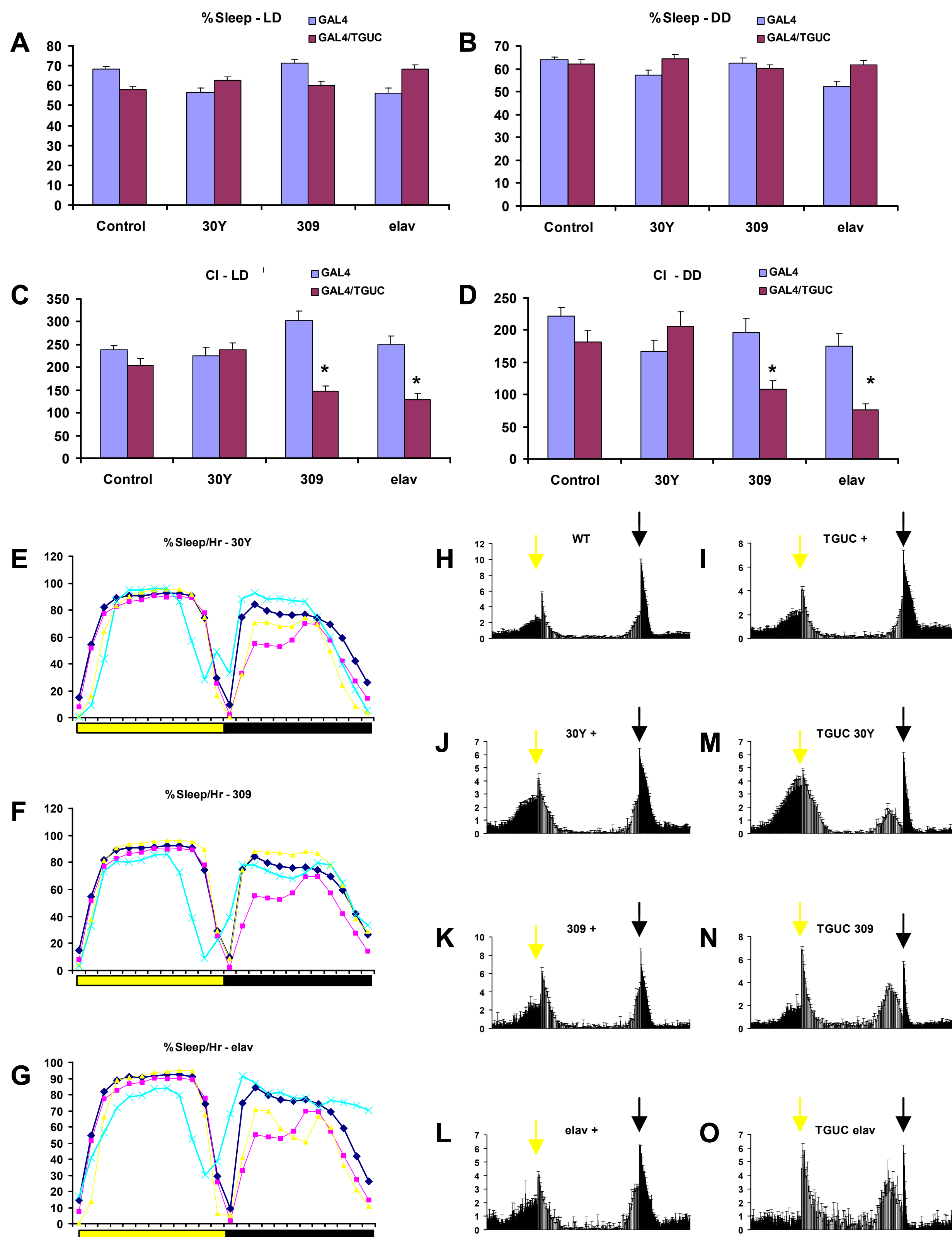
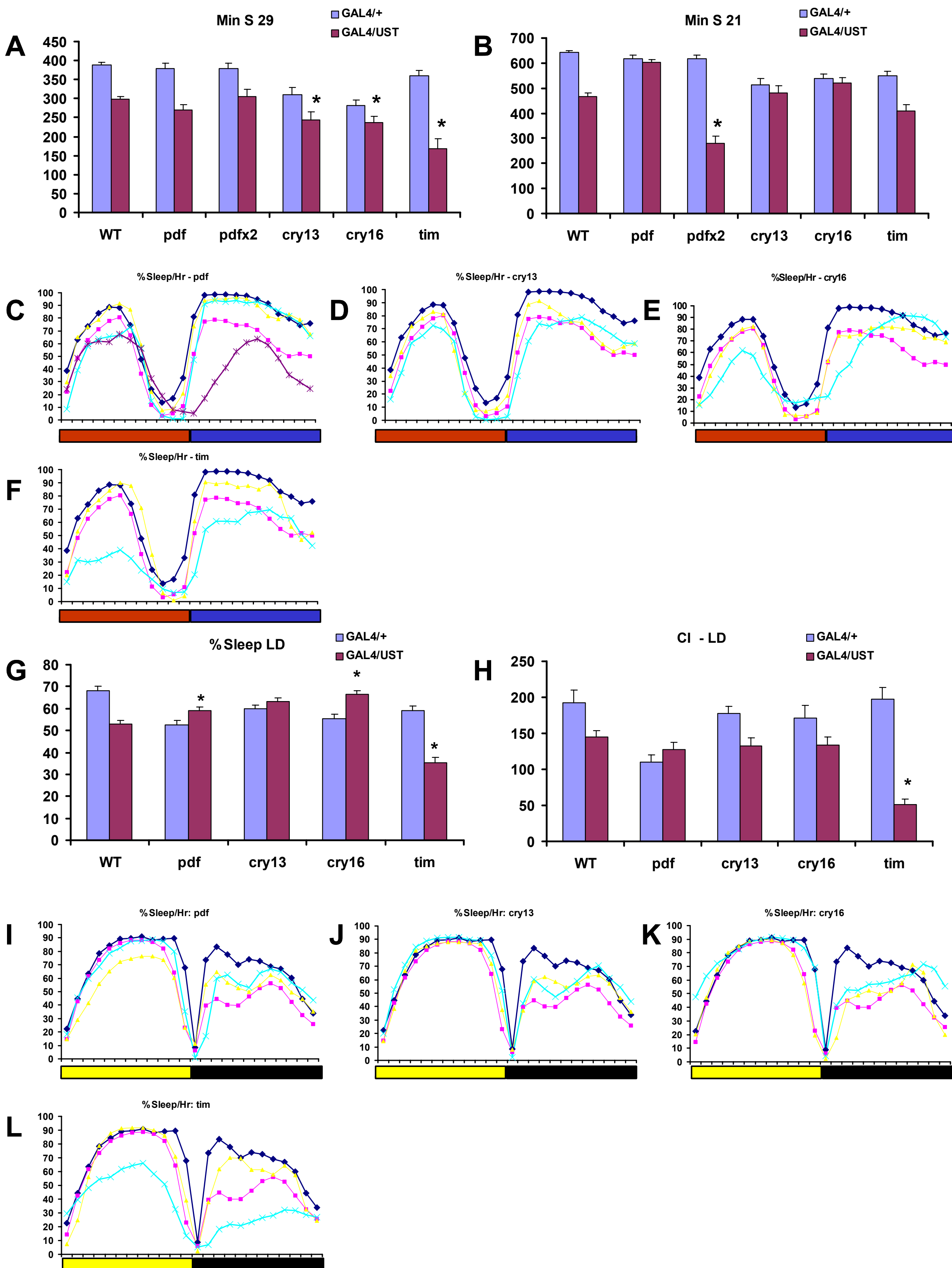

A
% Sleep LD
GAL4 (ar)
TGUC (ar)
WT
ar
30Y
309
elav
B
% Sleep DD
C
CI LD
NS
D
CI DD
E
%Sleep/Hr - 30Y
F
%Sleep/Hr - 309
G
%Sleep/Hr - elav
H
% Sleep D
I
CI D
J
WT
K
ar
L
TGUC (ar)
M
30Y (ar)
N
309 (ar)
O
elav (ar)
P
TGUC 30Y (ar)
Q
TGUC 309 (ar)
R
TGUC elav (ar)

