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Circuitry Underlying Sleep in Drosophila Melanogaster: Anatomy and the Role of Octopamine

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Abstract
Almost 20 years ago, the gene underlying fatal familial insomnia was discovered, first suggesting the concept that a single gene can regulate sleep. In the two decades since, there have been many advances in the field of behavioral genetics, but it is only in the past 10 years that the genetic analysis of sleep has emerged as an important discipline. Major findings include the discovery of a single gene underlying the sleep disorder narcolepsy, and identification of loci that make quantitative contributions to sleep characteristics. The sleep field has also expanded its focus from mammalian model organisms to Drosophila, zebrafish, and worms, which is allowing the application of novel genetic approaches. This thesis picks up on current sleep research to understand sleep, using Drosophila as our model organism. In Drosophila we have the unique opportunity to study at a single neuron level, how it regulates sleep and by doing this try to understand why we sleep. This work is devoted primarily to the neurotransmitter octopamine, which is the invertebrate homolog of norepinephrine. We show that octopamine is a wake-promoting signal in the fly, as is its counterpart in mammals. Behavioral changes in the animal are seen with modifications of a single octopamine-producing cell and this effect is used to understand both the anatomical and cellular pathways involved in this signal. We find that octopamine exerts its arousal properties through cAMP/PKA-dependent mechanisms in the Pars Intercerebralis (PI) neurons of the brain. Its actions are independent of the mushroom body, which we have also shown to be an important sleep regulating structure in the fly. This understanding of the anatomical circuitry driving wakefulness in the fly paves the way for finer dissection of the cellular and molecular mechanisms underlying sleep.

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CIRCUITRY UNDERLYING SLEEP IN *DROSOPHILA MELANOGASTER:* ANATOMY AND THE ROLE OF OCTOPAMINE.

Amanda Crocker

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Circuitry Underlying Sleep in *Drosophila Melanogaster*: Anatomy and the Role of Octopamine.

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ABSTRACT

CIRCUITRY UNDERLYING SLEEP IN *DROSOPHILA MELANOGASTER:* ANATOMY AND THE ROLE OF OCTOPAMINE.

Amanda Crocker

Thesis Advisor: Amita Sehgal

Almost 20 years ago, the gene underlying fatal familial insomnia was discovered, first suggesting the concept that a single gene can regulate sleep. In the two decades since, there have been many advances in the field of behavioral genetics, but it is only in the past 10 years that the genetic analysis of sleep has emerged as an important discipline. Major findings include the discovery of a single gene underlying the sleep disorder narcolepsy, and identification of loci that make quantitative contributions to sleep characteristics. The sleep field has also expanded its focus from mammalian model organisms to *Drosophila*, zebrafish, and worms, which is allowing the application of novel genetic approaches. This thesis picks up on current sleep research to understand sleep, using *Drosophila* as our model organism. In *Drosophila* we have the unique opportunity to study at a single neuron level, how it regulates sleep and by doing this try to understand why we sleep. This work is devoted primarily to the neurotransmitter octopamine, which is the invertebrate homolog of norepinephrine. We show that octopamine is a wake-promoting signal in the fly,

as is its counterpart in mammals. Behavioral changes in the animal are seen with modifications of a single octopamine-producing cell and this effect is used to understand both the anatomical and cellular pathways involved in this signal. We find that octopamine exerts its arousal properties through cAMP/PKA-dependent mechanisms in the Pars Intercerebralis (PI) neurons of the brain. Its actions are independent of the mushroom body, which we have also shown to be an important sleep regulating structure in the fly. This understanding of the anatomical circuitry driving wakefulness in the fly paves the way for finer dissection of the cellular and molecular mechanisms underlying sleep.

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CHAPTER I:

INTRODUCTION AND OVERVIEW

This introduction consists of two main parts. The first is an overview of sleep research and the use of genetics to understand sleep, and the second is a more in-depth introduction to the invertebrate neurotransmitter octopamine, the major subject of this thesis. The first part was published as a review in 2010 (Crocker and Sehgal, 2010).

Part 1: Genetics of Sleep

Sleep is one of the great mysteries of science. It is a fundamental phenomenon with no known molecular function despite the fact that it spans genetically diverse eukaryotes, from higher order phyla such as mammals, to lower phyla such as arthropods (Tobler, 2005). Within individual species many characteristics of sleep are tightly regulated. These include, but are not limited to, the timing of sleep onset, depth of sleep, and average duration. Since all these organisms show regulation of the same sleep-associated processes, the thinking is that conserved genetic mechanisms underlie sleep across species (Allada and Siegel, 2008).

The recognition that sleep may be regulated by conserved genetic mechanisms has not yet led to a unified understanding of it. A closely related process- the generation of circadian rhythms- is now explained on the basis of a universal model, largely because of mechanistic studies done in phylogentically

very diverse organisms. Studies of sleep have been primarily descriptive, consisting of lesion studies that have identified relevant anatomic areas in mammals, and pharmacological data that have pinpointed effects of different neurochemicals. However, even these have not provided specific loci/foci to the extent known for the circadian field, perhaps because these do not exist for sleep. For instance, there is still no specific anatomical area that can be lesioned to completely eliminate sleep. Likewise, if there is a specific neurotransmitter for sleep, it is still hypothetical. Thus, sleep does not appear to be controlled by a single locus or dedicated genes. It is better understood as a broad system wide phenomenon.

Hypotheses for sleep include somatic theories (healing of the body and other endocrine functions), cellular metabolic theories (removal of reactive oxidative species and energy replenishment), brain specific functions such as synaptic plasticity (in adults, this would underlie memory consolidation) or synaptic downscaling. One needs to be careful with some of these hypotheses since they are often based on detrimental effects of sleep deprivation, which is both a cellular and organismal stress due to the fact that it exceeds our normal physiological time awake. Given that wake-promoting pathways are involved in other biological functions excess activity of these pathways could produce effects independent of sleep. Also, the sleep field is split between those who want to associate sleep function with specific aspects of the electroencephalogram (EEG) (described below) and those who want to understand what happens to the brain when it is offline, independent of the EEG.

Genetics provides a new way to address the function of sleep. While for the past 20 years genetics has been used primarily to verify lesion and pharmacological studies through targeted gene approaches, it can now be used to probe more intricate questions in sleep. Thus, forward genetic screens, inducible and anatomical specific genetic mutations, genetic alterations in synaptic signaling and excitability, genetic lesioning of cells, microarrays and other genetic manipulations can be employed to identify novel mechanisms underlying sleep, and also to test specific hypotheses for sleep function.

The first part of the sleep introduction focuses on the use of genetic and molecular techniques in model organisms to understand sleep. The first section provides the basic background of sleep research and introduces the study of sleep from a genetic perspective. The second section focuses on the heritability of sleep traits and the genes underlying these traits. The third describes the use of genetic manipulations in model organisms to understand the neurochemistry of sleep. The introduction ends with a discussion of recent studies designed to identify novel sleep-regulating genes, all of which have the ultimate goal of identifying sleep function.

Background:

The Definition of Sleep

In the broadest sense, sleep is defined as a period of inactivity. This period is accompanied by an increase in arousal threshold, often in a stereotypical body position, and, if disrupted, is followed by a period of sleep

rebound (Hendricks et al. 2000; Huber et al. 2004). This definition is applied to organisms as genetically simple as *Caenorhabditis.elegans* as well as to more complex organisms such as mice and even humans.

To understand sleep, two distinct aspects of it must be addressed. These are the timing of sleep and the length/quality of sleep (Borbely, 1982). Both are maintained to be approximately the same from day to day. The timing of sleep is well established as a function of the circadian system in the brain. The circadian system is important for driving many aspects of behavior and physiology with a ~24 hour period through a set of molecular oscillators. How our body knows how much sleep we need is less understood. Based upon the rebound, or compensatory sleep, that follows sleep deprivation, sleep is thought to be an essential process whose amount is controlled by a homeostatic system (Dauvilliers et al., 2005).

In mammals, sleep is identified empirically by physiological markers. In humans, as well as monkeys, rats and mice, changes in brain activity during sleep and wakefulness can be monitored using an electroencephalograph (EEG), and different stages of these behavioral states have come to be identified by characteristic EEG patterns (wave forms) (Table 1-1) (Allada and Siegel 2008; Ambrosius et al. 2008). These patterns are best defined in humans where a typical sleep EEG consists of 1-3 NREM (non-rapid eye movement) stages and a rapid eye movement (REM) stage (Table 1-1). Stage 1N is characterized by the transition from faster oscillations in the 8-13 Hz range during wakefulness to oscillations in the 4-7Hz range. Stage 2N is characterized by sleep spindles (fast

oscillations in the 12-14 Hz range) on top of the slower oscillations. Stage 3N is the deepest stage of sleep and is composed of at least 20% slow, large amplitude oscillations in the 0-4 Hz range known as delta waves; at its deepest points this stage of sleep could consist of >50% delta waves. (Aeschbach and Borbely, 1993; Dumermuth et al., 1983). Depth of sleep is often characterized by the term “delta power” which refers to the frequency and amplitude of the delta waves produced. Delta power is hypothesized to be a readout of the homeostatic drive, so the higher the delta power the greater the sleep pressure in the animal (Tobler and Borbely, 1986; Webb and Agnew, 1971). In other mammals the sleep stages are less well defined, and generally fall into the categories of NREM, REM or wakefulness (Tobler and Borbely, 1986).

Genetic Approaches to the Study of Sleep

The first clue that human sleep could be genetically regulated came from twin studies conducted in the 1930s (Reviewed in (Dauvilliers et al., 2005)). These studies showed that monozygotic twins are more likely to have similar sleep amounts and sleep onset times than dizygotic twins. After the invention of the EEG it was found that monozygotic twins also show similarities in their EEG spectrum. Despite the fact that there is great variation in EEG spectrum from individual to individual, within monozygotic twins it is highly correlated (Ambrosius et al., 2008; Anokhin et al., 1992; Steinlein et al., 1992; van Beijsterveldt and Boomsma, 1994). As discussed below more recent studies

have validated the genetic basis of the EEG pattern and even identified genetic loci that underlie these traits.

Mice have approximately 85 % genetic similarity to humans and so provide an excellent model for the genetic analysis of sleep (Church et al., 2009). They display similar EEG traces, and periods of sleep that are regulated by the same homeostatic and circadian mechanisms as in humans (Allada and Siegel, 2008). However, there are several drawbacks to using a mouse model: They have a long generation time. They are likely to show compensation or redundancy in genes critical for the survival of the mouse, many of which may be important for sleep. Also, until recently, genetic tools were not available to alter the expression of genes over time or spatially within the mouse brain (Rossant and McMahon, 1999).

Simpler organisms such as *C.elegans*, *Drosophila melanogaster* (fruit fly), and *Danio rerio* (zebrafish) are all also proving to be excellent models for sleep (Allada and Siegel, 2008; Cirelli, 2009). They share the advantage that they are all genetically tractable, and have relatively simple genomes (the worm and the fly also have very short generation times). In addition they all have the ability to generate both simple and complex behaviors (Sokolowski, 2001). Lower redundancy of the genome in these organisms makes it easier to identify genes important for sleep. Research in these model organisms has also seen a burgeoning of genetic tools that can be used to probe sleep, ranging from technology that allows for precise temporal and spatial control of genes to reagents that can bypass the need for electrophysiology.

While the utility of the model organisms listed above is unquestionable, it is important to note that the genetics of sleep is not restricted to these organisms. Family-based linkage studies and genome wide association studies are pinpointing sleep genes in humans (Hallmayer et al., 2009; Winkelmann et al., 2007). In addition, advances in the genetics of sleep have sometimes come from unexpected systems. For instance, a dog model for narcolepsy led to the identification of a gene underlying this disorder and a circadian rhythm mutation, tau, was found fortuitously in a hamster (Lin et al., 1999; Ralph and Menaker, 1988).

Genetic analysis of Sleep Traits:

Natural Variations in Sleep Traits.

While earlier work focused primarily on wake EEG, more recent studies have examined EEGs during sleep (Ambrosius et al., 2008; De Gennaro et al., 2008). De Gennaro et al. (2008) showed recently that frequencies of 8-16 Hz during NREM sleep show a high amount of heritability, regardless of sleep-need or intensity. Despite these studies, very little familial linkage work has been done on EEG sleep traits.

Large scale mapping studies of genetic differences in sleep architecture between inbred mouse strains (QTL mapping) has allowed researchers to isolate genes that underlie subtle differences across strains (O'Hara et al., 2007). One study focused on theta oscillations, which vary in frequency across inbred strains, but very little within a strain. Tafti et al. (2003) looked specifically at the difference between the Balb/cByJ mice which have “slow” theta frequencies on

the EEG and c57Bl/6J which have “fast” theta rhythms (Tafti et al., 2003). In mice, hippocampal-derived theta rhythms in the 6-10 Hz range are seen during REM sleep and exploratory behavior, including wheel running. This is different from the cortical theta rhythms seen in humans in stage N1 of sleep that are in the 4 to 7Hz range. Tafti et al. (2003) were able to narrow down the region of interest to a single gene on chromosome 5 known as *Acads* (short chain acyl-coenzyme A dehydrogenase). They found that Balb/cByJ mice have a deficiency in *Acads*, which underlies the slowing of the theta rhythm.

Another successful QTL study identified a gene on chromosome 14, *Rarb*, that contributes to the 1-4hz delta frequency in mice (Maret et al., 2005). Taking a reverse genetic approach to specifically target the *Rarb* gene, Maret et al. (2005) showed that retinoic acid signaling (the pathway *Rarb* functions in) is important for modulating cortical synchrony during NREM sleep.

Later in this review we will touch on some of the ion channels that have been mutated to alter the EEG pattern. Since the channels probably account more directly for the oscillatory bursting of the sleep EEG, they may be regulated by some of the genes discussed above. However, while these studies are important for the insights they provide regarding the genetic control of the sleep EEG, they do not reveal the significance of these waves. Nor, for that matter, do they allow association of EEG patterns with sleep function.

Circadian influences on sleep

As mentioned above, the circadian regulation of sleep is much better defined than the homeostatic regulation. In addition, the molecular basis of circadian control is quite well understood, as a result of cross-disciplinary approaches which included organisms as simple as cyanobacteria and *Neurospora* (Sehgal, 2004). Molecular mechanisms of the circadian clock are indeed conserved from cyanobacteria to humans. From *Drosophila* to humans, the molecules are also largely conserved and have even been implicated in human circadian disorders. The mammalian molecular clock mechanism described below does not do justice to the current state of knowledge in this field; its brevity can be attributed to the fact that it has been covered in countless other reviews, and to the need to focus this writing on the homeostatic regulation of sleep.

The circadian system in mammals and invertebrates involves molecular feedback loops within cells that can maintain a ~24 hour rhythm (Siepka et al., 2007). In all these organisms, the core components of the clock are broken into positive and negative regulators. In mammals, *BMAL1* and *NPAS2* /*CLOCK* are the positive regulators that drive the transcription of *Per* (*Period*) and *Cry* (*Cryptochrome*), which feed back and inhibit the transcription of *BMAL1* and *CLOCK*/*NPAS2*, thereby forming the negative regulators. Following degradation of the negative regulators, a new cycle begins. Mice mutant for any of these genes, or combinations of these genes, generally display aberrant rest: activity patterns although redundancy often results in weaker phenotypes than predicted.