Figure 4.5 ***Clk*** **Expression in broadly expressing GAL4 drivers rescues sleep and/or circadian *Clk*^{ar} mutant phenotypes.** (**A-D**). Sleep and sleep consolidation phenotypes of controls (WT blue bar, *ar* blue (ar homozygous stock) and purple (TGUC(*ar*) bars, 30Y*(ar)*, 309*(ar)*, elav*(ar)* blue bars) and *Clk* rescue (30Y*(ar)*/TGUC*(ar)*, 309*(ar)*/TGUC*(ar)*, elav*(ar)*/TGUC*(ar)*, purple bars) flies in entrained, 29°C, 12hr light:12hr dark (LD, 4 days), and non-entrained constant darkness (DD, 4 days) conditions. Average %Sleep/24 hr LD (**A**), DD (**B**), and sleep consolidation (CI: weighted average sleep bout length, min) LD (**C**) and DD (**D**). (**E-G**) Sleep profiles (24hr profile of 4 day average %Sleep/Hr) for 30Y (**E**), c309 (**F**), and *elav* (**G**) control and rescue genotypes in LD. Thick dark blue line = WT control, yellow line = TGUC (*ar*) control, pink line = *ar* homozygote control, light blue line = GAL4 (*ar*) control, thick purple line = GAL4(*ar*)/TGUC(*ar*) rescue genotype. Yellow bars = 12 hr light phase, black bars = 12 hr dark phase. (**H,I**) Average %Sleep/12 hr D (of LD) (**H**), and sleep consolidation (CI: weighted average sleep bout length, min) D (of LD) (**I**). (**J-R**) Activity profiles (24hr profile of 4 day average normalized activity/5min) of control (**J-O**) and rescue (**P-R**) flies in LD. Lights on time is indicated by yellow arrow and lights off time is indicated by black arrow. WT = *yw* CS outcross, TGUC = *tubulin*GAL80^{ts};UAS*Clk.* With the exception of WT, all flies are in a homozygous *ar* mutant background. Rescue flies were compared to WT using 2-tailed t-test, if comparison is not significantly different (P<0.05), the bar is labeled “NS”. N = 25-111 (LD), N = 25-84 3-6 (DD), N expt = 3-7 (LD), 3-6 (DD). Error bars indicate SEM.



A
% Sleep - LD
GAL4
GAL4/TGUC
Control
30Y
309
elav
B
% Sleep - DD
GAL4
GAL4/TGUC
Control
30Y
309
elav
C
CI - LD
GAL4
GAL4/TGUC
Control
30Y
309
elav
D
CI - DD
GAL4
GAL4/TGUC
Control
30Y
309
elav
E
% Sleep/Hr - 30Y
F
% Sleep/Hr - 309
G
% Sleep/Hr - elav
H
WT
I
TGUC +
J
30Y +
M
TGUC 30Y
K
309 +
N
TGUC 309
L
elav +
O
TGUC elav

Figure 4.6 ***Clk*** **Over-expression in broadly expressing GAL4 drivers results in reduced sleep consolidation and an advanced evening activity peak.** (**A-D**). Sleep and sleep consolidation phenotypes of controls (WT blue bar, TGUC/+, purple bar, 30Y/+, c309/+, *elav*/+ blue bars) and *Clk* over-expression (30Y/TGUC, c309/TGUC, *elav*/TGUC, purple bars) flies in entrained, 29°C, 12hr light:12hr dark (LD, 4 days), and non-entrained constant darkness (DD, 4 days) conditions. Average %Sleep/24 hr LD (**A**), DD (**B**), and sleep consolidation (CI: weighted average sleep bout length, min) LD (**C**) and DD (**D**). (**E-G**) Sleep profiles (24hr profile of 4 day average %Sleep/Hr) for 30Y (**E**), c309 (**F**), and *elav* (**G**) control and over-expression genotypes in LD. Thick dark blue line = WT control, pink line = TGUC/+ control, yellow line = GAL4/+ control, thick light blue line = GAL4/TGUC over-expression genotype. Yellow bars = 12 hr light phase, black bars = 12 hr dark phase. (**H-O**) Activity profiles (24hr profile of 4 day average normalized activity/5min) of control (**H-L**) and over-expression (**M-O**) flies in LD. Lights on time is indicated by yellow arrow and lights off time is indicated by black arrow. WT = *yw* CS outcross, TGUC = *tubulin*GAL80^{ts};UAS*Clk.* "**+**" = wild type allele – homozygous GAL4 or TGUC was crossed to *yw* control, heterozygous progeny represented. Over-expression flies were compared to TGUC/+ and GAL4/+ controls using 2-tailed t-test, significant ($P<0.05$) comparisons are indicated with asterisk (*). N = 25-111 (LD), N = 25-84 3-6 (DD), N expt = 1-7 (LD), 1-6 (DD). Error bars indicate SEM.



A
Min S 29
GAL4/+
GAL4/UST
WT
pdf
pdfx2
cry13
cry16
tim
B
Min S 21
C
%Sleep/Hr - pdf
D
%Sleep/Hr - cry13
E
%Sleep/Hr - cry16
F
%Sleep/Hr - tim
G
%Sleep LD
H
CI - LD
I
%Sleep/Hr: pdf
J
%Sleep/Hr: cry13
K
%Sleep/Hr: cry16
L
%Sleep/Hr: tim

Figure 4.7 Circadian neurons may promote sleep. (**A-F**) Average total sleep (average min sleep/12 hr) at 29°C (**A**) and 21°C (**B**) sleep profiles (24hr profile of 4 day average %Sleep/Hr) for pdf (**C**), cry13 (**D**), cry16 (**E**) and tim (**F**) control and circadian cell inhibited (GAL4/UST) flies during temperature cycling (TC). Thick dark blue line = WT control, pink line = UST/+ control, yellow line = GAL4/+ control, thick light blue line = GAL4/UST genotype. Thick purple line on pdf profile represents data for homozygous pdf;UST flies (pdfx2). Red bars = 12 hr 29°C phase, blue bars = 12 hr 21°C phase. (**G-L**) Sleep and sleep consolidation phenotypes in control and circadian cell inhibited flies in entrained, 29°C, 12hr light:12hr dark (LD, 4 days) conditions. Sleep (%sleep/24hr) (**G**) and sleep consolidation (C**I**: weighted average sleep bout length (min)) (**H**), sleep profiles (24hr profile of 4 day average %Sleep/Hr) for *pdf* (**I**), *cry13* (**J**), *cry16* (**K**) and *tim* (**L**). Thick dark blue line = WT control, pink line = UST/+ control, yellow line = GAL4/+ control, thick light blue line = GAL4/UST genotype. Yellow bars = 12 hr light phase, black bars = 12 hr dark phase. WT = *yw* CS outcross, UST = UASshi^{ts}. "**+**" = wild type allele – homozygous GAL4 or UST was crossed to *yw* control, heterozygous progeny represented. GAL4/UST flies were compared to UST/+ and GAL4/+ controls using 2-tailed t-test, significant ($P<0.05$) comparisons are indicated with asterisk (*). N = 14-97, N expt = 1-8 (**A-F**), N = 24-90, N expt = 2-7 (**G-L**). Error bars indicate SEM.

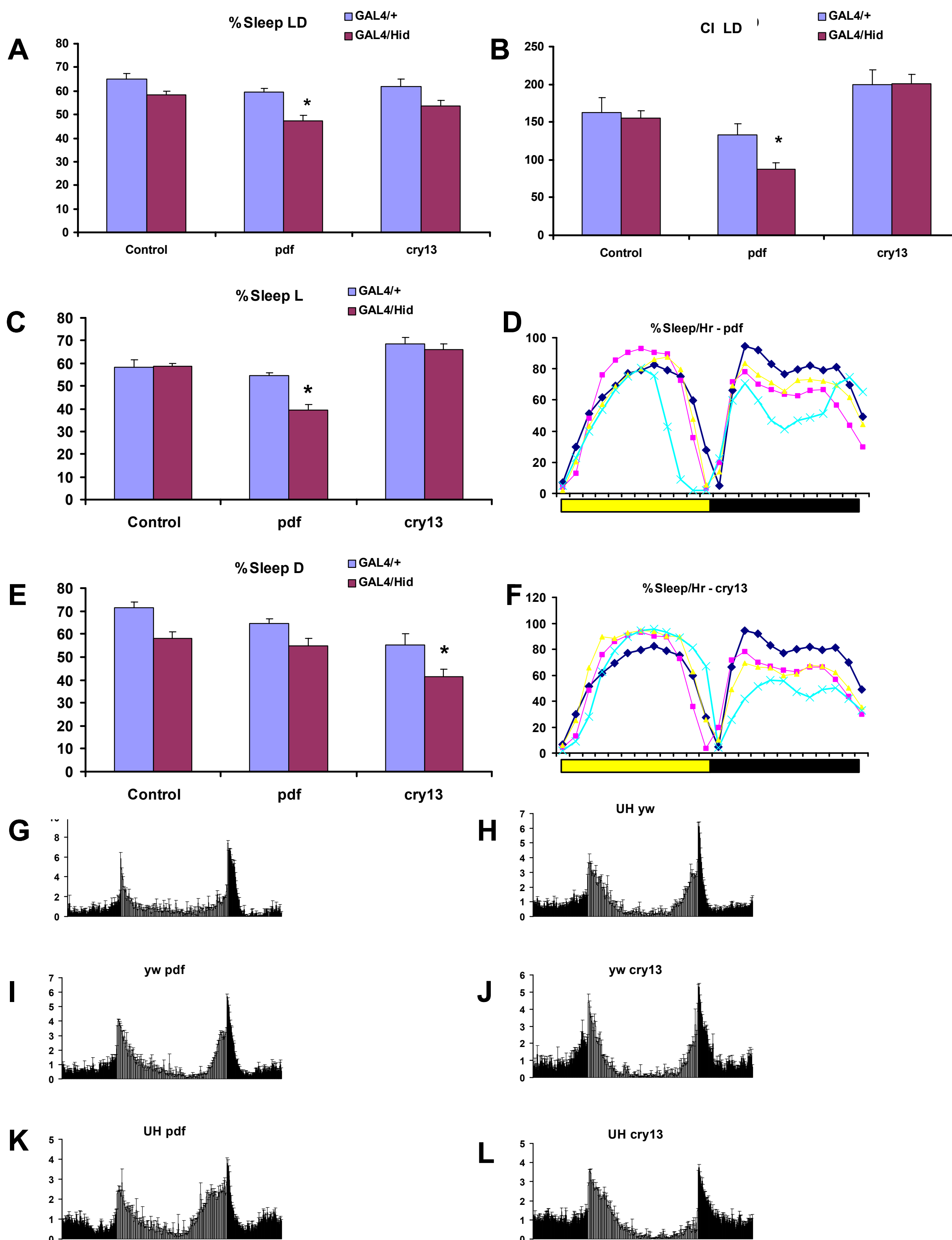

A
% Sleep LD
GAL4/+
GAL4/Hid
Control
pdf
cry13
B
CI LD
C
% Sleep L
D
% Sleep/Hr - pdf
E
% Sleep D
F
% Sleep/Hr - cry13
G
H
UH yw
I
yw pdf
J
yw cry13
K
UH pdf
L
UH cry13

Figure 4.8 Separate groups of circadian neurons may promote sleep during light and dark. (**A-F**) Sleep and sleep consolidation phenotypes in control and circadian cell ablated (GAL4/UH) flies in entrained, 25°C, 12hr light:12hr dark (LD, 4 days) conditions. (**A**) Sleep (%sleep/24hr) and (**B**) sleep consolidation (CI: weighted average sleep bout length (min)). (**C**) Sleep (%sleep/12hr) during L only, sleep profile (24hr profile of 4 day average %Sleep/Hr) for *pdf* (**D**), and sleep (%sleep/12hr) during D only (**E**) sleep profile for *cry*13 (**F**). Thick dark blue line = WT control, pink line = UH/+ control, yellow line = GAL4/+ control, thick light blue line = GAL4/UH genotype. Yellow bars = 12 hr light phase, black bars = 12 hr dark phase. (**G-L**) Activity profiles (24hr profile of 4 day average normalized activity/5min) of control (**G-J**) and circadian cell ablated (**K,L**) flies in LD. Lights on time is indicated by yellow arrow and lights off time is indicated by black arrow. WT = *yw* CS outcross, UH = UAS*hid.* "**+**" = wild type allele – homozygous GAL4 or UH was crossed to *yw* control, heterozygous progeny represented. GAL4/UH flies were compared to UH/+ and GAL4/+ controls using 2-tailed t-test, significant ($P<0.05$) comparisons are indicated with asterisk (*). N = 14-23, N expt = 1-2. Error bars indicate SEM.

Table 4.1 Rhythmicity is reduced in *Clk* mutants. Average period and rhythmicity (P-S: power-strength) for *Clk* mutants in constant darkness (25°C, 7 days) with standard error (SE), and including %Rhythmic (%R) value. P = p-value: genotypes were compared to WT using 2-tailed t-test, significance level is set at P<0.05. N = number of flies, NE = number of experiments.

	PERIOD			P-S					
Genotype	**AVE**	**SE**	**P**	**AVE**	**SE**	**P**	**%R**	**N**	**NE**
WT	24.2	0.1	1.00	80.7	4.3	1.00	100.0	75	6
ar +	24.2	0.1	0.86	48.0	6.7	**0.00**	69.2	52	4
jrk +	24.4	0.1	0.23	4.7	1.5	**0.00**	17.5	40	4
D1 +	24.8	0.1	**0.00**	15.0	3.2	**0.00**	39.5	43	3
ClkP +	24.9	0.1	**0.00**	77.8	6.6	0.71	97.6	41	3
ar				2.4	0.7	**0.00**	0.0	50	4
jrk				2.6	0.8	**0.00**	0.0	59	5
ClkP	26.2	0.1	**0.00**	8.1	2.1	**0.00**	24.0	25	2
ar jrk	24.5			3.8	1.4	**0.00**	2.5	40	3
ar D1				2.0	0.8	**0.00**	0.0	45	3
ar ClkP	26.1	0.2	**0.00**	56.7	7.4	**0.00**	83.8	37	3
jrk D1				0.2	0.2	**0.00**	0.0	16	2
jrk ClkP				1.2	0.6	**0.00**	0.0	43	3
ClkP D1				2.9	1.0	**0.00**	0.0	37	4

Table 4.2 Circadian phenotypes are influenced by genetic background in *Jrk* mutants. Average period and rhythmicity (P-S: power-strength) for backcrossed *Jrk* mutants in constant darkness (25°C, 7 days) with standard error (SE), and including %Rhythmic (%R) value. P = p-value: *Jrk* and *Jrk*/+ flies were compared to WT using 2-tailed t-test, significance level is set at P<0.05. N = number of flies, NE = number of experiments.

	PERIOD			P-S				
Genotype	**AVE**	**SE**	**P**	**AVE**	**SE**	**P**	**%R**	**N**
WT	24.1	0.1	1.00	95.8	13.3	1.00	79.2	24
jrk +	23.7	0.6	0.53	23.6	5.0	**0.00**	58.3	36
jrk	AR	AR		1.2	0.7	**0.00**	0.0	8

Table 4.3 Period and rhythmicity in the *Clk*^{ar} mutant is partially rescued by broad *Clk* expression. Average period and rhythmicity (P-S: power-strength) for controls and *Clk* rescue flies in constant darkness (29°C, 4 days) with standard error (SE), and including %Rhythmic (%R) value. P = p-value: rescue flies were compared to WT using 2-tailed t-test, significance level is set at P<0.05. WT = *yw* CS outcross, TGUC = *tubulin*GAL80^{ts};UAS*Clk.* With the exception of WT, all flies are in a homozygous *ar* mutant background. N = number of flies, NE = number of experiments.

	PERIOD			P-S					
Genotype	**AVE**	**SE**	**P**	**AVE**	**SE**	**P**	**%R**	**N**	**NE**
WT	24.0	0.0	1.00	62.5	2.7	1.00	95.2	84	6
ar				0.2	0.1	**0.00**	0.0	41	4
TGUC (ar)	25.7	0.6	**0.00**	3.3	1.3	**0.00**	10.9	46	5
30Y (ar)				0.2	0.2	**0.00**	0.0	37	3
TGUC 30Y (ar)	23.8	0.3	0.26	14.1	3.2	**0.00**	42.1	38	3
309 (ar)	20.5			1.0	0.4	**0.00**	2.3	43	3
TGUC 309 (ar)	23.6	0.2	**0.01**	6.3	1.1	**0.00**	24.6	57	3
elav (ar)	24.5			0.7	0.5	**0.00**	4.0	25	3
TGUC elav (ar)	22.6	0.2	**0.00**	5.7	2.0	**0.00**	14.3	49	3

Table 4.4 Period is shortened and rhythmicity is reduced by broad *Clk* expression. Average period and rhythmicity (P-S: power-strength) for controls and *Clk* over-expression flies in constant darkness (29°C, 4 days) with standard error (SE), and including %Rhythmic (%R) value. P = p-value: over-expression flies were compared to WT using 2-tailed t-test, significance level is set at P<0.05. WT = *yw* CS outcross, TGUC = *tubulin*GAL80^{ts};UAS*Clk.* "**+**" = wild type allele – homozygous GAL4 or TGUC was crossed to *yw* control, heterozygous progeny represented. N = number of flies, NE = number of experiments.