As one would expect, the circadian system is important in determining the timing of sleep. This is best demonstrated in a disorder known as Familial Advanced Sleep Phase Syndrome (FASPS) which results in very early sleep and wake times. Genetic studies have identified a mutation in the *Casein kinase 1 delta* gene in one family afflicted with FASPS and a mutation in the *period* gene, which affects its interaction with Casein kinase 1, in yet another family (Toh et al., 2001; Xu et al., 2005). Remarkably both these genes were first identified in *Drosophila* as part of the circadian clock, thereby attesting to the conservation of molecular mechanisms (Kloss et al., 1998; Konopka and Benzer, 1971). Interestingly the *Tau* mutation in the hamster which causes a very short period is an allele of Casein Kinase 1 epsilon. (Lowrey et al., 2000)

The FASPS mutations do not change the overall length of any of the sleep parameters or homeostat but do alter (advance) the timing of sleep onset. The same phenotype is produced when either *Per2* or *Casein kinase 1 delta* is mutated in the mouse, but in flies the equivalent mutation in *Casein kinase 1 delta* results in a phase delay (Xu et al., 2005). Thus, this particular amino acid is also important in flies, although the regulation may be somewhat different.

Naturally occurring polymorphisms in circadian clock genes do not cause extreme phenotypes like FASPS, but can have effects on the timing of sleep. Indeed, several studies have attempted to correlate such polymorphisms with preferences for early wake-up times (seen in “morning” types or “larks”) or late sleep times (“evening” types or “owls”) (Archer et al., 2003; Carpen et al., 2005; Carpen et al., 2006; Viola et al., 2007). The C allele of the T2434C polymorphism

in *Per*1 is associated with morningness and disruptions in sleep timing (Carpen et al., 2006).

It is clear that circadian genes affect the timing of sleep. What still remains debatable is whether they have a role in the homeostatic regulation of sleep i.e. in determining seep amount. There is some evidence to this effect. Mice with mutations in some core circadian genes such as *CLOCK*, *BMAL* and *Cry,* as well as other circadian regulators, show changes in sleep amount. *Clock* mutant mice sleep on average 2 hours less than their wild-type littermates (Naylor et al., 2000). The *BMAL* knockout mice and the *CRY1/CRY2* double knockout mice both show increases in their total sleep time (Laposky et al., 2005; Wisor et al., 2002). A knockout of *Prokineticin* 2, which is a possible output signal from the SCN (suprachiasmatic nucleus, center for circadian rhythms in mammals), shows reduced total sleep and attenuated sleep rebound following a period of deprivation (Hu et al., 2007). Likewise, mutations in some circadian genes in fruit flies also have disruptions in the sleep homeostat (Chung et al., 2009; Donlea et al., 2009; Hendricks et al., 2003; Shaw et al., 2002) .

A recent study implicated yet another circadian gene in the regulation of sleep length. *Dec*2, a basic helix loop helix (bHLH) protein, is thought to function in the clock as a repressor of *Clock/Bmal1* (Honma et al., 2002). The recent study found a point mutation in the *Dec2* gene in a family of short sleepers (He et al., 2009). These people fall asleep at a normal time, unlike people with advanced phase syndrome, discussed above, but wake up early so that their average amount of sleep is about 6 hours (He et al., 2009). In mice, knockout of

Dec2 did not result in a decrease in sleep like the point mutation found in humans. But, when the specific point mutation was introduced into the mouse, it decreased sleep time, without affecting circadian period, suggesting that it has a dominant effect. Interestingly, a role for *Dec2* is conserved in flies (Lim et al., 2007).

Flies generated to express the mouse Dec2 gene carrying the P385R mutation showed a sleep phenotype similar to that seen in mammals (He et al., 2009).

Even with these effects of circadian genes on sleep amount, it remains unclear as to whether the circadian clock affects sleep homeostasis. For one, the effects are small. For another, they have not been reported for all clock genes. Finally, for the genes that have been implicated, the sleep phenotypes could reflect pleiotropic or non-circadian effects of these genes.

The Genetics of Sleep Neurochemistry:

The first book on the anatomy of sleep was published in the 1840s (Edelson, 1992). Since that time, most of our understanding of sleep neurochemistry has relied on physiological and pharmacological studies. But over the past 10-20 years, genetics has emerged as a major tool to investigate sleep neurochemistry as well as the circuitry associated with it. Indeed, much of the earlier physiological/pharmacological work in mammals is now supported by genetic approaches and has been summarized in many excellent reviews and anatomical maps (Andretic et al., 2008; Saper et al., 2005). More recently there

has been a surge of genetic studies identifying sleep-regulating areas and neurochemicals in the fly brain (see Figure 1-1).

The neurochemical analysis of sleep has involved characterization and manipulation of the major neurotransmitter systems, as well as their receptors. Genetic approaches have classically created and/or characterized targeted knockouts of candidate molecules and identified new roles or confirmed old roles for many of these in sleep. We provide below a brief outline of how genetic modifications in the biosynthetic or signaling pathways of different neurotransmitters have provided insights into sleep neurochemistry. A list of these neurotransmitters, as well as other genes, that affect sleep across species is provided in Table 1-2.

Hypocretin/Orexin:

The discovery of the orexin, also known as hypocretin, gene represents one of the most significant advances in sleep research in the past 20 years. Its role in narcolepsy was discovered independently by two labs, one studying canine narcolepsy and the other studying feeding behavior. The focus of Dr. Mignot's group at Stanford University (Lin et al. 1999) was on cloning the narcolepsy gene. Using a breed of dogs afflicted with narcolepsy, they were able to map the relevant mutation to the gene encoding the hypocretin receptor (*hcrtrt*2) (Lin et al., 1999). Dr. Yanagisawa's group at University of Texas Southwestern (Chemelli et al. 1999), on the other hand, had identified ligands of orphan G-protein coupled receptors and in studying the phenotype of a knockout

mouse lacking one of these ligands, orexin, found that it showed narcoleptic behavior (Chemelli et al., 1999). It became clear from these and subsequent studies that orexin plays a critical role in stabilizing sleep and wake cycles, by influencing both wake-promoting and sleep-promoting areas of the brain (Mochizuki et al., 2004; Saper et al., 2005). Its major role is as a wake-promoting signal; in its absence, animals have trouble maintaining wakefulness and lapse rapidly into REM sleep. People with narcolepsy typically lack orexin-producing neurons for reasons that may have to do with altered immune function (Hallmayer et al., 2009).

Orexin has also been studied in zebrafish, but its role there is slightly more controversial, with conflicting studies reporting insomnia-like phenotypes of both the over-expression as well as knockout models (Prober et al., 2006; Yokogawa et al., 2007). There is also controversy over the projection pattern of orexin neurons and the localization of the fish orexin receptor (Kaslin et al., 2004; Yokogawa et al., 2007). Further studies in zebrafish will be needed to tease apart the role of orexin.

Flies do not have orexin, but it is hypothesized that Pigment Dispersing Factor (PDF), which is released from central clock neurons, is the fly equivalent of orexin. Some of these neurons (the large cells) play an important role in promoting wakefulness in the fly in the early morning and this is done through PDF (Donlea et al., 2009; Parisky et al., 2008; Shang et al., 2008; Sheeba et al., 2008a) (Figure 1-1). It is hypothesized that PDF acts much as orexin does in mammals, as a stabilizer of sleep and wake. Flies that lack PDF signaling, either

by disruption of the PDF gene, ablation of PDF neurons, or lack of the PDF receptor, show defects in their ability to respond to the 'lights on' transition and thus have reduced activity levels at the beginning of their day (Chung et al., 2009; Shang et al., 2008). They also show increases in overall sleep amounts with increased transitions from wake to sleep. Some of this action appears to be mediated through GABAergic inputs onto PDF neurons (Chung et al., 2009; Parisky et al., 2008). Additionally, flies with hyperexcitable PDF neurons show lower levels of sleep (Shang et al., 2008; Sheeba et al., 2008a; Sheeba et al., 2008b). Thus, the effect of PDF on sleep levels and consolidation is similar to that of orexin in mammals.

Acetylcholine:

Genetic analysis of acetycholine has been difficult in most organisms due to the many biologically essential functions of this neurotransmitter. In addition there are a very large number of nicotinic and muscarinic acetylcholine receptors and receptor subtypes. Thus, the role of acetylcholine in sleep has, to date, been better studied using pharmacological approaches, which indicate that acetylcholine is part of the arousal system critical for the waking EEG and REM sleep. For the few nicotinic receptor mutants analyzed for sleep, only minor changes in sleep architecture have been reported (Fonck et al., 2005; Lena et al., 2004). On the other hand, a mutation in the muscarinic acetylcholine receptor M3 results in a decrease in REM sleep (Goutagny et al., 2005). Targeted genetic disruption of the acetylcholine receptor subtypes within small

populations of neurons, such as with RNAi technology or genetically designed viruses, may be more informative. It is also possible that acetylcholine does not actually regulate sleep, but that sleep stages regulate acetylcholine, as suggested by (Gais and Born, 2004).

The Biogenic Amines:

The role of norepinephrine, dopamine, serotonin and histamine in sleep has been covered exhaustively (Berridge, 2008; Cirelli, 2009; Dzirasa et al., 2006; Monti and Monti, 2007; Monti et al., 2008; Saper et al., 2005). For this reason we have limited our discussion of these neurotransmitters. In brief, targeted genetic disruptions in the biogenic amines, specifically norepinephrine, dopamine, and histamine, have confirmed the wake-promoting action of these neurotransmitters suggested by lesion and pharmacological approaches (Hunsley et al., 2006; Hunsley and Palmiter, 2003; Monti and Monti, 2007; Ouyang et al., 2004; Popa et al., 2005; Qu et al., 2008; Vallone et al., 2002; Waddington et al., 2005; Wisor et al., 2001). Serotonin on the other hand has had a more complicated history since it may have different effects on REM versus NREM sleep. It is clear from genetic and pharmacological studies that it inhibits REM sleep (Boutrel et al., 1999; Boutrel et al., 2002), but it may actually promote NREM sleep (Jouvet, 1968).

One area in which genetic targeting studies have provided novel insight is in the role of histamine in narcolepsy. Mice carrying a mutation in *histamine decarboxylase* (*HDC*) show altered levels of sleep-hypersomnolence-and are

unable to maintain wakefulness during times of normally high vigilance, such as light transitions and cage changes (Parmentier et al., 2002). This phenotype is similar to that of narcolepsy and, in fact, a role of histamine in this disease is being investigated. Both orexin knockout mice and *HDC* knockout mice show sleep fragmentation and increased REM, but the *HDC* knockout mice show increased REM during the light phase when mice normally sleep (Anaclet et al., 2009), whereas orexin mutants display REM during waking hours. In support of the role histamine may play in narcolepsy, patients with narcolepsy have decreased levels of histamine in their cerebral spinal fluid (Nishino et al., 2009). These data begin to underscore the more complicated aspects of sleep-wake regulation.

Many environmental cues and inputs can promote wakefulness, and similarly there appear to be many neurotransmitter systems important for the response to each of those cues. Mice have periods of wakefulness following introduction to a novel environment such as new cages or new lighting, as well as increased arousal during and following locomotor tasks. Histamine may play a critical role in the EEG spectrum of sleep during the day when mice sleep, as well as in arousal induced by novel environments (Anaclet et al. 2009). Orexin on the other hand is connected to the wakefulness seen during the night (the mouse's active period) and is necessary to maintain arousal during and after locomotor tasks (Anaclet et al. 2009).

Unfortunately, both pharmacological studies and genetic studies conducted to date suffer from potential drawbacks. For one, pharmacological

studies rely on injections that may not be very specifically targeted. In addition, an agonist or antagonist can often have extraneous effects. On the other hand, genetic deletions, particularly those that occur during development, are frequently compensated by the animal. Also, since molecules can have different functions in different regions, analysis of global knockouts typically does not yield clear cut results. Perhaps for this reason, sleep phenotypes of genetic knockouts are often complicated, and sometimes controversial (Alexandre et al., 2006; Boutrel et al., 1999; Boutrel et al., 2002; Frank et al., 2002; Hedlund et al., 2005; Monti and Monti, 2007; Waddington et al., 2005; Wisor et al., 2003) . In order to address the role of each receptor in sleep inducible and anatomically specific knockouts need to be generated. Moreover, to control for redundancy it may be necessary to generate animals lacking multiple receptors.

Interestingly virtually all these neurotransmitters, e.g. dopamine, serotonin and octopamine (invertebrate counterpart of norepinephrine), regulate sleep in other model organisms such as the fly (Chang et al., 2006)(Yuan et al., 2006) (Andretic et al., 2008b; Crocker and Sehgal, 2008; Crocker et al., 2010; Kume et al., 2005; Lebestky et al., 2009; Wu et al., 2008) (Chapter 2,3 of this thesis) (Figure 1-1). While the work on dopamine and octopamine has confirmed that they constitute wake-promoting signals as they do in mammals, serotonin in the fly provides a sleep-promoting signal (Yuan et al. 2006). In general, analysis in the fly is simplified because, thus far, only one sleep state is known, there are fewer receptors for each neurotransmitter, and there is also less compensation and redundancy. It is also possible to map sleep-regulating effects of a molecule

to specific subsets of neurons through an unbiased genetic approach. Not only does this provide anatomical information, it also allows visualization of phenotypes produced by manipulation of just those cells. Using this approach we recently showed that only a subset of octopamine-producing cells is responsible for its wake-promoting signal (Crocker et al. 2010)(Chapter 3).

Although wake-promoting neurotransmitters are clearly important in determining sleep amount, there is much more interest in sleep-promoting molecules since these could more directly be part of the sleep homeostat. At the very least, they are required for implementation of sleep drive. Known sleep-promoting neuromodulators are discussed below.

GABA:

GABA is a major sleep-promoting neurotransmitter that, when released from the ventral preoptic area (VLPO) in mammals, inhibits wake-promoting areas (Gong et al., 2004a). In addition, release of GABA from the nucleus reticularis of the thalamus, and its action on other thalamic nuclei, promotes the transition from a wake to a sleep EEG (Cope et al., 2005). While pharmacological studies have implicated GABA-A receptor in sleep generation, genetic mutants of the pathway show minimal phenotypes. Mice carrying a point mutation in the GABA-A receptor, a1-a3, show no changes in sleep amount (Kopp et al., 2003; Kopp et al., 2004; Tobler et al., 2001). The GABA-A receptor a3 subunit knockout mice display normal sleep amounts, but have reduced spindle activity (10-15Hz range during NREM-REM transitions) (Winsky-Sommerer et al., 2008).

The knockout of the GABA-A receptor d subunit has only been examined after drug treatment and it shows normal EEG patterns (Winsky-Sommerer et al., 2007). The role of the GABA-A receptor b3 subunit has been more controversial with conflicting results on whether there is an effect on sleep and delta power (Laposky et al., 2001; Wisor et al., 2002a). Redundancy in the GABA signaling pathway likely accounts for these questionable phenotypes.