	PERIOD			P-S					
Genotype	**AVE**	**SE**	**P**	**AVE**	**SE**	**P**	**%R**	**N**	**NE**
WT	24.0	0.0	1.00	62.5	2.8	1.00	95.2	84	6
TGUC +	23.8	0.1	**0.03**	72.4	4.3	**0.05**	100.0	33	3
30Y +	23.9	0.1	0.30	76.5	3.0	**0.01**	100.0	26	2
TGUC 30Y	23.7	0.1	**0.00**	58.0	3.4	0.39	100.0	27	2
309 +	23.7	0.1	**0.00**	53.0	6.7	0.12	91.7	24	2
TGUC 309	23.3	0.1	**0.00**	35.6	4.8	**0.00**	91.7	24	2
elav +	23.3	0.1	**0.00**	83.1	8.8	**0.01**	100.0	15	1
TGUC elav	23.1	0.2	**0.00**	12.3	6.2	**0.00**	43.8	16	1

CHAPTER 5

Discussion and Future Directions

We have presented data implicating three regions of the *Drosophila* brain in sleep regulation, including the mushroom bodies, the central complex, and circadian neurons. We have also further characterized the role of the gene, *Clock*, in sleep, and have found that *Clock* may function to promote sleep within circadian neurons, mushroom body neurons, or both. These data contribute significantly to current sleep research, since they offer a means to more easily identify sleep regulated genes, through the search for mushroom body expressed genes and *Clock* target genes. Focusing research on these two classes of genes should aid the elucidation of the molecular mechanisms governing sleep, and thus, sleep function.

A. Mushroom Bodies and Central Complex

A1. A Role for the Mushroom Bodies and Central Complex in Sleep Regulation

The majority of our data supports a role for the mushroom bodies in sleep promotion. We have shown that inhibition of mushroom body output results in reduced sleep whether inhibited acutely or chronically (Figure 2.7, Figure 2.1), and results in reduced sleep consolidation (Figure 2.5), without altering clock function (Figure 2.6) or increasing activity (Figure 2.5). We showed that mushroom body function in particular was responsible for this phenotype by ablating the mushroom bodies in wild-type or MB inhibited flies (Figure 2.8), and blocking the short-sleep

phenotype with MBGAL80 (Figure 3.1, 3.4). In addition, we show that flies in which MB output is blocked, and sleep is reduced, exhibit an exaggerated response to sleep deprivation (Figure 2.12), and reduced lifespan (Figure 2.11). Finally, we suggest that the α/β lobes in particular may be responsible for sleep promotion (Figure 3.3-3.5), and that different groups of MB cells may be required for sleep promotion during the sleep and wake phase (Figure 3.2), although these cells have not been characterized. Together, these results support a role for mushroom body activity in sleep promotion, resulting in the dissipation of sleep need.

We cannot however exclude the hypothesis that some mushroom body neurons either promote wake, or contain no sleep/wake function at all. The majority of the work of Joiner et al. (2006) is in support of a role for the MB in wake-promotion. Their most convincing conclusions were based on data from one GAL4 line, the MB-Switch, RU486 inducible GAL4 (Mao et al., 2004). When neuronal activity is increased in these cells, sleep is reduced, and when activity is inhibited, sleep is increased (Joiner et al., 2006). We have repeated some of these data (Table 3.3) and have also presented indirect evidence that the MB may promote wake. Many GAL4 lines that expressed strongly in the MB did not effect, or had weak effects on sleep when inhibited with *shibire* (Figure 2.4, Figure 3.5), or when MB activity was inhibited or disrupted by other means (Table 3.3). Increasing GAL4 dosage, and/or combining neutral MB GAL4 lines together did not result in a sleep phenotype when inhibited, suggesting that this was not the result of weakly-expressing GAL4 lines (Figure 3.5). We conclude based on these data that either the MB GAL4 lines we tested contain both sleep-promoting and wake-promoting neurons, whose effects may cancel each other, or that a number of MB neurons do not regulate sleep or wake at all.

We have also presented evidence that MB α'/β' lobe neurons, and/or neurons of the central complex may be directly wake-promoting (Figure 3.6). Long-sleep GAL4 lines c320 and c305a (Krashes et al., 2007) contain α'/β' lobe expression, and c596a contains MB expression, but this has not been localized to either α/β or α'/β' lobes (Armstrong and Kaiser, 1996). All long-sleep GAL4 lines contain CC expression. The most specific long-sleep GAL4 line, c547, expresses in a small subset of ellipsoid body R-type neurons, the R2 and R4m neurons (Renn et al., 1999a). Three additional long-sleep GAL4 lines, c320, c596a, and c305a contain CC expression specifically, the ellipsoid body (c320 and c305a), fan shaped body (c320, c596a), and noduli (c320), in addition to MB expression as mentioned above (Armstrong and Kaiser, 1996; Krashes et al., 2007). These data are very preliminary, and should not be over-interpreted, since we experienced difficulty reproducing long-sleep phenotypes, these experiments represent a relatively low number of flies, and we found many of the long-sleeper lines to increase adult and developmental lethality. Also, with the exception of c547, long-sleep GAL4 lines did not have consistent long-sleep phenotypes under temperature cycling and constant temperature conditions. Excessive sleep could easily be the indirect result of sickness, and as such, additional measures to assess the health of long-sleep flies such as lifespan, walking/ eating/ grooming/ courtship ability/ response to stressors should be performed. As a first pass filter, flies with significantly lower waking activity should be excluded as potentially "sick". The long-sleep GAL4 lines discussed here did not exhibit reduced waking activity.

It should be noted that we did not observe large changes in waking activity in either sleep-regulatory or non-sleep regulatory GAL4 lines (Figure 2.5 and data not shown). Martin et al., (1998; 1999) determined a role for the MB and CC in walking activity, specifically they concluded that both regions regulate walking bout length, the MB suppress bout length, and the

CC enhance walking bout length. Experimental differences between our two experiments may explain these results. Martin et al. examined activity over a short period of time (4.5 hr vs many days), in female flies (vs. males), in total darkness, in a different container (rectangular, vs. circular, with water but not food), and in finer detail (sampling rate of 1 Hz, as opposed to activity counts/min). They also inhibited MB and/or CC function using a *tetanus toxin* transgene, expressed throughout development, rather than an adult expressed temperature sensitive *shibire* transgene. While these reasons alone could account for observed differences in activity between our experiments and theirs, it is also interesting to note that MB GAL4 lines that they found to regulate activity (H24, 17D, 201Y) were not found to regulate sleep amount (Figure 3.5). This may indicate that specific regions of the MB could regulate activity, separate from those areas that regulate sleep. Another possibility is that they were able to observe phenotypic effects of *tetanus toxin* expression in these cells that were not observed following *shibire* expression, which may be explained by the fact that the efficiency of these two transgenes differ depending on the properties of the targets, and time of transgene induction (Thum et al., 2006).

A2. MB and CC: Future Directions

Although we currently have no concrete evidence to the contrary, these data suggest that it may be too simple to classify the MB as a “sleep promoting center”. Many areas in the mammalian brain that are active during sleep are also active during waking (see introduction), and it is possible that the MB acts in a similar manner. Given the relative simplicity of the *Drosophila* nervous system, it is likely that discrete regions within the MB are involved in sleep-promotion, wake-promotion, and sleep/wake state stability in some capacity; or even that the same cells may perform all functions. Sleep negative/neutral GAL4 lines may contain both sleep

promoting MB and wake promoting MB and/or CC cells, resulting in a neutral effect on sleep. Alternatively, the same group of MB cells may both promote sleep and wake at different times of day in negative GAL4 lines, resulting in no overall effect on sleep when examined over 24hr. Thus, it will be crucial to design experiments to functionally and anatomically sub-divide the MB into sleep/wake/neutral circuitry.