In summary, genetic analysis of neurotransmitters that are widely distributed and necessary for life, such as GABA and glutamate, has proven to be very difficult in mammals. However, the *Drosophila*, *C.elegans* and recently zebrafish models have provided insight into how these systems are involved in sleep and its underlying circuitry. For instance, GABA is a major sleep-promoting signal in flies . Recent work shows that the wake-promoting large central clock cells, mentioned above, are the primary recipients of the GABA signal relevant for sleep (Agosto et al. 2008; Parisky et al. 2008; Chung et al. 2009). This creates a system similar to that seen in mammals, where the sleep-promoting neurons become active and shut down the wake-promoting centers of the brain.

Somnogens

Very early in sleep research, researchers showed that cerebral spinal fluid from a sleep-deprived animal could induce sleep in a rested animal (Legendre and Peiron, 1913). Since those early experiments the hunt has been on for a specific circulating somnogen that tells your body to go to sleep. Many found the idea that it could be adenosine very intriguing. The thinking goes that as one

uses more energy during the day, more and more of the body's ATP is converted to adenosine, signaling a need for sleep, which then restores energy levels (Benington and Heller, 1995).

The data on the role of adenosine are complicated. Mice that carry mutations in either the A2A or the A1 adenosine receptor do not show profound changes in sleep (Bjorness et al., 2009; Huang et al., 2005; Stenberg et al., 2003; Urade et al., 2003). This has been problematic for the field since caffeine is thought to promote arousal by blocking the A2A receptor (Huang et al., 2005). To date, the best evidence for a sleep-promoting effect of adenosine comes from pharmacological studies. However, recently mice expressing a dominant negative SNARE protein (this blocks the release of neuroactive molecules) in astrocytes were found to have reduced cortical slow wave oscillations, characteristic of NREM, and also decreased sleep pressure following periods of deprivation (Fellin et al., 2009; Halassa et al., 2009). This reduction was thought to be due to decreased ATP release from astrocytes, and thereby attenuated build up of extracellular adenosine. The idea is that typically adenosine would act through the A1 receptor to suppress synaptic transmission and promote slow wave activity (Fellin et al. 2009). These studies did not map the site of adenosine action, but they provide a basis for further investigation of a function for adenosine in sleep.

Caffeine action has also been studied in the fruit fly where it acts through the cAMP pathway, rather than the adenosine receptor, to promote wakefulness. In addition, using a cAMP reporter expressed in all neuronal tissue in the fly, Wu

et al. (2009) found that the effect of caffeine was quite global and not restricted to a specific region (Wu et al., 2009). Thus, while pharmacological treatment of flies with an adenosine agonist was shown to promote sleep (Hendricks et al., 2000a), there are as yet no genetic data to support a role of adenosine in fly sleep.

Interestingly, cAMP phosphodiesterase (PDE) is a known target of caffeine, but had been excluded as a possible mechanism to explain effects of caffeine on sleep in mammals because of the relatively lower affinity of caffeine for PDE. However, in light of a report indicating that effects of caffeine on immune function in mammals are mediated by PDE inhibition, as well as the recent fly data, it is worth re-exploring a role for PDE (Horrigan et al., 2006; Wu et al., 2009). This is particularly important given that the adenosine receptor knockouts have little to no sleep phenotype, and cAMP signaling is clearly involved in sleep regulation (see below). It should also be noted that the fly experiments involved chronic treatment with caffeine while the mammalian studies usually deliver it acutely. There may be differences in the mechanisms used under these different conditions.

Identification of Genes required for sleep homeostasis:

The big question remains -why do we sleep? There is also now the growing sense that the function of sleep may fall out of its molecular analysis. Since few sleep-regulating molecules are known, studies are under way to identify novel genes required for sleep. These studies include forward genetic

screens and genetic manipulation of candidate genes required for sleep, by focusing on changes in sleep amount as a readout of sleep homeostasis. In some cases, the candidate genes are based upon hypothesized sleep functions, so as to assess how loss or gain of a specific function affects sleep quantity. (See also Table 1-3).

Genes based upon somatic theories of sleep function

Sleep and the immune response

Is sleep necessary for normal body function such as the immune response and balanced metabolic activity (Van Cauter et al., 1997)? The idea that immune modulators like cytokines promote sleep has anecdotal support, since the mounting of an immune response by the body usually results in fatigue and sleepiness. Researchers have focused mainly on two cytokines, interleukin -1B (IL-1B) and tumor necrosis factor -α (TNF-a) as sleep-promoting molecules. Mice lacking IL-1B show decreased NREM sleep during their active time while mice lacking TNF-a show decreased NREM during their sleep time (Fang et al., 1998; Krueger et al., 1998). In addition, double knockouts lacking both IL-1B and TNF-a show a greater magnitude of slow wave delta power following sleep deprivation (Baracchi and Opp, 2008). From these data though, one cannot conclude a causal relationship between the immune system and sleep drive. These mutant animals still sleep and the lowered NREM may just reflect an overlap in immune and sleep circuitry.

Expression profiles undertaken to identify genes whose expression changes with sleep state have also identified genes in the immune response pathway (Cirelli et al., 2005b; Williams et al., 2007). In *Drosophila*, NFKappa B (*Relish*) and other immune response genes have been identified in such screens and are upregulated during wakefulness and prolonged wakefulness. In addition, flies with decreased levels of *Relish* have reduced nighttime sleep (Williams et al., 2007). In the rat cortex also, expression of genes in the immune response pathway is upregulated during wakefulness, as indicated by microarray studies. These include lysozyme, COX-2, and I-kappaB α (Cirelli et al., 2005b). However, while the microarray studies support the genetic mutant data, they also do little to address causality. They are nevertheless useful for identifying associations between gene expression profiles and behavioral states.

Sleep and metabolism

There have long been theories that sleep is important for metabolism (Benington and Heller 1995). This is supported by the potential role for adenosine and by reports showing associations between glycogen levels and sleep (Kong et al., 2002). In addition, there appears to be anatomic overlap in the regulation of sleep and metabolism. For instance, the mammalian hypothalamus is an important control structure for both processes. Recent work in the fly also implicates its major hormonal and metabolic center as an important place for the regulation of sleep (Crocker et al., 2010; Foltenyi et al., 2007) (Chapter 3) (Figure 1-1).

Epidermal growth factor receptor (EGFR), a receptor important for cell proliferation and growth through different signaling pathways, is implicated in sleep regulation in *Drosophila* and in *C.elegans* (Foltenyi et al., 2007; Van Buskirk and Sternberg, 2007). Foltenyi et al. (2007) found that increased signaling of the EGFR pathway results in increased sleep. Signaling was increased through gain of function mutations of the EGFR ligands in an area of the fly brain homologous to the hypothalamus, known as the Pars Intercerbralis (PI) (Figure 1-1), and was correlated with changes in downstream ERK signaling. Our own recent work shows that wake-promoting effects of octopamine are mediated by insulin-producing cells in the fly brain, which are also located in the PI.(Crocker et al., 2010)(Chapter 3).

The EGFR pathway is also implicated in *C. elegans* sleep. The *C. elegan*s model for sleep focuses on a developmentally regulated state of quiescence, called lethargus, that occurs in conjunction with larval molts. Interestingly, lethargus is regulated by the worm homolog of the circadian gene *per*, and is associated with synaptogenesis (a hypothesized function of sleep), suggesting that it represents a primordial sleep-like state (Raizen et al., 2008; Van Buskirk and Sternberg, 2007). Over-expression of *lin-3* (ligand for EGFR) induces lethargus-like behaviors (Van Buskirk and Sternberg, 2007). The receptor *Let-23* (EGFR) is found in only a handful of neurons, of which the ALA neurons are responsible for the effect of *lin-3* on lethargus (Van Buskirk and Sternberg, 2007). These neurons are neuroendocrine in nature, similar to the neurons through which EGF affects sleep in flies. Thus, in the mouse, the fly and

in the worm, metabolic and endocrine functions appear to be tied to sleep. Whether it is just an anatomical overlap or a functional overlap still needs to be addressed.

Many mouse mutants with altered metabolic function also show changes in sleep. For instance, mouse knockouts of the ghrelin gene show a slight increase in sleep (Laposky et al., 2008). Correspondingly, leptin deficient mice have a decrease in NREM sleep and increased fragmentation (Szentirmai et al., 2007). Mice that do not make growth hormone releasing hormone (GHRH) and its receptor show significantly less NREM sleep, whereas mice overexpressing growth hormone sleep more (Obal et al., 2003; Obal et al., 2001). Unfortunately these studies do not address the fundamental question of whether increased hunger in these animals overrides the sleep signal.

More recently genes important for dealing with cellular stress have been implicated in sleep regulation. Through both differential expression profiles and targeted gene approaches, the gene *Bip* is implicated as a sleep-promoting factor. *Bip* is important for the unfolded protein response in the ER and is upregulated following periods of sleep deprivation in mice (Cirelli et al., 2005b). In addition, flies with altered *Bip* levels show changes in their homeostatic response to sleep deprivation (Naidoo et al., 2007).

Genes Important for Synaptic Modulation:

One of the current hypotheses for why we sleep is that it allows for or even promotes synaptic downscaling (Tononi and Cirelli, 2006). This hypothesis

is based upon the presumption that during wakefulness interaction of animals with their environment leads to the strengthening of some synapses while others remain the same. It postulates that synaptic downscaling during sleep promotes efficiency in terms of energy and space while maintaining the relative ratios of the strength of synapses. This hypothesis has been supported in recent years by differential expression studies of genes whose expression changes with sleep/wake state. Many immediate early genes and genes that regulate synaptic strength were identified in these studies. These include NARP and Homer1a in the awake rat cortex (Cirelli et al., 2005b). In addition, knockouts of c-Fos (another immediate early gene marking neuronal activity) and Gria3 (AMPA-receptor GluR3 subunit) in mice show alterations in their sleep (Shiromani et al., 2000; Steenland et al., 2008). The c-Fos null animals have more wakefulness and reduced slow wave sleep and the Gria3 animals show dampened EEG powers across waking and NREM sleep but no changes in total sleep amount (Shiromani et al. 2000; Steenland et al. 2008). The *Homer1*a gene was also identified in QTL analysis as a strong candidate for a gene underlying sleep homeostasis and magnitude of delta power (Mackiewicz et al., 2008; Maret et al., 2007). More recent work by Gilestro et al. (2009) monitored genes known to target the synapse and used them to monitor changes in synapses following across normal sleep wake stages as well as periods of sleep deprivation. They found sleep was necessary for declining synaptic marker strength (Gilestro et al., 2009).

While these results are tantalizing, it may be premature to conclude that synaptic downscaling is a function of sleep. In order to definitively address this question, genetic tools that allow one to better visualize circuitry and synapses need to be employed. Zebrafish provide an ideal model organism for such approaches since they are translucent and thus allow one to visualize changes in synapses.

Ion Channels and Channel-Regulating Molecules

Forward genetic screens in the fruit fly have identified sleep-regulating genes that are important for K^+ channel activity. The *Minisleep* (mns) fly line, isolated in a genetic screen, carries a mutation in the *Shaker* K^+ channel (Cirelli et al., 2005a). Based upon the mns phenotype, a mutation in the β subunit of *Shaker*, *hyperkinetic*, was tested and also found to reduce sleep (Bushey et al., 2007). An independent genetic screen isolated a short sleeping mutant known as *sleepless* which also affects activity of the Shaker K^+ channel (Koh et al., 2008)(Wu et al., 2010). Both the *sleepless* and the *Shaker* mutants sleep very little at night. Interestingly neither of these mutations has been rescued in a specific area of the fly brain, suggesting that a global change in synaptic properties underlies sleep.

These mutants have other phenotypes as well, such as shorter lifespan, ether sensitivity and in the case of *Shaker* a learning and memory deficit (Bushey et al., 2007; Koh et al., 2008). In the case of *sleepless* it was found that the ether sensitivity could be rescued independent of the sleep phenotype (Koh et al.

2008). It would be interesting to know whether lifespan can also be rescued independently.

In mice the large number of ion channels, subunits and distribution has made it difficult to identify the role these play in sleep. Indeed, based upon the redundancy of K^+ channels in mammals, it is unlikely that mutations in these would have been found through forward genetic screens since such screens typically require strong phenotypes. However, reverse genetic approaches have allowed the detection of subtle sleep phenotypes in mice mutant for *Shaker*-like channels (Douglas et al., 2007; Espinosa et al., 2004; Espinosa et al., 2008; Vyazovskiy et al., 2002). In addition, mice lacking an N-Type calcium channel α 1b subunit have a sleep phenotype. This subunit is important in many of the major anatomical regions important for arousal including the locus coeruleus and the dorsal raphe (Beuckmann et al., 2003). Accordingly, mice lacking this subunit show hyperactivity (increased consolidation of REM and increased NREM to Wake transitions). These mice also show decreased power during NREM sleep, implying decreased sleep drive. When the α 1G subunit of the T-type Ca^{++} channel is knocked out globally in mice they display decreased NREM cortical EEG oscillations, due to the inability of thalamical relay neurons to go into a bursting mode, and increased fragmentation of sleep (Anderson et al., 2005; Lee et al., 2004). The same phenotype is observed when this subunit is knocked out specifically in the thalamus, thereby verifying lesion studies which implicate the thalamus in arousal and in the generation of the sleep EEG (Anderson et al. 2005). Along the same lines, mice lacking the SK2 channel (a K^+ channel

specific to the dendrites of the nucleus reticularis of the thalamus) show weakened delta waves and spindles in the EEG, which results in very fragmented sleep (Cueni et al., 2008). This K^+ channel couples with the T-type Ca^+ channels described above. These animals provide an ideal model to determine how specific electrical attributes of cortical neurons correlate with sleep EEG.

Genes Involved in Learning and Memory

In both mice and flies many genes important for learning and memory have been targeted for sleep analysis. These include but are not limited to CREB, Protein Kinase A (PKA), cAMP, ERK, cGMP and some of the ion channels listed above.

Manipulations of CREB, a transcription regulator, influence total sleep and NREM in mice (Graves et al., 2003b). Thus mice lacking either one of two CREB isoforms in the entire brain show altered sleep. These animals spend less time awake and have longer bouts of NREM sleep. They also have altered memory formation and reduced long term potentiation (Graves et al., 2002). The effect on long term potentiation (LTP) and hippocampal-dependent memory formation is similar to what is seen following a period of sleep deprivation (Graves et al., 2003a). More recently, effects of sleep deprivation on LTP were rescued by an inhibitor of a specific phosphodiesterase, supporting the idea that effects of sleep on hippocampal plasticity are mediated by cAMP signaling (Vecsey et al., 2009). Another molecule implicated in learning and memory and sleep is brain derived

neurotrophic factor (BDNF) (Monteggia et al., 2004). Levels of BDNF increase with increased exploratory behavior which also increases the depth of delta power during sleep (Huber et al., 2007). The role of BDNF may not be independent of CREB since BDNF is a major target of CREB.

The mammalian work linking sleep with learning and memory genes has been largely limited to analysis of BDNF and CREB. In the fly this is not the case. There are many tools available to fly researchers that allow one to probe very specific parts of these pathways. Two important intracellular pathways implicated in sleep are the cAMP-PKA pathway and the ERK pathway (Foltenyi et al., 2007; Hendricks et al., 2001; Joiner et al., 2006). It is thought that the cAMP pathway provides a wake-promoting signaling pathway, whereas the ERK pathway, at least within a subset of neurons, is sleep-promoting. Mutants and transgenic flies carrying manipulations of these different signaling pathways have also helped to locate anatomical regions important for sleep regulation. In the case of cAMP and PKA, an area known as the mushroom body is important (Joiner et al., 2006; Pitman et al., 2006) (Figure 1-1). The mushroom body is similar to the hippocampus in mammals in that it is involved in memory formation (Heisenberg, 2003).

Bushey et al. (2007) correlated short sleep with short term memory deficits in flies (Bushey et al., 2007). They specifically looked at *hyperkinetic* flies and variations of the *Shaker* mutation and found that regardless of other behaviors, the sleep phenotype (short sleep) and decreased memory are associated. It is currently unknown whether short sleep causes a memory deficit or vice versa.

Following a period of sleep deprivation learning is impaired, but the converse has also been argued (Cirelli, 2009). Genetically manipulating the mushroom body in the fly, as described above, can produce either short or long sleepers depending on the region targeted. In addition flies exposed to an enriched environment sleep more (Donlea et al., 2009; Ganguly-Fitzgerald et al., 2006). This increase in sleep is dependent on cAMP presumably because it involves memory consolidation, and can be rescued within the central clock cells in the fly (Donlea et al. 2009) (Figure 1-1).

As in flies and mammals, cyclic nucleotide pathways affect lethargus in worms.. Worms deficient in *egl-4* (cGMP-dependent kinase) have reduced periods of quiescence, whereas gain of function mutants of egl-4 show enhanced quiescence (Raizen et al., 2008). Similar sleep phenotypes are observed in flies that have alterations in PKG (similar to *egl-4*) (Raizen et al. 2008). Also a mouse conditional brain knockout of cGMP-dependent protein kinase type 1 causes increased sleep fragmentation, exaggerated delta rebound following deprivation and reduced REM sleep (Langmesser et al., 2009). Thus a role for cGMP in sleep is conserved across evolution. cGMP protein kinase mutants also have defects in learning and memory, in both mice and flies (Feil et al., 2009).

While the analysis of genetic mutants may not have provided major breakthroughs in our knowledge of sleep up to this point, it has been critical in many respects. First, even where the studies have been purely confirmatory, they have served to unequivocally establish a particular mechanism or a role for a specific molecule in sleep. This extends also to genes underlying diseases

associated with sleep problems: Sleep disturbances have been reported in people with Angelman syndrome and Fragile X syndrome, and in both cases knockout f the relevant gene in mice and/or flies has produced a circadian/sleep phenotype (Dockendorff et al., 2002; Wu et al., 2008; Zhang et al., 2008). In many cases, genetic mutants have helped to resolve controversies. Moreover, forward genetic screens in model organisms are already identifying new molecules and will likely also lead to paradigm-shifting findings. Such screens immediately associate gene with function and importantly, they are done in a completely unbiased fashion which arguably is the best approach for a process about which little is known. Finally, the anatomic studies conducted in invertebrates are already indicating overlap between sleep and other aspects of physiology e.g. metabolism. These findings could be invaluable for what they suggest about sleep function. Importantly, they can be easily followed up with experiments designed to address specific hypotheses

The future potential of genetic approaches is also tremendous, given the rapid development of novel genetic tools and technology. For example the use of optogenetic tools has already provided insight into the orexin pathway (Adamantidis et al., 2007; Zhang et al., 2007) In addition, inducible and tissue-specific gene expression, which will allow precise targeting of genetic manipulations, will undoubtedly clarify the sleep function of genes whose role is currently controversial. In an interesting merge of pharmacological methods and genetic tools, viruses have been developed which can be introduced into specific anatomic areas (Adachi et al., 2008; Fuller et al., 2008) .. The fly provides many

unique genetic techniques, some of which have been discussed in the course of this review. Recently there has been an explosion of tools which allow scientists to alter activity/signaling within a subset of neurons; this includes but are not limited to expression of Na^+ channels, K^+ channels, Ca^{++} channels and vesicular release blockers (for review see (Hodge, 2009)). This is a huge advance for behavioral studies because electrophysiological stimulation of neurons to alter behavior has to be done in a very artificial controlled environment in mammals, but in flies since this is done genetically they are able to behave normally in an unrestricted environment. cAMP monitors have also been developed that can be specifically expressed in certain cells and do not rely on bath application or injection (Shafer et al., 2008). Finally, in flies and mammals techniques have been developed to specifically tag a subset of neurons in the brains and determine their expression profile (Miller et al., 2009; Zong et al., 2005).

Conclusion

Genetics can tell us a lot about what sleep does for organisms, but the potential of this approach has only just started to be recognized in the sleep field. With the generation of conditional and anatomically restricted knock-outs (or knock-ins) in mice, we are on the verge of answering many questions. These include determining the roles of adenosine and BDNF in sleep and memory. In flies anatomically and/or temporally-restricted expression of sleep-regulating transgenes has already been performed. These approaches have provided great insight into the role specific signaling pathways play in sleep. In the future this

technology will be used to rescue sleep mutants in a region-specific manner although some of these mutations, such as in ion channels, may turn out to have global effects that cannot be rescued in specific areas. However, the real power of the fly, worm and fish models lies in their amenability to unbiased genetic screens. With a process like sleep, about which little is known, we suggest that the best approach is one that is not associated with any preconceived assumptions, since it allows the identification of completely novel mechanisms and pathways. Thus far it appears that redundancy and/or compensation in mammals will make it difficult to detect strong phenotypes through genetic screens. The fly work, on the other hand, has already demonstrated that mutants with strong phenotypes can be identified.

While forward genetic screens in mouse may not be realistic (or cost-effective), QTL analysis and microarray approaches are yielding potential sleep-regulating genes. The use of new genetic tools described above will allow researchers to investigate whether or not these genes specifically affect sleep. An example of this is provided by the *Rarb* story, where the gene was identified through QTL mapping, and then specific targeted disruptions of this gene were undertaken.

At this point, there is no evidence that a single gene or subset of genes acting in specific subset of neurons is responsible for sleep. It is more likely that a sleep is a network phenomenon. It is also likely that there will be many hypotheses for why we sleep and strong evidence for each, since many of the neurotransmitters and signaling pathways that keep us awake serve other

functions. For instance, orexin is apparently involved in both feeding behavior and maintaining wakefulness. Sleep deprivation results in several impaired processes, some of which may turn out to reflect consequences of increased wakefulness rather than indicating an actual function of sleep. With the advancement of new genetic tools, it is likely that we will soon see experiments directly testing some of these hypotheses such as cellular metabolic function and synaptic scaling.

From the data discussed in this review it is likely that sleep is important for overall homeostatic regulation of the entire organism, possibly down to within the cell homeostasis. It is clear that sleep is a very basic process and that studying it in model organisms will provide significant insight into why we sleep. In general, advances in genetics in all model organisms will provide a wealth of knowledge for the sleep field in the coming years.

Introduction Part 2: Octopamine

The focus of this thesis is on the neurotransmitter, octopamine, the insect homolog of norepinephrine. I chose to work on octopamine because it is a major invertebrate neurotransmitter and also because receptors for octopamine are highly expressed in the Mushroom Body (an area known to regulate sleep and wake) (Joiner et al., 2006; Roeder, 2005). In the fruit fly as well as other invertebrates, octopamine is synthesized and regulated through pathways that are distinct from those that produce dopamine, unlike in mammals where

dopamine is a precursor in the synthesis of norepinephrine (Roeder, 1999). Octopamine is known to play a role in memory formation, larval locomotion, wing beating, ovulation and aggression (Cole et al., 2005; Hoyer et al., 2008; Lee et al., 2003; Roeder, 2005; Saraswati et al., 2004; Schwaerzel et al., 2003), but its effects on sleep have not been examined.

Octopamine Biochemistry:

Octopamine is synthesized through a series of enzymatic reactions beginning with the molecule tyrosine. This is unlike the synthesis of mammalian norepinephrine, which is synthesized from dopamine via the enzyme dopamine β hydroxylase (Livingstone and Tempel, 1983; Roeder, 1999). In flies dopamine is also synthesized from tyrosine, but through an enzyme known as tyrosine hydroxylase, whereas the first step in octopamine synthesis is catalyzed by the enzyme tyrosine decarboxylase 2 (*Tdc2*). *Tdc2* synthesizes tyramine (the precursor to octopamine), thus, disruption of *Tdc2* results in low levels of octopamine and tyramine (Cole et al., 2005). The second step involves an enzyme known as tyramine β-hydroxylase *(TβH)* which results in the synthesis of octopamine from tyramine. Loss of *TβH* reduces octopamine levels but increases tyramine levels tenfold (Monastirioti et al., 1996). It is generally thought that *TβH* is the fly homolog of mammalian dopamine β-hydroxylase (*DbH)*. Since synthesis of both amines (tyramine and octopamine) requires *Tdc2*, the cellular distribution of *Tdc2* should reflect cells that potentially produce both tyramine and octopamine, but not dopamine.

Octopamine Receptors:

In flies there are two classes of octopamine receptors, which are categorized as either α or β receptors (Evans and Maqueira, 2005; Evans and Robb, 1993; Han et al., 1998; Maqueira et al., 2005; Robb et al., 1994; Roeder, 1992; Verlinden et al., 2010). This classification stems from their homology to mammalian α and β adrenergic receptors. Alpha receptors in both mammals and in flies cause increases in Ca^{++}. Increases in cytosolic Ca^{++} attributable to activation of the octopamine α receptor results from the opening of channels in the endoplasmic reticulum and the release of internal stores. This is accomplished through activation of the phospholipase C (PLC) pathway (For review see (Verlinden et al., 2010). Unlike their mammalian counterparts, the *Drosophila* isoforms of the α receptors also couple to cAMP. This is true for the OAMB receptor (octopamine receptor first found in the MBs) and the OA1 receptor. OAMB specifically has two isoforms: the K3 and the AS (Lee et al., 2009). The K3 isoform couples to both Ca^{++} and cAMP and in some ways behaves more like the octopamine β receptor than the α receptor. The AS isoform only couples to Ca^{2+} and is a true octopamine α receptor. It behaves similar to the α adrenergic receptor.

The octopamine β receptors known as octB1R (also OA2), OctB2R and OctB3R, act like mammalian β receptors and all lead to increases in cAMP (Maqueira et al., 2005). Interestingly, most octopamine receptors are also

sensitive to tyramine, the other amine produced in *Tdc* cells, though at a much lower affinity (Roeder et al., 1995). Tyramine also has its own class of receptors which cause a decrease in cAMP when stimulated by tyramine, but an increase in Ca^{2+} when stimulated by octopamine (Roeder, 2005). These receptors are all thought to be G-protein-coupled receptors and support a role for agonist-receptor trafficking schemes, where receptors change their conformations in response to different agonists. This allows for signaling via different second messenger pathways.

Actions of octopamine in invertebrates:

Octopamine has been extensively studied in invertebrates other than *Drosophila*. Like norepinephrine in mammals, octopamine is implicated in stress, aggression, locomotion, learning, memory and other behaviors (Chentsova et al., 2002a; Saraswati et al., 2004; Schwaerzel et al., 2003; Sitaraman et al., 2010; Zhou and Rao, 2008) (For review see (Verlinden et al., 2010). In mammals, norepinephrine is an important contributor to stress (For review see (Goddard et al., 2010). Norepinephrine is derived from specific cell populations in the Locus Coeruleus (LC) and the brainstem groups known as A1 to A7 (Sara, 2009). The LC neurons are important for coordinating the stress response, and a subgroup of LC neurons along with the A1/A2 neurons regulate corticotrophin-releasing neurons (CRH neurons) of the paraventricular nucleus. The CRH neurons are important for mediating the metabolic and adaptive response to acute stress in mammals (Lightman, 2008). In invertebrates, octopamine may play a similar

role. Octopamine levels rise in response to many stresses, including immune challenge, heat, starvation, mechanical stress and others forces, in honeybees, locusts, cockroaches and *Drosophila* (Chen et al., 2008; Davenport and Evans, 1984; David and Coulon, 1985; Evans and Robb, 1993; Harris and Woodring, 1992; Hirashima and Eto, 1993; Orchard et al., 1993). Worker honeybees show an increase in octopamine when their legs have been clamped to prevent movement. This result demonstrates the sensitivity of this biogenic amine to handling stresses during experimentation (Harris and Woodring, 1992). Studies of stress in the cockroach employed vibrations, flashing lights or immersions in 60°C heat baths. Each of these stresses resulted in an increase in octopamine levels (Hirashima and Eto, 1993). In locusts, the stress of long flights also increases octopamine levels (Orchard et al., 1993). Interestingly, the two studies that examined heat stress in *Drosophila* showed that *TDC* activity dramatically decreases in females and only modestly decreases in males in response to heat (Chentsova et al., 2002b; Gruntenko et al., 2004). This result is contradictory to previous work showing an increase in octopamine in response to stress in other invertebrates.

Octopamine in worker honeybees is also an important foraging signal, with levels highest during the peak annual foraging season, June through September (Harris and Woodring, 1992). Octopamine also regulates job position in the honeybee hive colony (Schulz and Robinson, 2001b). Worker bees begin their lives maintaining the hive and the Queen. Following increases in octopamine and

juvenile hormone (JH) levels, they transition to foraging outside the hive(Schulz et al., 2002) .

Octopamine also modulates other basic biological functions such as metabolism in invertebrates. In locust and crickets, octopamine affects global metabolism, including the release of Adipokinetic Hormone (AKH), which is activated in response to starvation and acts to liberate fat stores (for review see (Roeder, 2005). Octopamine itself also acts on the fat body of these insects to liberate fat stores synergistically with AKH. This effect has been observed during the energy-demanding migratory flights of locusts(Orchard et al., 1993). A similar situation is found in mammals where norepinephrine breaks down glycogen and releases fat stores.

In many invertebrate species octopamine has been shown to induce locomotion and arousal (Barron et al., 2002a; Roeder et al., 2003; Saraswati et al., 2004). In fact, it is used in pesticides where it induces a 'walk off leaf' phenotype and thereby promotes pest removal (Roeder et al., 2003). A recent study showed how the venom of parasitic wasps acts on the octopamine system in cockroaches to cause hypokinesia and prevent the cockroach from fleeing the wasp (Rosenberg et al., 2007). This allows the wasp to lay its eggs inside the cockroach. The hypokinesia caused by the venom can be rescued through octopamine injections to the brain of the cockroach.

More recent research has demonstrated a role of octopamine in even more complex behaviors such as aggression, learning and memory, and cocaine sensitivity (Baier et al., 2002; Hardie et al., 2007; Hoyer et al., 2008; Schwaerzel

et al., 2003; Sitaraman et al., 2010; Zhou and Rao, 2008). Indeed, there is a current surge of studies on the role of octopamine in complex behaviors in *Drosophila*. To this end, many octopamine-signaling mutants have been generated, along with methods to target and modulate the neurons that produce octopamine. These reagents have been used to demonstrate that octopamine signaling in the mushroom body (the olfactory learning center in the fly) is necessary for the fly to acquire appetitive (sucrose-associated) memories but not place memories (Schwaerzel et al., 2003; Sitaraman et al., 2010). In addition, the ventral unpaired medial group of octopaminergic neurons in the fly brain is necessary for a male fly to show aggression (Zhou and Rao, 2008).