As covered extensively in the introduction, neither the MB nor the CC should be considered homogeneous structures. Dendrites and axons from different MB lobes are segregated anatomically, and it seems likely that inputs onto MB cell bodies, dendrites, or axons, will further functionally sub-divide lobes (Lin et al., 2007). In addition, since vertical (α/α') and medial (β/β'/γ) MB lobes contact extrinsic output neurons that project to distinct neuropil regions (Ito et al., 1999), this suggests another level of complexity in MB organization. Similarly in the CC, dendrites of EB neurons are segregated, small field neurons link different regions of the CC together, and large field neurons link the CC to the rest of the brain in a manner that is poorly understood (Renn et al., 1999a), but suggests a high degree of complexity.

As a starting point for dividing the MB and CC into sleep/ wake/ neutral circuitry, it will be necessary to quantify the extent of MB and CC expression in each sleep-relevant GAL4 line, by using α/β, γ, and α'/β' lobe and CC specific antibody staining (Crittenden et al., 1998; References within Renn et al., 1999a). We could also employ more finely detailed mapping techniques (Lin et al., 2007) to map GFP expression patterns within the MB and CC. Lin et al. (2007) have developed a technique called "warping" to map GFP expression patterns onto a "standard MB", using anatomical landmarks. Using their pioneering work as a template, we may be able to "warp" our sleep relevant GAL4 lines (eg. 30Y) onto the standard MB, to determine the precise location of GFP labeled cells within MB lobes. We could develop a similar "standard

CC" to map GFP expression onto as well. Since all but one long-sleep GAL4 contains ellipsoid body (EB) expression, it is tempting to conclude that the EB promote wake, however, much more detailed anatomical and behavioral analysis needs to be applied to these lines before making this conclusion. In fact, an analysis from Krashes et al. (2007) suggests that the EB neurons labeled by c305a and c320 are not the same cell types. This does not invalidate a role for the EB in wake-promotion, but does suggest that the EB and/or entire CC may play a complicated role in this process. These anatomical techniques ideally should be applied to all GAL4 lines used to assess sleep, including sleep promoting, wake promoting, and sleep-neutral GAL4 lines. In this way, conflicting results of MB GAL4 lines promoting sleep, wake, both, or neither, may be partially resolved.

Additionally, the anatomical and functional refinement of sleep and wake-promoting cells will be greatly aided by developing a variety of MB and CC specific GAL80 lines. These can either be generated by enhancer trapping techniques, or by exchanging GAL4 with GAL80 in sleep-relevant GAL4 lines, a strategy which is currently being attempted in our lab (unpublished data, Gang Liu). There is some evidence that the 30Y and c309 GAL4 lines may contain both wake promoting and sleep promoting cells (Chapter 3, Joiner et al., 2006), and that cells within these GAL4 lines may employ different molecular mechanisms in sleep/wake promotion (Figure 4.5, Joiner et al., 2006). The elucidation of these sub-groups could be aided by generating 30YGAL80 and c309GAL80 lines. For instance, if 30YGAL80 blocked GFP expression and sleep-promoting properties of c309GAL4 or vice versa, this would indicate that these GAL4 lines were expressed in the same neurons. If 30YGAL80 did not block the sleep-promoting properties of c309GAL4, this would indicate that the two GAL4 lines label anatomically distinct regions of the MB. Another possibility is that 30YGAL80 could block both

the sleep and wake promoting functions of c309, or vice versa, indicating that these cells are dual-function, and not anatomically distinct groups of cells. As mentioned, long-sleep GAL4 lines contain both MB and CC expression. As a first step in determining whether the MB or CC cells are wake-promoting, MB expression in long-sleep GAL4 lines should be blocked by MBGAL80, or newly generated CC-specific GAL80 lines, and the long-sleep phenotype reassessed.

It will also be important to determine neurotransmitter profiles within MB and CC neurons to determine the precise relationship between these groups, and the nature of the sleep/wake circuit. For example, the function of the VLPO in sleep was aided by learning not only that it was active during the sleep phase (Sherin et al., 1996; Szymusiak et al., 1989; Szymusiak et al., 1998), but that it used the inhibitory neurotransmitter, GABA (gamma-aminobutyric acid), to inhibit areas active during wake (Sherin et al., 1998; Steininger et al., 2001). The neurotransmitters used by most MB and CC neurons are unknown, however there is evidence that at least some α'/β' lobe and EB neurons may be cholinergic, since a GAL80 that specifically blocks cholinergic cells blocks α'/β' lobe and EB neuron GFP expression in c305GAL4 (Krashes et al., 2007). Antibody staining also suggests that other EB neurons may be GABAergic (Hanesch et al., 1989). Since at least one type of large-field CC neuron has been identified that could link regions of the MB (α/α' lobes) to the CC (Martin et al., 1999; Liu et al., 2006), this could provide an anatomical circuit by which the potentially wake-promoting CC cells could inhibit sleep-promoting MB cells. To further elucidate neurotransmitter identities of MB and CC neurons, RNAi against different neurotransmitter precursor molecules can be targeted within sleep or wake-promoting cells, and the effect on behavior can be observed.

A3. MB and CC: An Anatomical Link Between Sleep and Memory

One of the most significant findings of the preceding work is that regions important for memory formation are also involved in sleep regulation, including both the mushroom bodies (particularly olfactory memory) (Review: Keene and Waddell, 2007) and central complex (visual memory) (Liu et al., 2006). It is unlikely to be a coincidence that tissues involved in synaptic plasticity are also critical for sleep regulation, especially given the accumulating behavioral (Review: Walker and Stickgold, 2006), electroencephalographic (Huber et al., 2004a; Marshall et al., 2006; Huber et al., 2007), and genetic (Hendricks et al., 2001; Cirelli et al., 2004; Cirelli et al., 2006; Ganguly-Fitzgerald et al., 2006), evidence suggesting a link between the two processes. The two sensory modalities shown to be most important for the social-enrichment induced sleep increase in *Drosophila* were vision and olfaction (Ganguly-Fitzgerald et al., 2006), the two regions thought to provide the primary synaptic input to the mushroom bodies (olfaction) (Oleskevich et al., 1999), and central complex (visual) (Hanesch et al., 1989). Data presented in this thesis provides the first evidence that sleep and plasticity/memory behaviors are co-localized within the same regions in the brain, (although a link between the CC and sleep is very preliminary) and suggests that molecular pathways regulating plasticity may serve as part of the homeostatic signal for sleep need.

We cannot yet make a strong conclusion of which cells are responsible for sleep and wake-promotion, however, parallels between anatomical functions of the α’/β’ and α/β lobes in learning and memory suggests one potential model: Mushroom body regions thought to store memory traces, the α/β lobes (Dubnau et al., 2001; McGuire et al., 2001; Pascual et al., 2001; Schwaerzel et al., 2002 Yu et al., 2006) may contain sleep-promoting neurons (Chapter 2, 3), and mushroom body regions whose activity is required to consolidate memories, the α’/β’ lobes

(Krashes et al., 2007), may contain wake-promoting neurons (Figure 3.6). This suggests that that α/β lobe neurons may function downstream of α'/β' lobe neurons in memory processing, and we propose a similar relationship for sleep/wake regulation. In this model, α'/β' lobe neuronal activity during wake results in the accumulation of synaptic changes, which are stored within α/β lobe neurons. The accumulation of synaptic changes itself may act as the "sleep need" signal, perhaps through increased levels of phosphorylation, calcium levels, AMPA receptors, brain derived neurotrophic factor, CREB, or any other molecule known to increase during synaptic potentiation (Review: Beninigton and Frank, 2003). The synaptic plasticity induced sleep need signal could then act as a trigger to activate α/β lobe neurons, whose intrinsic activity promotes sleep (Figure 5.1). This model not does necessarily support a role of further memory consolidation in sleep, but instead, suggests that synaptic changes during wake may serve as the "sleep need" signal. The model predicts that increasing α'/β' lobe output acutely should result in a subsequent increase in sleep. It also predicts that sleep need continues to accumulate in flies in which α/β lobe neuron output has been chronically inhibited. In fact, we observed that lifespan was dramatically reduced, and sleep rebound was enhanced in MB-inhibited short-sleep flies (Figure 2.11), which GFP expression patterns indicate express primarily in the α/β lobes. This would be the expected result of a "chronically sleep deprived" animal who is unable to reduce sleep need, as in chronically sleep-deprived rats (Rechtschaffen and Bergmann, 1995).