Prior to this thesis work, the role of octopamine in wakefulness in the fly remained unknown.,

Figure 1-1

Figure 1-1. Brain regions in the fly important for sleep–wake regulation. Many of the major neurotransmitter systems within the fly have been analyzed for their role in sleep. In the biogenic amine category, only octopamine has been localized to a specific cell group relevant for its role in sleep–wake behavior (purple). Dopamine and serotonin play a role in the regulation of sleep, but the specific subgroups of cells have not been mapped, and thus the major cell groups that produce these transmitters are shown in this figure (black and yellow). PDF-producing large ventral lateral neurons (LNv) are important for promoting arousal in response to light and are shown in blue. Despite not knowing where the neurotransmitter signals originate, areas that receive these signals have been identified (PI neurons or mushroom body). Manipulation of intracellular signaling pathways has also implicated these areas (PI neurons and mushroom body).

Table 1-1

Table 1. *Terms commonly used to describe sleep*

	Glossary
Electroencephalogram (EEG)	Measures electrical potentials on the surface of the head. It is thought to be a readout of activity in the underlying cortical neuronal populations.
Wake EEG	Small-amplitude, high-frequency oscillations. These waves are produced as a result of desynchrony in firing between cortical neurons.
NREM EEG	Larger-amplitude, lower-frequency oscillations that are thought to reflect the transition of these neurons to a more synchronous and bursting firing pattern.
REM EEG	Small-amplitude, high-frequency oscillations that look like the waking EEG, but are accompanied by loss of muscle tone and the presence of REM.
Slow wave sleep	Stage N3 and the deepest stage of sleep. This is when the synchrony in neuronal firing is thought to be at its highest. There is also the presence of delta waves, which are the slowest- and largest-amplitude EEG waves seen during sleep.
Delta power	The density of delta waves; this is thought to reflect one's need for sleep.
Circadian component of sleep	The timing of sleep.
Homeostatic component of sleep	The depth or length of sleep.

Table 1-2

Table 2. *Genes implicated in homeostatic regulation of sleep through genetic studies across species*

	Mammals	Fruit fly (*D. melanogaster*)	Zebrafish (*D. rerio*)	Worms (*C. elegans*)
Circadian genes	***Period 1,2,3***	***Period***		***Lin-42*** (*per* homolog)
	Bmal1	***Cycle***		
	NPAS2/Clock	*Pigment-dispersing factor (PDF)*		
	Prokineticin 2			
	Cry1/Cry2			
	CK1δ			
	Dec2			
Neurotransmitters				
Serotonin	*Sert*	*TRH*		
	5-HT1a	***5-HT1a***		
	5-HT1b			
	5-HT2a			
	5-HT2c			
	5-HT7			
Dopamine	***DAT***	***Fumin***		
		DopR1		
Norepinephrine (octopamine)	***DbH***	***TbH***		
		Tdc		
Histamine	*HDC*			
Acetylcholine	*Chrm3 (muscarinic AchR M3)*			
	Chrm2/4 (muscarinic AchR M2/4)			
Orexin/hypocretin	***Orexin/Hcrtrt2***	***PDF*** (hypothesized to function similarly)	***Hcrt* overexpression** ***Hcrtrt2***	
Adenosine	*A2a receptor*			
	A1 receptor			
GABA		*Rdl* (GABA receptor)		
Cytokines/immune or stress response genes	***NFκB1***			
	Bip	***Relish*** (***NFκB***)		
	TNF-α	***Bip***		
	IL-1b,IL-6,IL-10			
Synaptic transmission genes	***Homer1a***	***Homer***		
	dnSNARE			
	c-Fos			
	Gria3			
Ion channels				
K^+ channels	***Kv1.2***	***Shaker***		
	Kv3.1	***Hyperkinetic***		
	Kv3.3	*Sleepless*		
	Kv3.2			
Ca^{++} channels	*Cav2.2*			
	Cav3.1			
Signal				
Metabolic/cellular growth genes	*Ghrelin*	***Rho (enzyme in EGFR Pathway)***		***EGFR* (*Let-23*)**
	Leptin	***Spitz* (*TGF-a*)**		***EGF* (*Lin-3*)**
	Dwarf (GHRH)	*Star*		
	GHRHR			
	MT-rGH (growth hormone)			
Learning and memory genes	***Prkg1 (PKG)***	***Foraging* (*PKG*)**		***Egl-4* (*PKG*)**
	CREB	***dCREB2b***		
	α/δ	*Dunce (PDE)*		
		PKA		
		Rutabaga (cAMP)		
Genetic disorders	***Ube3A***	***dUbe3a***		
	FMR1	***dFMR1***		

All of the genes listed here have been analyzed for effects on sleep through genetic analysis, knockout, knock-in, or other mutants. Genes in bold are implicated in sleep in multiple species.

Table 1-3

Table 3. *Theories for sleep function*

Theory	Description	Genetic evidence
Somatic function	Sleep is for the body, such as for normal immune function and for normal metabolic activity.	Animals with mutations in the NFκ B gene, TNF-α, IL-B, ghrelin, and leptin display sleep phenotypes. Also, immune genes are up-regulated following periods of wakefulness.
Cellular metabolic function	Sleep is for repairing cells; e.g., removing reactive oxygen species or replenishing ATP levels.	Oxidative stress pathway genes are up-regulated following sleep deprivation.
Brain functioning		
Synaptic downscaling	Mechanisms that scale down synaptic strength to maintain relative strength of different synapses and yet conserve energy and space in the cell.	Genes involved in synaptic scaling are up-regulated during sleep. Mutations in cFos and GRIA3 show alterations in sleep. In QTL analysis for sleep homeostatic genes, Homer1a was found.
Memory consolidation	Sleep is important for consolidating memory in the hippocampus.	Alterations in CREB, PKA, and cAMP signaling alter sleep, as do alterations in PKG and BDNF. PDE4 is increased following sleep deprivation, and pharmacologically blocking this will rescue deprivation-induced impairments in LTP.

Chapter II:

Octopamine regulates sleep in *Drosophila* through protein kinase A-dependent mechanisms

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Abstract

Sleep is a fundamental process, but its regulation and function are still not well understood. The *Drosophila* model for sleep provides a powerful system to address the genetic and molecular mechanisms underlying sleep and wakefulness. Here we show that a *Drosophila* biogenic amine, octopamine, is a potent wake-promoting signal. Mutations in the octopamine biosynthesis pathway produced a phenotype of increased sleep, which was restored to wild type levels by pharmacological treatment with octopamine. Moreover, electrical silencing of octopamine-producing cells decreased wakefulness, where as excitation of these neurons promoted wakefulness. Because protein kinase A (PKA) is a putative target of octopamine signaling and is also implicated in *Drosophila* sleep, we investigated its role in the effects of octopamine on sleep. We found that decreased PKA activity in neurons rendered flies insensitive to the wake-promoting effects of octopamine. However, this effect of PKA was not exerted in the mushroom bodies (MB), a site previously associated with PKA action on sleep. These studies identify a novel pathway that regulates sleep in *Drosophila.*

Introduction

Sleep is a core process that spans genetically diverse eukaryotes from mammals to arthropods (Allada and Siegel, 2008; Tobler, 2005) . Disrupting

sleep in any of these organisms is detrimental to their performance, memory and health (Rechtschaffen, 1998). Extreme loss of sleep can even lead to death (Shaw et al., 2002). Thus, it must serve a very important function. Its conservation over evolution supports this claim for another reason: the need to sleep must outweigh selection pressure to eliminate it as a risk to predation. However, the function of sleep is unknown and the molecular regulation underlying it is poorly understood.

Sleep can be monitored through electroencephalograms (EEG) and electromyograms (EMG), but when such electrophysiological recordings are technically difficult, as in the case of *Drosophila*, it is monitored through analysis of behavior. *Drosophila* show a sleep state characterized by changes in position, increased arousal threshold, and periods of inactivity which can last several hours (Hendricks et al., 2000a; Shaw et al., 2000). Although little is known about the regulation of this sleep state, effects of some neurotransmitters have been described. Thus, GABA and serotonin promote sleep, the latter by acting through the 5-HT1A receptor expressed in the mushroom body (MB) (Yuan et al., 2006). The only arousal-promoting signal identified in *Drosophila* is dopamine (Andretic et al., 2005; Kume et al., 2005).

In mammals, dopamine and norepinephrine are associated with states of arousal (Aston-Jones and Bloom, 1981). However, results regarding the effects of norepinephrine on total sleep and wake amounts have been mixed (Hunsley and Palmiter, 2003; Ouyang et al., 2004), in part because of differences in experimental protocols and also perhaps attributable to the effects of

norepinephrine manipulation on dopamine levels (Schank et al., 2006). The insect equivalent of norepinephrine, octopamine, is synthesized and regulated through pathways that are distinct from those that produce dopamine. However, although octopamine is known to play a role in memory formation, larval locomotion, wing beating, ovulation and aggression (Roeder, 2005), it has not been examined for effects on sleep. Here we demonstrate a novel role for octopamine in the regulation of sleep and wake in *Drosophila*. Feeding octopamine to flies leads to a protein kinase A (PKA)-dependent decrease in total sleep, whereas removal of octopamine from the food is followed by a sleep rebound. In addition, flies mutant for octopamine show an increase in total sleep, which can be restored to control levels with the administration of octopamine. We show that electrical excitation of octopamine-producing cells decreases total sleep, whereas electrical silencing of these cells increases sleep. Other parameters of sleep such as sleep latency and arousal threshold are also altered. Last, we demonstrate an activity-promoting role for the octopamine precursor, tyramine, which is independent of the effects of octopamine.

Methods

Fly Strains Used

Wild-type fly strains include *w;RC1;RC1 (*isogenized chromosome 1 from the w^{1118} stock; isogenized chromosomes 2 and 3 from the *RC1* strain*)*, w^{1118}, *Canton S*, *Iso31 (*Isogenic w^{1118} strain*).* Octopamine production mutants include $Tdc2^{RO54}$ (*Tyrosine decarboxylase 2* mutant), $T\beta H^{nM18}$/*FM7* (*Tyramine* β

Hydroxylase mutant). Gal4 lines included *Tdc2-Gal4 (*neuronal *Tdc2* expression pattern), *Tdc1-Gal4* (non-neuronal *Tdc1* expression pattern), *Elav*GeneSwitch (pan-neuronal expression during adulthood), *MB*Switch (mushroom body specific expression during adulthood). Upstream Activating Sequence (*UAS*) lines include , *UAS-B16B* (*NaChBac*) (bacterial Na^+ channel), *UAS-Kir2.1(*inward rectifying K^+ channel)*, UAS-BDK33* (*PKAr*) (*Drosophila* inhibitory subunit of PKA with mutated cAMP binding site), *UAS-Tdc2, UAS-Tdc1*. The following lines were ordered from the Bloomington Stock center: *Tdc2-Gal4* (9313), *Tdc1-Gal4* (9312), *UAS-NaChBac* (9466), *UAS-Tdc2* (9315), *UAS-Tdc1* (9314), *UAS-GFPnls* (7032), Iso31 (5905). *UAS-Kir2.1* and *UAS-B16B* were a gift from Dr. B. White (National Institutes of Health, Bethesda, MD). *Elav*GeneSwitch (Osterwalder et al., 2001), *MB*Switch (Mao et al., 2004) and *UAS-BDK33* (Rodan et al., 2002) were used previously in the laboratory (Hendricks et al., 2001; Joiner et al., 2006). The wild-type isogenic line *w;RC1;RC1* was a gift from W. Joiner (University of Pennsylvania, Philadelphia, PA). *Tdc2*^{RO54} lines and *w*^{1118} background line were a gift from Dr. G. Schupbach (Princeton University, Princeton, NJ) and Dr. J. Hirsh (University of Virginia, Charlottesville, VA). The *TβH*^{nM18}*/FM7*, *TβH*^{m6}*/FM7*, and *Canton S* background control were a gift from Dr. E. Kravitz (Harvard University, Boston, MA).

All *Gal4* and *UAS* lines were outcrossed 7 times into the *w;RC1;RC1* or *Iso31* background. The *w;RC1;RC1* background was chosen for the *Tdc2-Gal4* expressing *Kir2.1* because it shows lower levels of nighttime sleep (Table 2-1) and thus affords the potential to avoid a ceiling effect.

Sleep Analysis

Sleep analysis was performed as previously described (Joiner et al., 2006). All flies were kept on a 12h light/dark (LD) cycle at 25˚C schedule unless otherwise noted. Female and male flies 4-8 d old were placed in 65mm x 5mm tubes containing 5% sucrose and 2% agar and entrained for 24-36 h before the sleep recording. Baseline sleep was determined by monitoring activity for at least 3 d with no disruptions in an LD cycle. Locomotor activity was monitored using the DAMS/Trikinetics system as described previously (Joiner et al., 2006). Sleep was defined as a 5 m bout of inactivity as described previously (Joiner et al., 2006; Shaw, 2003) . Latency to sleep was defined as the time in minutes from the moment lights were turned off to the first bout of sleep. Sleep consolidation scores were generated based on the amount of fragmentation seen in sleep, as measured by brief awakenings and the length of sleep bouts.

Arousal Threshold

Arousal threshold was measured at three times of the night (2 h after lights off, 6 h after lights off and 10 h after lights off). Increasing levels of mechanical stimulation were applied to determine the minimum stimulus for arousal. The levels were then labeled weak, medium, and strong, with weak being the lowest level of stimulation and strong being the maximum. Animals were scored based on their response to these three levels (Hendricks et al., 2000a).

Feeding Octopamine and Tyramine

Wild-type (*w;RC1;RC1, Iso31*) female animals were loaded into monitors as described above and given 24-36 h to acclimate. One day of baseline data were collected, and then, at the lights-on transition, the flies were transferred either to tubes containing 5% Sucrose/2% Agar plus 10mg/ml octopamine or onto sucrose- agar alone. Octopamine administered at 10mg/ml orally, was shown previously to be optimal for rescuing egg-laying (Monastirioti et al., 1996). A dose-response curve of *Iso31* flies on octopamine is shown in Fig. 2-10a. The animals were left for 3 d on or off octopamine and removed at the lights-on transition. Rebound was determined and analyzed as previously described for sleep deprivation (Joiner et al., 2006). The males were examined in the same manner; however 10mg/ml octopamine was fatal for them over three d so the octopamine concentration was reduced to 5mg/ml (data not shown). Other concentrations of octopamine were tested, but 10mg/ml was found to be the optimal amount for 3 d for wild-type females (Fig 2-10a). A blue food assay was performed as described (Edgecomb et al., 1994) to ensure that the animals were eating the food (data not shown). Because of the lethality seen with 10mg/ml octopamine, the $T\beta H^{nm18}$ females and controls were placed on 7.5mg/ml octopamine.

Tyramine was also fed to wild-type flies. Similar to what was seen with octopamine, tyramine at higher concentrations produced increased lethality so wild-type male flies were fed 5mg/ml tyramine in 5%sucrose/2%agar. Females were placed on 10mg/ml tyramine in 5%sucrose/2%agar. We tested other

amounts of tyramine and determined that 10mg/ml was the optimal concentration (data not shown).

Mianserin was also used to address octopamine signaling. Based upon the work of Maqueira et al. (2005), who used this compound to block cAMP increases attributable to octopamine in vitro (Maqueira et al., 2005), it was used at 0.2mg/ml. All mianserin experiments were done with *Iso31* control flies. Following the same protocol outlined above for octopamine, we placed flies on either mianserin alone, mianserin + 10mg/ml octopamine, control food or control + 10mg/ml octopamine.

Hydroxyurea Analysis

Ablation of mushroom bodies with hydroxyurea (HU) was performed as described previously (de Belle and Heisenberg, 1994). First instar larvae of *Iso31* flies were collected and placed on either a yeast paste and water mixture, or a yeast paste and 50mg/ml HU mixture for 4 h at 25˚C. They were then washed with water and placed in regular food vials until adulthood. At 4-8 d after eclosion, animals were loaded into monitor tubes as described above. They were given three d of acclimation and then transferred onto 7.5mg/ml octopamine, because high lethality was observed at 10mg/ml. After three d the animals were then transferred back onto normal 5% sucrose agar tubes. Sleep analysis was then performed as described above. Following the completion of the sleep analysis fly heads were dissected in 4% paraformaldehyde and fixed for 30 m, mounted on slides and analyzed for loss of the alpha and beta lobes of mushroom bodies.

PKA inhibition studies

For PKA inhibition studies, we crossed the *Elav*GeneSwitch transgene into *UAS-BDK33* flies. Octopamine at 10mg/ml was used as described above. Because the GeneSwitch construct can be turned on during adulthood using the drug RU486 (11β-(4-dimethylamino)phenyl-17β-hydroxy-17-(1-propynyl)estra-4,9-dien-3-one), we placed half the animals on 5% sucrose/2% Agar tubes containing either 500 μM RU486 dissolved in ethanol or ethanol alone (1%) for three d. Half of each group was then transferred to octopamine-containing food- either 5% sucrose/ 2% Agar + 500 μM RU486 + octopamine or 5% sucrose/2% Agar + ethanol (1%) + octopamine. Both groups were also simultaneously fed 10 mg/ml octopamine for 3 days. At the lights-on transition at the end of this period, animals were transferred off of octopamine onto 5%sucrose/2% agar containing either 500 μM RU486 or ethanol (1%). Sleep analysis was performed as described above.

Statistics

To compare multiple groups, 2-way ANOVA was used to determine significance for total sleep, nighttime sleep, daytime sleep, sleep bout number both day and night and latency to sleep. For non-Gaussian distributed data we used the Kruskal-Wallis Test; this included sleep bout length (daytime and nighttime), consolidation score, activity per waking minute and peak activity. Statistical significance is denoted by asterisks. * = $p \leq .01$, ** = $p \leq .001$, *** = $p \leq .0001$.

Results:

Mutants with reduced octopamine have increased sleep

To address a role for octopamine and its precursor, tyramine, in *Drosophila* sleep, we analyzed two known genes that affect biosynthesis of these amines (Certel et al., 2007; Cole et al., 2005; Monastirioti et al., 1996). As shown in the flow diagram in Figure 2-1A, the *Tdc2* enzyme synthesizes tyramine (precursor to octopamine) from tyrosine; thus, its disruption results in low levels of octopamine and tyramine (Cole et al., 2005). In contrast, *TβH* synthesizes octopamine from tyramine and therefore its loss reduces octopamine, but increases tyramine levels 10-fold (Fig.2-1a) (Monastirioti et al., 1996). Because synthesis of both amines requires *Tdc2*, the cellular distribution of *Tdc2* should reflect cells that potentially produce both tyramine and octopamine. We determined the expression pattern of *Tdc2* by using the well-known *UAS-Gal4* binary system to express green fluorescent protein (GFP) under the control of the *Tdc2* promoter (Fig. 2-1b). Expression of GFP was seen in discrete unilateral subsets of cells located along the ventral medial line of the brain, as well as in discrete bilateral clusters of cells in the lateral protocerebrum region and surrounding the oesophagus cavity.

We then assayed sleep in flies mutant for each of the two genes in the octopamine biosynthesis pathway. Baseline sleep levels were examined in flies carrying a point mutation in the *Tdc* gene ($Tdc2^{RO54}$) or a lesion, created by imprecise excision of a P transposable element, in the *TβH* gene ($T\beta H^{nm18}$). Under baseline conditions, we found that male and female animals of both

mutants displayed increased levels of sleep (Fig. 2-2a,b; Fig.2-3a,b). All male data are shown in Table 2-1 while female data are depicted in Figures 2-2 - 2-5. The increase in sleep in *Tdc2*^{RO54} and *TβH* ^{nm18} mutants occurred largely during the day (Fig.2-2a,b; 2-3a,b), perhaps because flies are already sleeping maximally during the night. The increase in total sleep was accompanied by a decreased latency to sleep (the time from lights off until the animal's first bout of sleep) in the *Tdc2*^{RO54} mutants (Fig. 2-2c), suggesting an increase in homeostatic drive to sleep. The *TβH* ^{nm18} mutation did not have a significant effect on latency, although there was a slight decrease in males (Table 2-1). The Canton S background strain that the *TβH*^{nm18} mutants were crossed into displays rapid onset of sleep compared to other wild type control lines used in this study (Table 2-1), which may occlude any decrease in latency caused by the mutation.

Analysis of sleep architecture indicated that the increase in sleep in *Tdc2*^{RO54} mutants was attributable to an increase in sleep bout number (Table 2-2), whereas the increase in *TβH* ^{nm18} mutants occurred from an increase in bout length (Table 2-2). Because both mutants affect octopamine similarly, this difference in sleep architecture is most likely attributable to differences in levels of tyramine. We also determined the arousal threshold in both mutants by measuring their response to a stimulus of increasing intensity. The arousal threshold during sleep was higher in both sets of mutants than in wild-type flies (Fig 2-2d and 2-3c). This suggests that these animals are in a deeper state of sleep than their controls.

An increase in sleep could result from the animals being sick and unable to move. To address this possibility, we measured locomotor activity in *Tdc2*^{RO54} and *TβH* ^{nm18}. We found that *Tdc2*^{RO54} and *TβH* ^{nm18} had peak activity levels that were not significantly different from those of controls (data not shown). However, , the *TβH* ^{nm18} flies showed significantly increased waking activity (Fig. 2-3d), as measured by total activity while awake; despite their increased total sleep time, the *TβH* ^{nm18} mutants showed a hyperactive phenotype when awake.. In contrast the average rate of movement was significantly decreased in *Tdc2*^{RO54} flies compared with the wild type controls (Fig. 2-3d) Because activity in the two mutants is affected in opposite directions, it is unlikely that the increased sleep phenotype of the mutants is secondary to effects on activity. More likely, the loss of octopamine (common in both mutants) underlies the decrease in sleep, whereas differences in tyramine levels account for effects on locomotor activity. In fact, our data are consistent with previously published data indicating that the increased (10 fold) levels of tyramine in the *TβH* ^{nm18} flies cause an increase in locomotor behavior when awake (Hardie et al., 2007).

It was shown previously that expression of *Tdc1* (the non-neuronal form of *Tdc*) in *Tdc2*-producing cells rescues the *Tdc2*^{RO54} mutant locomotor phenotype (Hardie et al., 2007). We found the same to be true for sleep. When *Tdc1* was expressed in *Tdc2*-producing cells in a *Tdc2*^{RO54} background, we found that we were able to rescue the baseline sleep phenotype (Fig. 2-2E), as well as sleep architecture and latency to sleep (data not shown).

Altering excitability of octopamine/tyramine-producing cells affects sleep

If octopamine and/or tyramine are released in the brain to regulate sleep, then blocking or increasing their release should also affect sleep. Thus, we sought to determine if electrical manipulation of the cells producing tyrosine decarboxylase, which should affect the release of octopamine and tyramine, produces a change in sleep. The *Tdc2-Gal4* line mentioned above, which expresses Gal4 in cells producing octopamine and tyramine, was crossed to flies carrying transgenes for ion channels under the control of a *UAS* element recognized by *Gal4* (*UAS-NaChBac* or *UAS-Kir2.1)* (Baines et al., 2001; Cole et al., 2005; Nitabach et al., 2005; White et al., 2001). The *UAS-NaChBac* transgene is derived from a gene encoding a bacterial Na^+ channel, which has the characteristics of high open probability and low inactivation, thus driving membrane voltage to a more depolarized and easily excited state. Expression of the Na^+ channel in *Tdc2*-positive cells resulted in a decrease in sleep of 56.5%, corresponding to a loss of ~346 min (Fig.2-4a,b). The loss of sleep was specific to the nighttime, with no significant sleep loss during the daytime, which may be indicative of normally high octopamine activity during the day. This hypothesis is consistent with mammalian studies where the noradrenergic cells of the locus coeruleus fire primarily during the active period (Aston-Jones and Bloom, 1981). We also found a corresponding increase in sleep latency in these flies. They took, on average, 74 min longer to fall asleep after lights off (Fig.2-4b). Flies expressing *NaChBac* in *Tdc2*-positive cells also showed a decreased arousal

threshold, suggesting that they are easily awakened during the night (Fig.2-4d). Bout analysis indicated that nighttime sleep bouts were shorter in duration (Table 2-2). In addition, there was a significant increase in daytime bout number (Table 2-2), which may reflect increased homeostatic drive resulting from the reduced sleep at night. However, the animals were unable to maintain long sleep bouts even under these conditions.

We also expressed a hyperpolarizing K^+ channel transgene under the control of the *Tdc2-Gal4* driver and found that this produced an increase in total sleep (Fig.2-5a,b). *Kir2.1* is an inward rectifying K^+ channel that has a high open probability and no inactivation. Expression of this channel hyperpolarizes neurons and decreases membrane resistance, thus making it more difficult for membrane potential to reach threshold for firing action potentials (Baines et al., 2001). Consistent with a previous report, we found that locomotor activity decreased when *Tdc2-Gal4* was used to express the inward rectifying K^+ channel (*UAS-Kir2.1*) (data not shown). Analysis of sleep parameters, however, revealed that it was actually an increase in sleep that accounted for the phenotype (Fig.2-5a,b). Flies expressing *UAS-Kir2.1* in *Tdc2* cells showed, on average, 174 min increase in sleep and also displayed changes in several sleep measures such as latency and arousal threshold. These flies also showed a decrease in latency to sleep (Fig. 2-5c) and an increased arousal threshold during sleep, requiring more stimulation to wake up compared with controls (Fig. 2-5d). In addition, they showed a trend toward longer bouts of sleep during the night, although the major increase in sleep came from the increased number of sleep bouts during the day

(Table 2-2). The relative lack of an increase in nighttime sleep length may result from a ceiling effect of sleep at night.

Although sleep levels and architecture are clearly affected in flies expressing sodium or potassium channels in *Tdc2* neurons, it is possible that altered activity levels contribute to the overall phenotype. Thus, we also examined peak activity levels and activity while awake. The *Tdc2-Gal4* females expressing *NaChBac* showed significantly lower peak activity (Table 2-1) as well as decreased activity while awake compared with the outcrossed background control (mean±SEM; *w;Tdc2-Gal4/NaChBac*;+, 1.7±0.06; *Iso31*, 2.09±0.06;$p \leq 0.01$, Kruskal-Wallis test). However, it is unlikely that a *decrease* in waking activity underlies a reduced sleep phenotype. We also found significantly reduced waking activity in animals expressing the K^+ channel under the control of the *Tdc2* driver (Table 2-1). This decrease in waking activity is similar to the decreased waking activity seen with the $Tdc2^{RO54}$ mutant, in which levels of both octopamine and tyramine are low (mean±SEM; *w;Tdc2-Gal4/kir2.1*;*RC1*, 2.14±0.06; *w;RC1;RC1*, 3.87±0.97;$p \leq 0.0001$, Kruskal-Wallis test). Thus, reducing electrical activity in octopamine-producing cells has the same effect on activity and sleep as a mutation ($Tdc2^{R054}$) that decreases levels of octopamine.

To ensure that the effect on sleep caused by loss of octopamine signaling is specific to neuronal *Tdc2* and not to a global loss of *Tdc*, we made use of a *Tdc1-Gal4* driver that is expressed in non-neuronal cells. Expression of the *UAS-B16B* transgene (*NaChBac* channel) under the control of this driver produced no significant change in sleep (Fig.2-6). Thus, we conclude that the

sleep phenotype observed in the *Tdc2*^{R054} mutant or produced by manipulations of *Tdc* cells is specific to the neuronal form of *Tdc*, *Tdc2*. Unfortunately the *UAS-Kir2.1* channel proved to be lethal with *Tdc1-Gal4*.

Given that the *NaChBac* channel increases excitability of *Tdc2* cells and thereby presumably stimulates release of octopamine/tyramine, we asked whether overexpressing *Tdc2* would have the same effect. As predicted, we found that overexpression of *Tdc2* in *Tdc2*-producing cells resulted in a 300 min decrease in nighttime sleep (Fig 2-7). As with the *NaChBac* channel this appears to be a nighttime specific sleep loss. There was also a decrease in sleep with the overexpression of *Tdc1* in *Tdc2*-producing cells (data not shown).

Oral administration of octopamine reduces sleep in flies

Because the *Tdc2*^{RO54} and *TβH* ^{nm18} mutants change levels of octopamine and tyramine, it is important to dissociate the effects of the two to identify the transmitter responsible for the sleep phenotype. As noted previously, *Tdc2*^{RO54} decreases levels of both tyramine and octopamine, whereas the *TβH* ^{nm18} decreases octopamine but increases tyramine. To determine whether a change in octopamine is sufficient to regulate sleep, we placed a wild-type isogenic line, Iso31, on octopamine-containing food for three d. It was shown previously that animals fed 10mg/ml octopamine have increased levels of this neurotransmitter, particularly in the brain (Barron et al., 2007). Supporting this finding, ingested octopamine rescues the egg-laying phenotype displayed by *TβH* ^{nm18} and *Tdc2*^{RO54} mutants (Cole et al., 2005; McClung and Hirsh, 1999; Monastirioti et

al., 1996). We found that flies fed 10 mg/ml octopamine had ~ 200 min less nighttime sleep than control flies maintained on sucrose-agar alone (Fig.2-8a) (Dose-response curve is shown in Fig 2-9a). Thus, similar to the *UAS-NaChBac* effect, this was a nighttime specific effect. Following the removal of octopamine, these flies showed a corresponding sleep rebound of ~70 min (mean±SEM; control, 289.9 ±13.92, n=40; 10mg/ml octopamine, 367.53 ± 16.91, n=40; $p \leq 0.001$, Two-way ANOVA)(Fig. 2-8c). In addition to the effect it had on wild type flies, a lower concentration of orally administered octopamine (7.5mg/ml octopamine) was able to restore the sleep phenotype of the $T\beta H^{nm18}$ mutant to control levels (Fig. 2-8c). This concentration of octopamine produced no significant change in sleep in the *Canton S* strain, which is the background of the $T\beta H^{nm18}$ mutants.

To exclude the possibility that the sleep phenotype was attributable to some non-specific toxicity associated with octopamine, we attempted to block the effect by inhibiting octopamine signaling. Thus, we co-administered 0.2mg/ml mianserin, which acts by inhibiting octopamine-induced cAMP increase (Fig. 2-8c) (Maqueira et al., 2005). Co-administration of mianserin almost completely blocked the effect of feeding 10mg/ml octopamine to flies. This demonstrates that the loss in sleep produced by octopamine feeding is attributable to the ingestion of octopamine itself and not attributable to toxic, non-physiological effects. In addition, these data suggest that the effects of octopamine on sleep are mediated by β receptors.