B. Circadian Neurons and Clock

B1. A Role for Circadian Neurons and Clock in Sleep-Promotion

We present data in support of a role of circadian neurons in sleep promotion (Chapter 3, 4). We show that sleep during the light phase is partially promoted by the sLNv and lLNv in 30YGAL4, and by the LNv, and LNd in c309GAL4, also during the light phase (Figure 3.1). Ablation of the LNv (with *pdf*GAL4) and Lv/LNd/DN1 (with *cry*13GAL4) results in reduced sleep (Figure 4.8), specifically, the LNv may promote sleep during the light phase, and the LNd/DN1 may promote sleep during the dark phase. Inhibition of circadian cell output, using the *tim*GAL4 driver, which expresses in all circadian neurons, dramatically reduces sleep (Figure 4.7). The interpretation of this experiment is complicated by non-circadian cell expression in this GAL4 line, however, that is partially testable by combining *tim*GAL4 with *pdf*GAL80 and/or *cry*GAL80, to suppress circadian cell expression. If a short-sleep phenotype remains following *cry*GAL80 block, then this suggests a role for non-circadian *tim*GAL4 cells in sleep promotion, but also may implicate circadian cells not blocked by *cry*GAL80, such as the DNs (Stoleru et al., 2004). To further differentiate between DNs and non-circadian cells would require the generation of new, DN-specific GAL80 lines, or GAL80 lines comprising all circadian neurons (eg. *tim*GAL80). Although the *pdf*, *cry*13, and *tim* drivers all show sleep effects following either inhibition or ablation, the *cry*16 driver only minimally affects sleep, decreasing sleep slightly when *cry16*GAL4 cells are inhibited under cycling conditions, and increasing sleep under constant conditions (Figure 4.7). This may be due to significant ellipsoid body expression in this line masking a sleep promoting effect of circadian cells, which could also be assessed using *cry*GAL80. It is unclear why *pdf* and *cry*13 GAL4 lines showed sleep effects when the cells were

ablated, but not when the cells were inhibited (as a single GAL4, in the case of *pdf*). One possibility is that this may reflect stronger driver strength during development, when *hid* is active, than in adulthood, when *shibire* is activated, resulting in total cell ablation in the first case, but inefficient cellular inhibition, in the second.

Data presented in this thesis (Chapter 4) further supports a role for *Clock* in sleep promotion and consolidation. Clk^{Jrk}, Clk^{ar}, and D1 mutants have reduced sleep amount and sleep consolidation in both LD entrained, and DD constant darkness conditions, and dramatically different sleep distribution in LD (Figure 4.2). Sleep amount is not correlated with activity in LD or DD (Figure 4.2). Phenotypes of *Clock* heterozygotes suggest that the Clk^{ar}, and D1 alleles semi-dominantly affect, and Clk^{Jrk} dominantly affects both sleep and circadian rhythmicity (Figure 4.2, Table 4.1). A new *Clock* allele, Clk^{p}, weakly affects both sleep and rhythmicity, most likely due to significant amounts of remaining *Clk* function (Figure 4.1). While we show that components of the Clk^{Jrk} phenotype may be modified by genetic background, such as sleep in DD, there is still a strong affect of Clk^{Jrk} on sleep in LD, and a trend towards a significant Clk^{Jrk} effect on consolidation in both LD and DD, suggesting that these effects are real (Figure 4.2). No other arrhythmic circadian mutant exhibits a similar sleep and consolidation phenotype, suggesting that the *Clock* sleep phenotype is relatively unique. In addition, we show that reduced sleep consolidation in DD is not an indirect effect of decreased circadian arrhythmicity, since consolidation and rhythmicity are weakly correlated (Figure 4.4). Given the contribution of genetic background to *Clock* sleep and rhythmicity phenotypes, we were hesitant to assign a function to *Clock* in sleep without first rescuing *Clock* function. Although the results of the rescue experiment were also complicated by genetic background effects (sleep, in one control line), we were able to completely rescue *Clock* consolidation and %sleep during the dark, and

partially rescue *Clock* period and rhythmicity phenotypes, with the 30YGAL4 driver (Figure 4.5, Table 4.3). Other drivers (c309 and *elav*, others not shown) partially rescued circadian phenotypes, but not consolidation. *Clock* over-expression in 30Y cells did not effect consolidation, whereas *Clock* over-expression in c309 and *elav* reduced sleep consolidation (Figure 4.6), suggesting either that elevated levels of CLK protein disrupt consolidation only in cells in which *Clock* is not normally required for this function, or that *Clock* levels were too high in these cells. Its not entirely clear why circadian specific drivers promoted sleep, but did not rescue the *Clock* sleep phenotype, but suggests a possible role for *Clock* in sleep promotion outside of circadian cells.

B2. Proposed Models and Future Directions

B2.1.Reassessment of the Opponent Process and 2-Process Models of Sleep Regulation

Our data support a role for circadian neurons, particularly the LNv, in sleep promotion, but prompt the question, how do circadian neurons regulate sleep amount? It was proposed following lesions of the circadian pacemaker (SCN) in monkeys, rats, and mice that circadian neurons actively oppose sleep need, since SCN lesions resulted in an increase in sleep (Edgar et al., 1993; Mendelson et al., 2003; Easton et al., 2004). Our data does not support a role for circadian neuron activity in opposing sleep need, and if anything, suggests the opposite. The 2-process model of sleep regulation (Daan et al., 1984) also cannot explain our data, since the 2-process model does not predict that sleep will change as a result of circadian cell ablation. Instead, the 2-process model suggests that the pacemaker sets the threshold for sleep. Presumably, this occurs at the cellular level in sleep regulatory tissues by an integration of circadian timing signals and the accumulation of homeostatic sleep need. The crux of the issue is, how can the pacemaker

oppose sleep (promote wake) in two species and oppose wake (promote sleep) in two other species? To resolve this discrepancy I propose the following model, which integrates features from both the opponent process and 2-process models, called the "sleep gradient" model. Assume that the circadian signal is a temporal gradient, as interpreted by target cells, for example, the cyclical transcription of a hormone within pacemaker tissues, or the direct or indirect pattern of synaptic activity from pacemaker to sleep-regulatory cells. In species with "sleep promoting" pacemakers (flies), the signal increases from morning to night, and at night at its highest levels, interacts with the sleep need signal in sleep target tissues to initiate sleep (for example, via activating an intercellular pathway, leading to the activation of sleep-promoting genes). In species with "wake-promoting" pacemakers (mice/monkeys/rats), secretion of the signal is highest at wake, and decreases throughout the day. When it is at its lowest levels, this interacts with the sleep need signal to initiate sleep (for example, by inactivating an intercellular pathway). In both cases, this signal can be thought of as a "sleep inhibitor", since sleep is not initiated until the sleep need signal passes some circadianly determined threshold; in the first case, a high threshold, and in the second case, a low threshold. Following circadian cell ablation then, sleep centers in "high gradient" species and "low gradient" species would no longer sense the timing signal, but would interpret this information very differently – in flies, the lack of a signal would tend to promote wake, and in monkeys/mice/rats, the lack of a signal would tend to promote sleep (Figure 5.2). This is only one of many possible explanations, but hopefully illustrates the point that the 2-process and opponent process models cannot explain this data set individually. It remains to be determined why and how a species-specific difference in the role of the pacemaker in sleep regulation exists. This model can be tested in flies by selectively increasing or decreasing circadian cell activity and observing the affect on sleep amount. Also,

the model could eventually be verified by identifying peptides released from pacemaker tissues, observing their sites of action and release patterns, and observing sleep phenotypes in loss-of function mutants. Alternatively, if the signal is via synaptic activity, connections between the pacemaker and sleep-regulatory regions could be traced, and the influence of pacemaker on sleep-regulatory targets could be assessed electrophysiologically.

B2.2. How/Where Does Clock Function in Sleep Regulation?