Wild-type flies fed tyramine did not show significant changes in sleep amount (Fig.2-9b). In addition, neither octopamine nor tyramine produced a significant change in activity while awake (Fig. 2-9c).

Octopamine acts through neuronal PKA to decrease sleep

cAMP-dependent protein kinase (PKA) signaling plays an important role in sleep in *Drosophila* (Hendricks et al., 2001) (Joiner et al., 2006). It is also known to be coupled to some of the octopamine and tyramine G-protein coupled receptors (Evans and Maqueira, 2005). Indeed, the blocker of octopamine used above inhibits cAMP signaling. To directly address whether the effect of octopamine on sleep is through PKA-dependent pathways, we expressed the regulatory subunit of PKA (*PKAr*), which inhibits activity of PKA, under the control of an *Elav*GeneSwitch driver (Joiner et al., 2006; Osterwalder et al., 2001). The use of the *Elav*GeneSwitch driver allowed us to inducibly express the regulatory subunit in all neurons. We found that expression of *PKAr* in adult neuronal tissue rendered the flies insensitive to the sleep-reducing effects of octopamine (Fig. 2-10a), supporting the idea that octopamine is acting through a PKA-dependent pathway to promote arousal.

Because the MB is implicated in the effects of PKA on sleep (Joiner et al., 2006), we sought to determine if this is also the site of octopamine action on sleep. Thus, we ablated the mushroom bodies with hydroxyurea as described previously (de Belle and Heisenberg, 1994) and then treated these flies with octopamine. The sensitivity to octopamine was intact despite the absence of the

MBs (Fig. 2-10b). As expected, the MB ablation itself reduced sleep, but there was an additional decrease produced by feeding octopamine (Fig. 2-10b). The decrease was 87 min, which was comparable to the amount of sleep lost when *Iso31* flies were fed 7.5mg/ml octopamine (82 minutes). To verify that the MBs were ablated, we followed up the behavioral analysis of all flies with anatomical analysis of the brain. All flies that still contained some α and β lobes were eliminated from analysis.

We also examined the effects of octopamine on flies expressing *PKAr* under control of an inducible mushroom body GeneSwitch driver (*MB*Switch) and found that these flies were still sensitive to octopamine (data not shown). Our inability to block effects of octopamine on sleep by inhibiting PKA signaling in MBs supports the finding that elimination of MBs by hydroxyurea does not block the wake-promoting effects of octopamine.

Discussion:

Biogenic amines play many important roles in mammals, with several having significant effects on sleep-wake states. Thus, dopamine, serotonin, and norepinephrine are all important for regulating states of arousal. We hypothesized that, like its counterparts in mammals, the invertebrate neurotransmitter octopamine would be important for arousal in *Drosophila*. That prediction was supported by the data reported here. We find that decreases in levels of octopamine increase sleep, while increasing octopamine causes a decrease in sleep. In addition, although the mammalian data have been

contradictory with respect to the role norepinephrine plays in total sleep, we find that octopamine decreases total sleep time. The mammalian data are complicated in part because perturbations of the norepinephrine pathway result in changes in the levels of dopamine (Ouyang et al., 2004). The use of *Drosophila* allows us to examine specifically the role of octopamine without perturbing dopamine signaling.

By modulating the excitability of octopamine-producing cells, we were able to manipulate the output of these cells. In mammals, one can record from specific cell populations to determine when the cells fire action potentials. Although this assay is difficult to do in flies, we were able to electrically modulate the cells through expression of K^+ and Na^+ ion channels. We found that when octopamine-producing cells were more depolarized (expression of a Na^+ channel), the animal was awake more and unable to stay asleep, whereas when the cells were hyperpolarized (expression of a K^+ channel), the animals slept more.

Based primarily upon larval crawling assays, octopamine and tyramine were implicated previously in locomotor behavior (Gong et al., 2004; O'Dell, 1994; Saraswati et al., 2004; Scholz, 2005). Specifically, larvae move slower through quadrants when they have decreased octopamine levels (the *TβH* ^{nm18} and *Tdc2*^{RO54} mutants). More recent work showed that adult *Tdc2*^{RO54} flies also have a decrease in locomotor activity attributable to the lack of tyramine (Hardie et al., 2007). Our data showing differences in activity in the *Tdc2*^{RO54} and the *TβH* ^{nm18} mutants support the claim that tyramine plays an important role in

locomotion. Thus, whereas increased levels of tyramine in *Tbh* mutants increase activity, decreased levels in *Tdc* mutants decrease locomotor activity. However, both mutations increase sleep, which is most likely attributable to the loss of octopamine. In addition to overall sleep, we find that other sleep parameters such as latency to sleep and arousal threshold are affected in flies carrying these mutations. We infer that tyramine plays a role in locomotion, but octopamine specifically affects arousal states.

Studies of other invertebrate species support a role for octopamine in arousal (Corbet, 1991a; Corbet, 1991b). In fact, octopamine agonists are potential natural pesticides because they cause insect species to “walk off” the leaves (Roeder, 1999). As in *Drosophila*, changes in octopamine levels affect behavior in honey bees, as demonstrated through feeding and injection of octopamine as well as through analysis of endogenous levels of octopamine. Fussnecker et al. (2006) showed that injections of octopamine promote flying in honeybees (Fussnecker et al., 2006). In addition, octopamine and tyramine regulate other behaviors in honeybees such as hive maintenance and foraging (Barron et al., 2002b; Schulz and Robinson, 1999, 2001a; Wagener-Hulme et al., 1999) . Octopamine and tyramine also modulate sensory input in honeybees (Kloppenburg and Erber, 1995a; Kloppenburg and Erber, 1995b; Scheiner et al., 2002). In the locust, octopamine mediates heightened arousal in response to new visual stimuli (Bacon et al., 1995). Bacon et al. found that a specific subset of octopamine-producing neurons in the brain of the locust fires during the presentation of new visual stimuli, causing dishabituation of the descending

contralateral movement detector (DCMD) interneuron. Interestingly, application of endogenous octopamine can mimic this state of heightened arousal. Our study suggests that octopamine serves to promote arousal in *Drosophila*. It is possible that the increased arousal we see with too much octopamine, or decreased arousal with too little, is a result of improper gating of sensory stimuli, but without electrophysiological data we are unable to draw any conclusions. Note also that the *Tdc2* cells important for sleep and arousal in the fly brain have not been identified yet.

In previous studies, octopamine was fed to flies to rescue or verify a phenotype of the $T\beta H^{nm18}$ flies. The ability of octopamine to rescue egg laying in $T\beta H^{nm18}$ mutants was assayed in this manner, as $T\beta H^{nm18}$ flies are unable to release eggs. Animals were placed on different levels of octopamine, and 10mg/ml octopamine over a period of 6 d provided maximal rescue (Monastirioti et al., 1996). Using the same concentration, we found that a steady increase in octopamine levels led to a decrease in nighttime sleep. Based on the specific effect on nighttime sleep, we speculate that octopamine levels are already high during the daytime, thereby precluding any effects of an increase. This analysis is supported by the Na^+ channel data in which a significant decrease in total sleep was found only during the nighttime sleep periods. We speculate that normally, activity of these cells is low at night, and so expression of the Na+ channel causes them to fire more and release octopamine at an abnormal time, thereby producing a decrease in sleep. Similar results, indicating nighttime-specific effects, were obtained with overexpression of *Tdc2*. Work in other

insects also supports the idea of modulated octopamine release. Pribbenow and Erber (1996) demonstrated that honeybees who are already in a heightened arousal state of antennae scanning, do not change scanning frequency in response to octopamine administration, but in animals scanning at a low frequency, injections of octopamine significantly increase scanning (Pribbenow and Erber, 1996).

Our data suggest that the effects of octopamine are mediated through PKA-dependent signaling. In mammals, there are 9 different adrenergic receptors, some of which signal through PKA (Hoffman and Lefkowitz, 1996a; Hoffman and Lefkowitz, 1996b). The α1 adrenergic receptor is the only receptor associated with a wake-promoting effect in that the agonist methoxamine causes an increase in waking (Hilakivi and Leppavuori, 1984; Monti et al., 1988). However, the antagonist has no effect on total sleep (Benington et al., 1995; Berridge and Espana, 2000). It is important to note that the α1 receptor in mammals is thought to be coupled to phospholipase C and Gq (Ramos and Arnsten, 2007). The β adrenergic receptors (which are coupled to cAMP and PKA) probably do not have specific effects on sleep in mammals because, contrary to known effects of norepinephrine, the agonist increases sleep and the antagonist decreases sleep (Monti et al., 1988). Studies in *Drosophila* may be better able to identify biogenic amine receptors relevant for sleep because of the ease of genetic manipulation Many G-protein-coupled receptors in *Drosophila* which display activity that allows their bona fide classification as octopamine receptors (Evans and Maqueira, 2005). Our data here suggest that receptors

sensitive to mianserin are likely to be involved in regulating fly sleep. Because mianserin inhibits cAMP signaling, these data not only further support a role for PKA, but also implicate β receptors in octopamine action. We note that none of these receptors is known to display a circadian cycling profile.

Given that PKA was shown previously to regulate sleep in *Drosophila*, we are starting to see a link between the various molecules that affect *Drosophila* sleep. Interestingly, however, octopamine does not appear to act through the MBs, a structure known to mediate effects of PKA on sleep and also to express a class of octopamine receptors. Because flies lacking MBs still have substantial amounts of sleep, it is clear that other parts of the fly brain can drive sleep. The current study shows that even PKA can affect sleep in regions outside the MB. Defining the site of action of sleep-regulating molecules such as octopamine should help to identify these other brain regions.

Figures and Tables

Figure 2-1

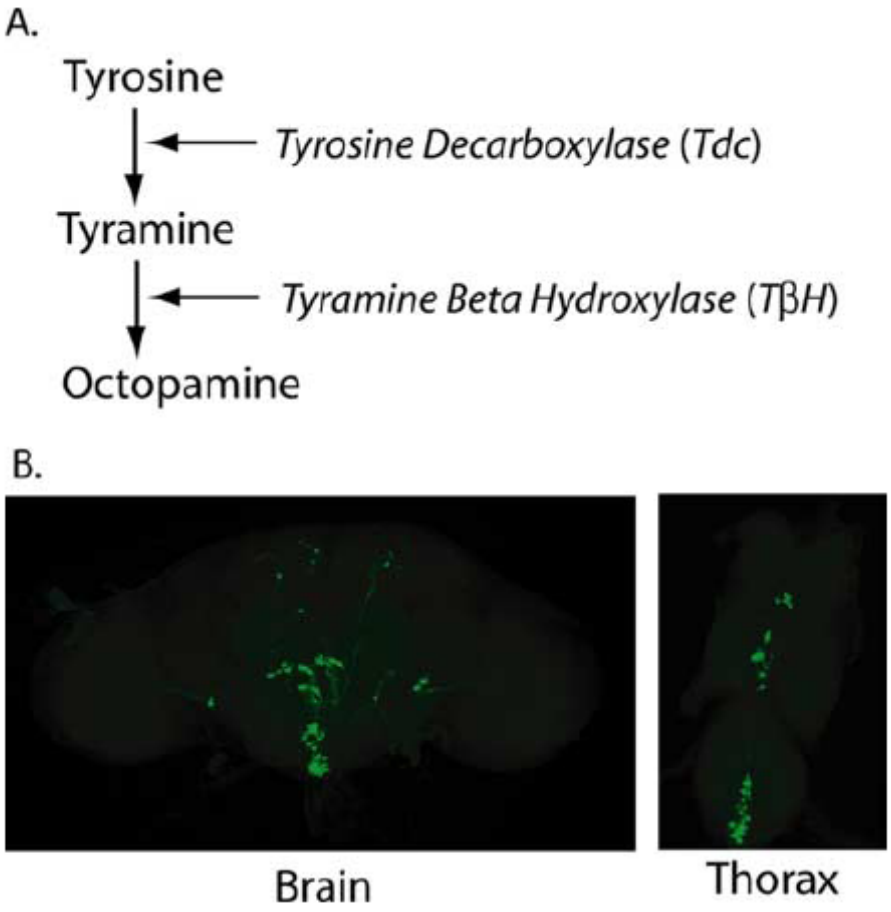


Figure 2-1. **The octopamine biosynthesis pathway and its distribution in the fly brain** A. Octopamine and tyramine are derivatives of tyrosine. Tyrosine is converted to tyramine by tyrosine decarboxylase (*Tdc*). Tyramine is then converted to octopamine by tyramine β hydroxylase (*TßH*). B. Expression pattern of the *Tdc2-Gal4* line as visualized with a GFP reporter. Octopamine is produced in a subset of neurons in the brain and thoracic ganglion. Expression was characterized by crossing *Tdc2-Gal4* with nuclear *UAS-GFPnls*.

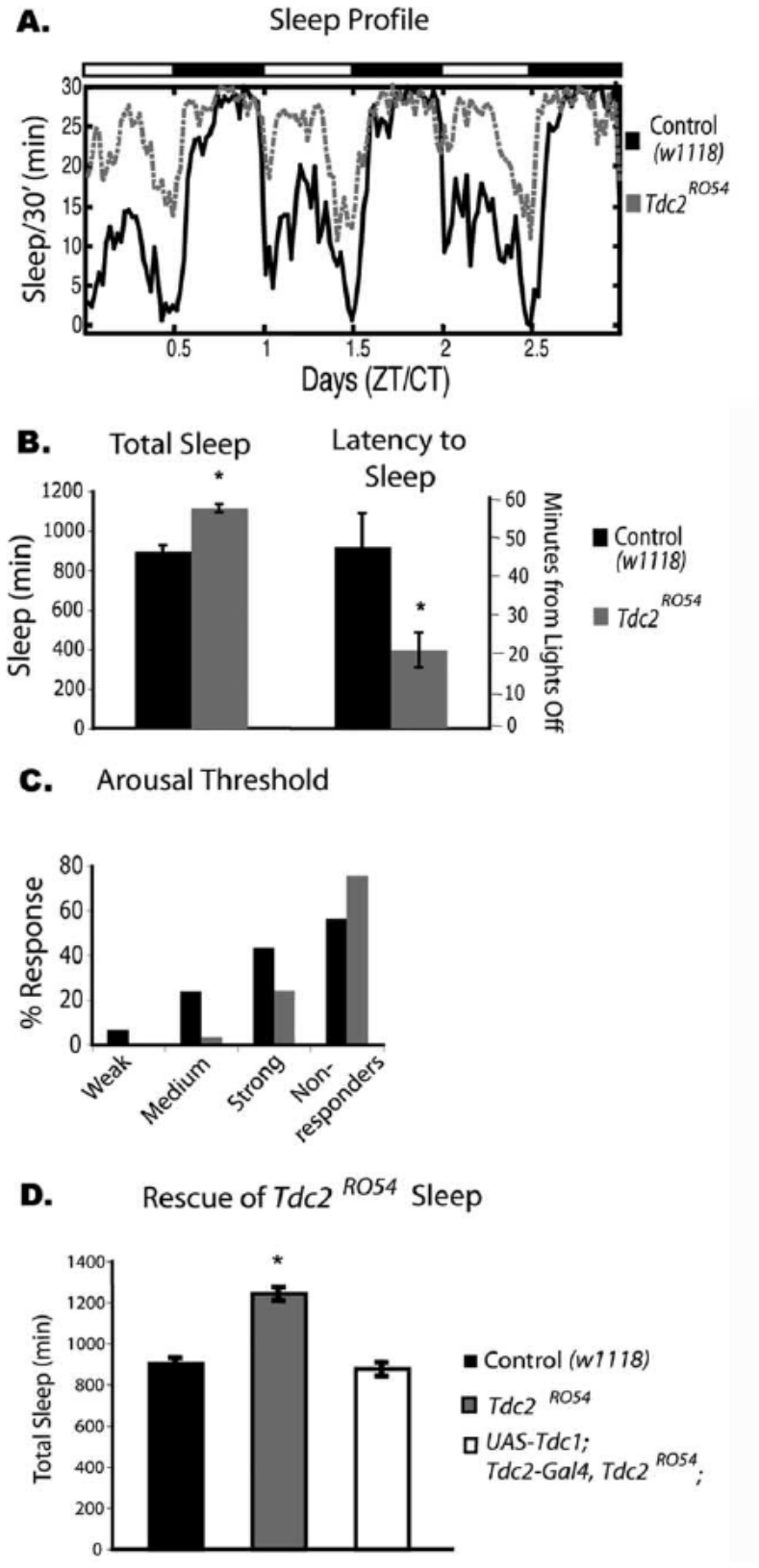

A.
Sleep Profile
Sleep/30' (min)
Days (ZT/CT)
Control (w1118)
Tdc2 RO54
B.
Total Sleep
Latency to Sleep
Sleep (min)
Minutes from Lights Off
Control (w1118)
Tdc2 RO54
C.
Arousal Threshold
% Response
Weak
Medium
Strong
Non-responders
D.
Rescue of Tdc2 RO54 Sleep
Total Sleep (min)
Control (w1118)
Tdc2 RO54
UAS-Tdc1; Tdc2-Gal4, Tdc2 RO54;