These data support a role for *Clock* in promoting sleep amount and consolidation, and localize the action of *Clock* to both circadian and mushroom body neurons, but these experiments have not provided conclusive evidence on how *Clock* performs this function. *Clock* could be working via three different mechanisms (Figure 5.3). First, it could be involved in generation of the timing/sleep promoting signal of circadian neurons. Second, it could be involved in generation of the homeostatic sleep need signal, in sleep tissues. Third, it could be involved in the integration between sleep timing and sleep need signals in sleep tissues, and responsible for activating the transcription of genes resulting in sleep promotion/sleep need dissipation.

To distinguish between a role for *Clock* in sleep need accumulation or dissipation, it should first be determined whether *Clock* mutants accumulate sleep need. Ways to examine this include assessing the response of *Clock* mutants to sleep deprivation, and examining lifespan. If sleep rebound is prevented or enhanced in *Clock* mutants, both abnormal responses to sleep deprivation, this could indicate a normal accumulation, but difficulty dissipating sleep need, and therefore, an improperly functioning sleep output. In the first case, sleep outputs cannot function at all to dissipate sleep need, and in the second case, sleep outputs function inefficiently to dissipate sleep need. If rebound is equivalent in controls and *Clock* mutants (for the same amount

of sleep loss), this could indicate a normally functioning homeostat, capable of dissipating sleep need. Published data on the *Clk*^{Jrk} mutant suggests that *Clock* mutants exhibit a hyper-rebound response to sleep deprivation (Shaw et al., 2002). If *Clock* mutants have a reduced lifespan, this could also indicate a chronically sleep deprived state due to an inability to reduce sleep need, culminating in early death. Other possibilities could explain this result, however, given that *Clock* is widely expressed, and alters the transcription of many genes (McDonald and Rosbash, 2001), some which are most likely not involved in sleep. Preliminary data on *Clk*^{Jrk} mutant lifespan not presented in this thesis suggests that lifespan is not reduced in these mutants. Sleep and lifespan should be measured congruently (not simply correlated), to determine if the increasing sleep trend that we saw in many *Clock* mutants in DD (Figure 4.2) continues throughout life, and results in an eventual abolishment of the *Clock* mutant sleep phenotype.

To distinguish between the possibility that *Clock* promotes the transcription of a sleep-promoting factor from within circadian neurons, or functions within sleep tissues, further tissue specific rescue experiments should be attempted, in parallel with tissue-specific *Clock* gene disruption. The 30Y driver expresses in both circadian and MB neurons, leaving open the possibility that *Clock* functions within either of these regions or both for normal sleep promotion. We can use MBGAL80 to block *Clock* rescue in MB neurons, or *pdf*GAL80/*cry*GAL80 to block *Clock* rescue in circadian neurons. If an intermediate rescue phenotype is observed with either GAL80, then this supports a role for *Clock* in both tissues. Since genetic background effects complicated the interpretation of previous rescue experiments, these experiments should either be attempted using a backcross/ genotype/ phenotype strategy as used for *Clk*^{Jrk} (Figure 4.3) or stocks used for rescue experiments should be backcrossed into the same genetic background. In parallel, we should examine the contribution of *Clock* to sleep independently, by both knocking

down *Clock* levels in tissue specific regions using RNAi, or by over-expressing a dominant negative *Clock* transgene, such as one similar to the Clk^{Jrk} mutation. Attempts to over-express a putative dominant negative *Clock* transgene (Tanoue et al., 2004) in MB and circadian neurons did not result in sleep phenotypes (not presented in this thesis), however these data represent a small number of flies, and many GAL4/UASClkDN combinations resulted in adult lethality. It is possible that ClkDN was not expressed at high enough levels to affect native CLOCK levels, and influence sleep amount. These and other possibilities should be assessed if using this transgene again, or re-attempting the strategy using a different transgene.

B2.3. Consolidation Differentiates Clk and Cyc from other Circadian Mutants

The preceding experiments present a role for *Clock* in promoting both sleep amount and sleep consolidation. None of the other circadian mutants examined shared both of these features (Figure 4.4). What is it about *Clock* that makes it unique? A simple answer might be that its functions in sleep are due to expression in non-pacemaker, MB cells (Houl et al., 2006). However, cyc^{01} mutants also exhibit reduced sleep consolidation, to a similar magnitude as *Clk* mutants. The exact distribution pattern of CYC in *Drosophila* is unknown, but the finding that CYC is in abundance of CLK by 1000X suggests that it may be ubiquitously expressed (Bae et al., 2000). Therefore, *cyc* may also function within pacemaker and/or non-pacemaker cells to promote consolidation. In addition to *Clk* rescue, we should attempt tissue-specific rescue of the *cyc* consolidation phenotype. It is possible that different tissues regulate sleep consolidation and sleep amount, for instance, consolidation (*Clk, cyc*) may be regulated by circadian neurons, and the mushroom bodies may regulate sleep amount (*Clk*), or vise versa. The observation that neither *per* or *tim* mutations reduce consolidation suggests that this may be a feature of the

positive arm of the circadian feedback loop, where CLK/CYC dimers are required in certain tissues to activate genes required for consolidated sleep. It will be interesting to examine whether CLK binds CYC, or a different partner in MB tissues, where they may together or separately activate genes required for sleep consolidation.

C. Uncovering the Function of Sleep: The Search for MB expressed, and Clock Target Genes

Taken as a whole, the data presented in this thesis serves as a foundation for targeted sleep gene discovery. Future experiments should be directed towards the search for novel mushroom body expressed and *Clock* target genes, or mining already established data sets (McDonald and Rosbash, 2001; Kobayashi et al., 2006), and testing or generating new mutant alleles. Once additional sleep-regulatory genes have been discovered and placed into current or novel molecular pathways we may finally be in a position to understand the function of sleep. The elucidation of sleep function may then lead to the development of new strategies to enhance sleep efficiency, and improve the quality of life for millions of people worldwide.

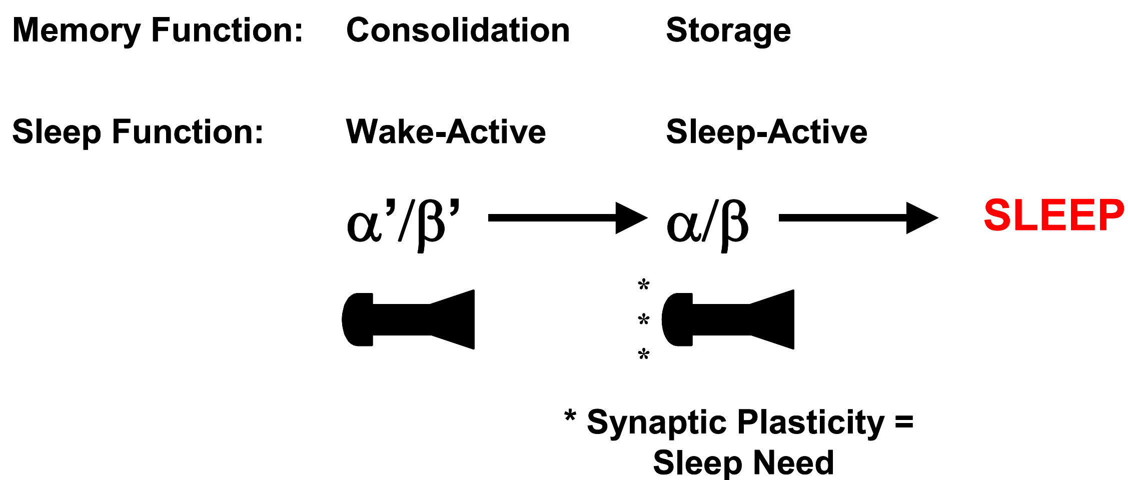
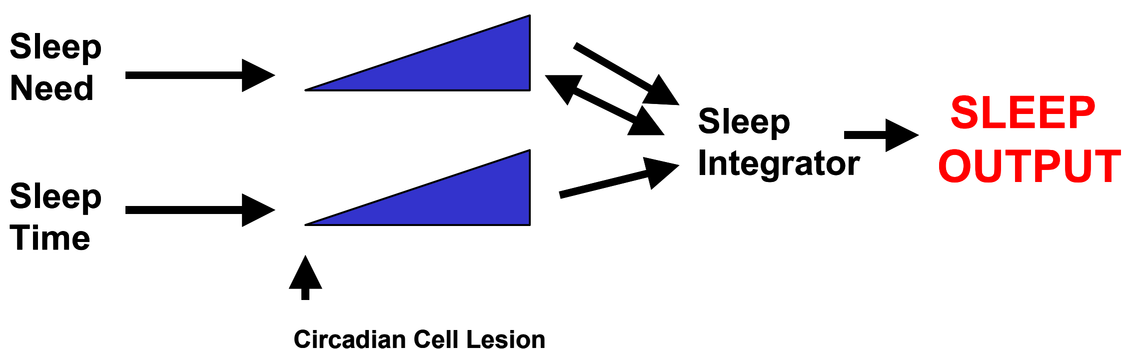


Figure 5.1 Proposed anatomical parallel between memory consolidation and sleep promotion. In this model, during wake, α'/β' neurons induce synaptic changes onto dendrites of α/β lobe neurons. Increased synaptic plasticity acts as a signal of sleep need, triggering activity of sleep-promoting α/β lobe neurons.



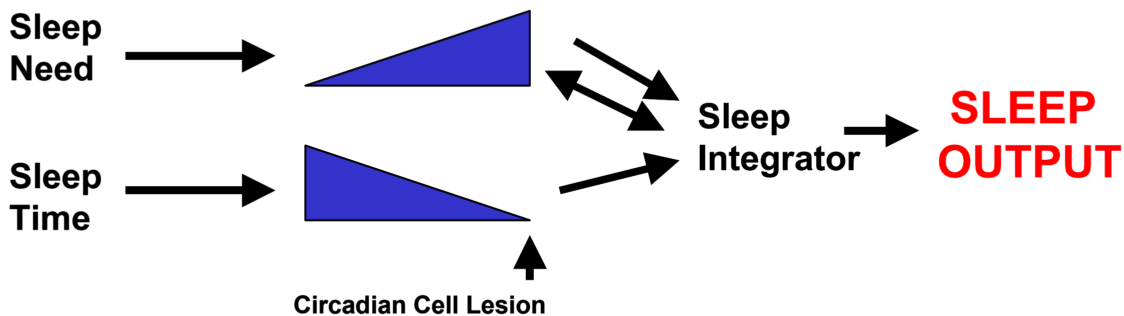


Figure 5.2 Proposed "sleep gradient" model of sleep regulation. In this model, the pacemaker either generates a sleep-opposing signal in either an increasing or decreasing temporal gradient. The timing gradient is integrated at the level of the sleep-regulatory region with a sleep need signal, which may be itself generated by sleep-regulatory tissues (double headed arrow), or other tissues in the brain. When sleep need has surpassed a circadianly determined threshold, sleep is initiated. "Circadian lesion" represents the location of the gradient following pacemaker cell ablation.

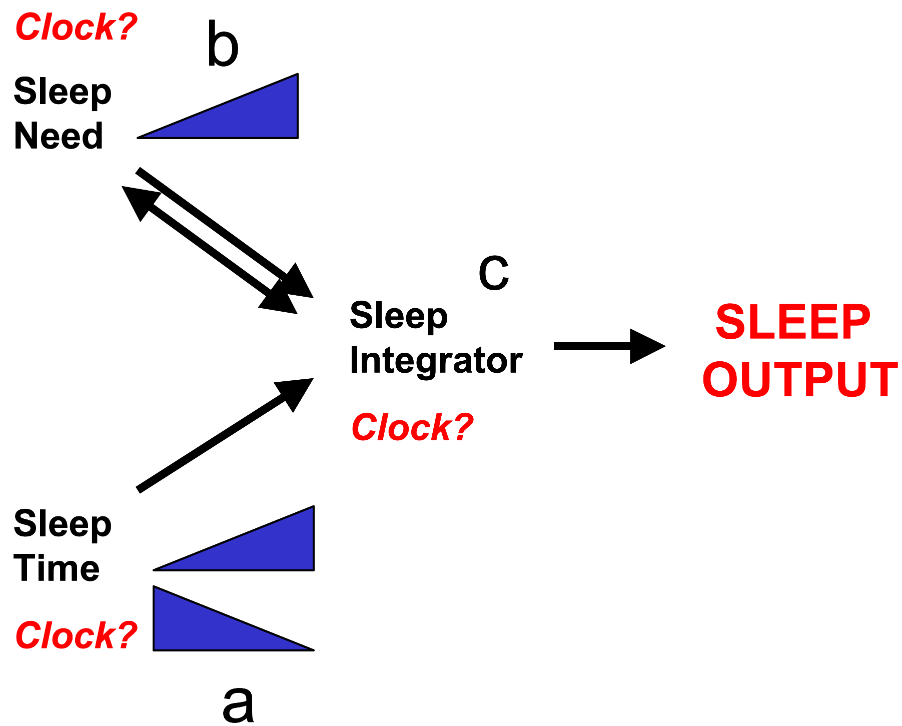


Figure 5.3 Three potential mechanisms of *Clock* action in sleep homeostasis. *Clock* may function to activate the transcription of genes required to a) generate the sleep-timing signal from pacemaker cells, b) generate the “sleep need” signal, or c) integrate sleep need and sleep timing signals and promote sleep initiation/ sleep need dissipation.

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2002-2007 **Ph.D. Thesis Research:**
"Genetic and Anatomical Regulation of Sleep in *Drosophila*: A Role for the Mushroom Bodies, Circadian Neurons, and the Gene, *Clock*, in Sleep Promotion"
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"EEG recording of sleep in a rat during 24 hr total sleep deprivation by a novel "slowly rotating wheel" method", **Dr. Fred Turek**
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Laboratory of **Dr. Benjamin Rusak**
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2003-2004 "Sensory Physiology Laboratory", Dr. Tracy Hodgson
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PUBLICATIONS:

Pitman, J.L., McGill, J.J., Keegan, K.P. & Allada R. (2006) A dynamic role for the mushroom bodies in promoting sleep in *Drosophila.* ***Nature.*** 441, 753-756.

Lim C.,* Chung, B.Y.,* **Pitman J.L**., McGill J.J., Pradhan S., Lee J., Keegan K.P., Choe J. & Allada, R. (2007) *Clockwork orange* encodes a transcriptional repressor important for circadian-clock amplitude in *Drosophila.* (*co-first authors). ***Curr Biol.*** 17(12), 1082-1089.

PRESENTATIONS:

Pitman JL, McGill JJ, Keegan KP, Allada R**,** "A Neural Locus Involved in Sleep in *Drosophila*." **Oral Presentation**, 20th Annual SLEEP meeting – June 2006, Salt Lake City, UT

Pitman JL, Jermaine J. McGill, Keegan K, Allada R, "A dynamic role for the mushroom bodies in promoting sleep in *Drosophila.*" **Nominated Northwestern University Graduate Student Representative, Oral Presentation**, Chicago Chapter Society for Neuroscience Meeting – March 2006, Chicago, IL

Pitman JL, Keegan K, Allada R, "Mushroom bodies promote sleep in *Drosophila.*" **Oral Presentation**, Neurobiology of *Drosophila* Meeting – October 2005, Cold Spring Harbor Laboratories, NY

Pitman JL, Keegan K, Allada R,"A learning and memory center promotes sleep in *Drosophila.*" **Poster Presentation**, 19th Annual Meeting of the Associated Professional Sleep Societies – June 2005, Denver, CO

Pitman JL, Keegan K, Allada R, "An anatomic connection between sleep and learning in *Drosophila.*" **Invited Graduate Student Speaker**, Annual Northwestern University Institute for Neuroscience Retreat – September 2004, St. Charles, IL

Pitman JL, Keegan K, Allada R, "The mushroom bodies may be a sleep-promoting center in *Drosophila.*" **Oral Presentation**, Midwest *Drosophila* Meeting – October 2004, Allerton, IL

Allada R, Burg E, **Pitman JL**, Kilman V, "Potent effects on circadian timing by targeted expression of a temperature sensitive allele of *shibire"*. **Poster Presentation**, Neurobiology of *Drosophila* Meeting – October 2003, Cold Spring Harbor Laboratories, NY

PRESS:

Web Exclusive, **Discover Magazine**, Reporter, Virginia Hughes, June 8th, 2006
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OTHER ACADEMIC ACTIVITIES

2006-2007 **Committee Member**, Interdepartmental Neuroscience Program Student Seminar Series search committee

2005-2007 **Member**, Sleep Research Society

2002-2007 **Presenter and Attendee**, weekly Allada Lab meeting, Journal Club

2002-2007 **Presenter and Attendee**, monthly Center for Sleep and Circadian Biology Journal Club

2002-2007 **Presenter and Attendee**, weekly Fly Club (research presentations)

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