Figure 2-2. **Baseline sleep phenotype of *Tdc2*^{RO54} mutants, which have decreased levels of octopamine and tyramine.** ***A***, Three days of baseline sleep recording in*Tdc2* females. The *Tdc2*^{RO54} line (gray dashed line) shows significantly more sleep than its control w^{1118} (black solid line). Dark bars and white bars on top indicate nighttime and daytime, respectively. n = 8 for *Tdc2*^{RO54} and w^{1118}. CT, Circadian time; ZT, Zeitgeber time. ***B***, Total sleep is significantly increased in*Tdc2*^{RO54} mutant females (mean ± SEM; *Tdc2*^{RO54}, 1115 ± 19, n = 23; w^{1118}, 895 ± 32, n = 32; $p \leq 0.0001$, two-way ANOVA). Latency to sleep is significantly lower in *Tdc2*^{RO54} mutants (mean ± SEM; *Tdc2*^{RO54}, 20.6 ± 4.6, n = 23; w^{1118}, 47.5 ± 8.7, n = 32; $p \leq 0.001$, two-way ANOVA). ***C***, Arousal threshold during sleep in the *Tdc2*^{RO54} mutants. The animals were given three levels of stimulation to determine whether they were arousable. All animals that responded to the first stimulation were also aroused on stronger stimulation. Compared with wild type, the *Tdc2* mutant line had a higher percentage of flies that did not respond to any of the three levels of stimulation. In addition, fewer *Tdc2*^{RO54} flies responded to the weaker stimuli (*Tdc2*^{RO54}, n = 23; w^{1118}, n = 32).***D***, Rescue of the baseline sleep phenotype in *Tdc2*^{RO54} with expression of the *Tdc1* gene. Total sleep was quantified, and *Tdc2*^{RO54} mutant females were significantly different from both wild-type and rescued animals. There was no significant difference between rescued animals and controls (mean ± SEM; w^{1118}, 895 ± 33, n = 32; *Tdc2*^{RO54}, 1238 ± 33, n = 16; $p \leq 0.0001$; *UAS–Tdc1*; *Tdc2–Gal4*, *Tdc2*^{RO54}, 871 ± 33; n = 16).

Figure 2-3

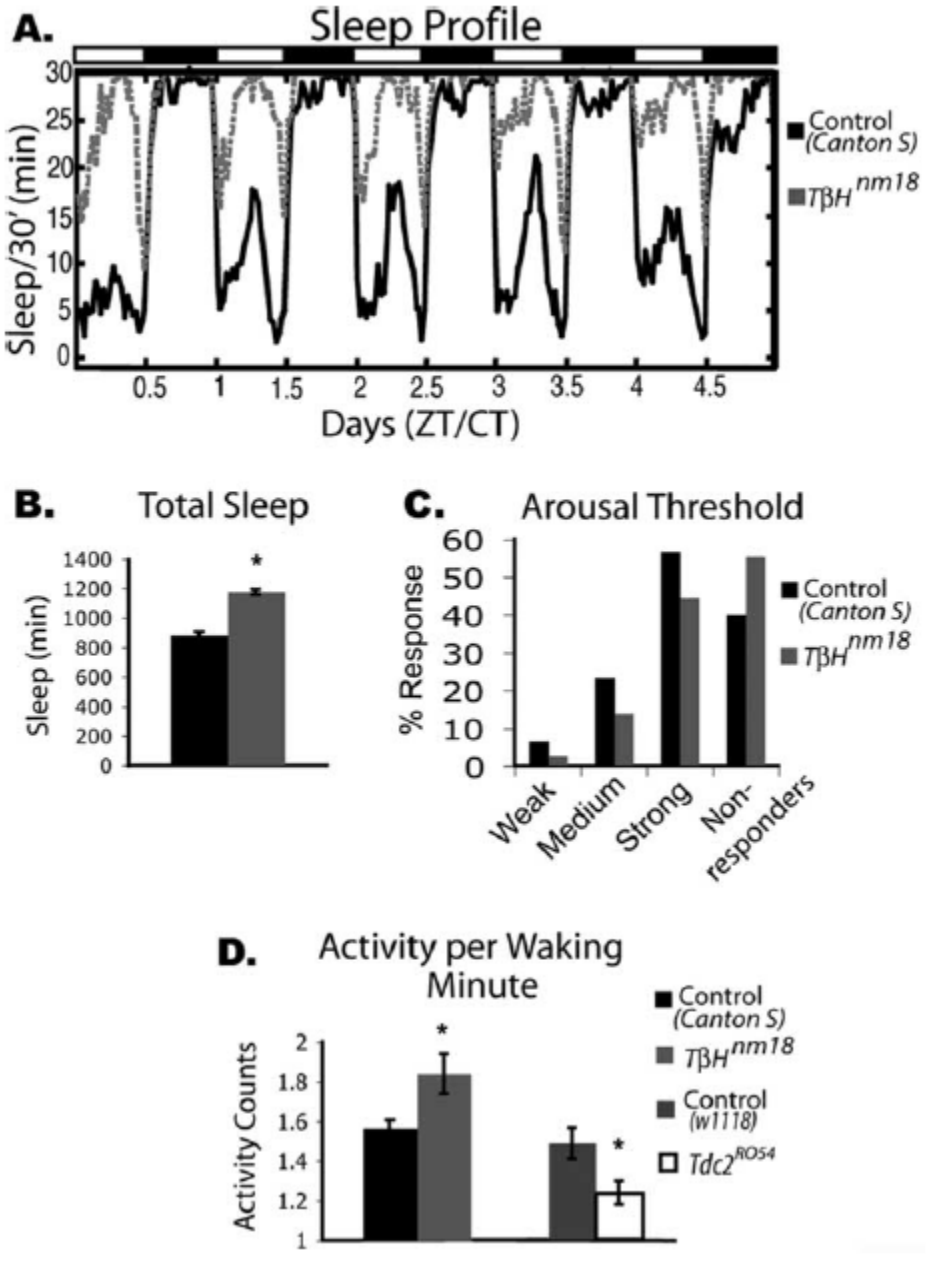

A.
Sleep Profile
Sleep/30' (min)
Days (ZT/CT)
Control (Canton S)
TβH nm18
B.
Total Sleep
Sleep (min)
C.
Arousal Threshold
% Response
Weak
Medium
Strong
Non-responders
D.
Activity per Waking Minute
Activity Counts
Control (Canton S)
TβH nm18
Control (w1118)
Tdc2 RO54

Figure 2-3. **Baseline sleep phenotype of *TβH* ^{nm18} mutants, which have decreased levels of octopamine and increased levels of tyramine**. ***A***, Five days of baseline sleep recording in *T*β*H* females. The *T*β*H*^{nm18} line (gray dashed line) shows significantly more sleep than its control, *Canton S* (black solid line). Dark bars and white bars on top indicate nighttime and daytime, respectively. Sixteen animals are shown for each group. CT, Circadian time; ZT, Zeitgeber time. ***B***, Total sleep is significantly increased in the *TbH*^{nm18} mutants (mean ± SEM; *T*β*H*^{nm18}, 1176 ± 17, *n* = 46; *Canton S*, 884 ± 20, *n* = 52; $p \leq$ 0.0001, two-way ANOVA). ***C***, Arousal threshold for the *T*β*H*^{nm18} mutants. The animals were given three levels of stimulation to determine whether they were arousable. All animals that responded to the first stimulation were also aroused on stronger stimulation. Compared with wild type, the*Tbh* mutant line had a higher percentage of flies that did not respond to any of the three levels of stimulation. In addition, fewer *Tdc2*^{RO54} flies responded to the weaker stimuli (*T*β*H*^{nm18}, *n* = 46; *Canton S*, *n* = 52). ***D***, Activity per waking minute, which quantifies activity during the time the animal is awake, was significantly increased in the *T*β*H*^{nm18} mutants (mean ± SEM; *T*β*H*^{nm18}, 1.84 ± 0.13; *Canton S*, 1.57 ± 0.05; $p \leq 0.01$, Kruskal–Wallis test) but significantly decreased in the *Tdc2*^{RO54} mutants (mean ±SEM; *Tdc2*^{RO54}, 1.2 ± 0.09; *w*^{1118}, 1.5 ± 0.08; $p \leq$ 0.01, Kruskal–Wallis test).

Figure 2-4

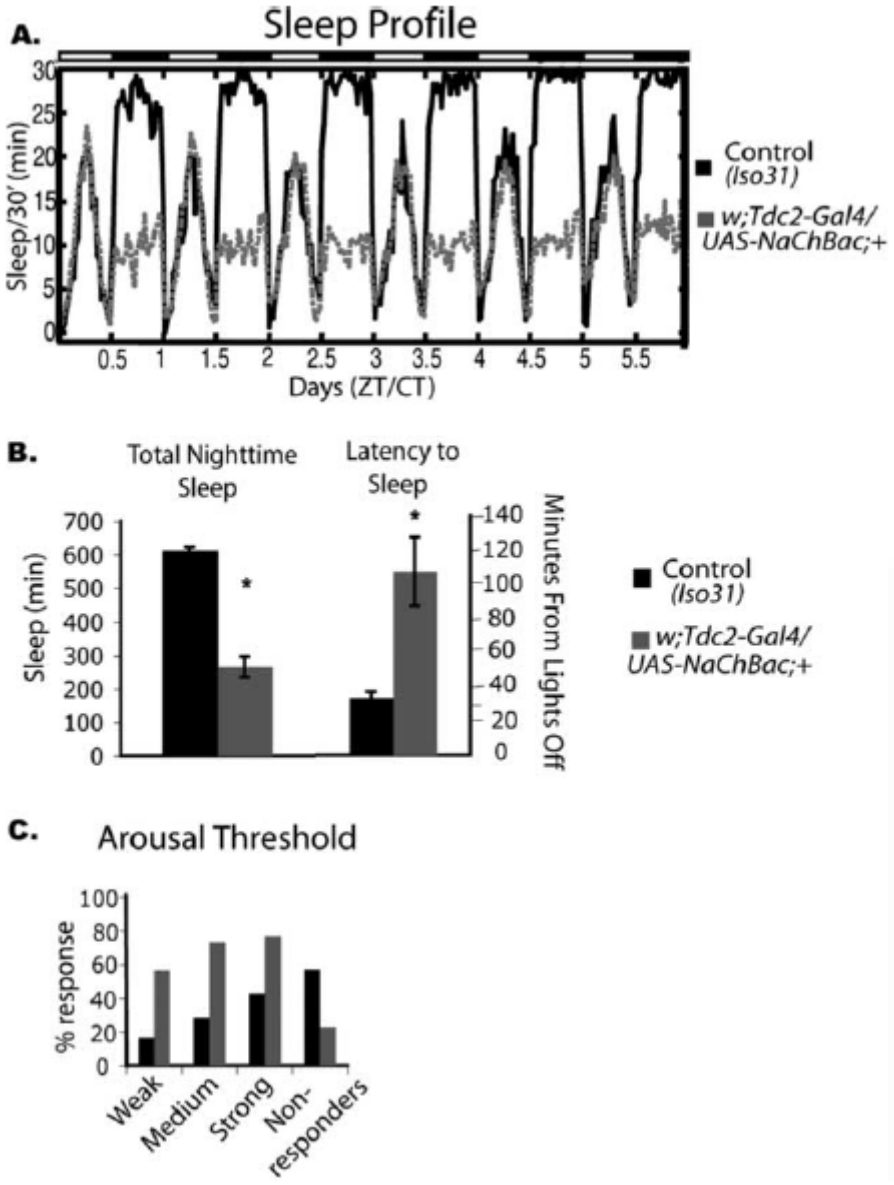

A.
Sleep Profile
Sleep/30' (min)
Days (ZT/CT)
Control
(Iso31)
w;Tdc2-Gal4/
UAS-NaChBac;+
B.
Total Nighttime
Sleep
Latency to
Sleep
Sleep (min)
Minutes From Lights Off
Control
(Iso31)
w;Tdc2-Gal4/
UAS-NaChBac;+
C.
Arousal Threshold
% response
Weak
Medium
Strong
Non-
responders

Figure 2-4. **Baseline sleep phenotype produced by depolarizing *Tdc2*-positive neurons.** Female flies carrying a *UAS-NaChBac* transgene under the control of *TDC2-Gal4* were assayed for sleep. ***A***, Six days of baseline sleep recording of eight animals each. *w*;*Tdc2–Gal4*/*NaChBac*;+ flies (gray dashed line) show significantly less sleep then *Iso31* controls (black solid line). Dark bars and white bars indicate nighttime and daytime, respectively. CT, Circadian time; ZT, Zeitgeber time. ***B***, Nighttime sleep is significantly decreased in *w*;*Tdc2–Gal4*/*NaChBac*;+ flies (mean ± SEM; *w*;*Tdc2–Gal4*/*NaChBac*;+, 267 ± 31, $n = 33$; *Iso31*, 613 ± 9, $n = 58$; $p \leq 0.0001$, two-way ANOVA). The latency to sleep is significantly longer in *w*;*Tdc2–Gal4*/*NaChBac*;+ flies (mean ± SEM; *w*;*Tdc2–Gal4*/*NaChBac*;+, 107 ± 20, $n = 33$; *Iso31*, 34 ± 3, $n = 58$; $p \leq 0.01$, two-way ANOVA). ***C***, Arousal threshold in the w;*Tdc2–Gal4*/*NaChBac*;+ flies. The animals were given three levels of stimulation to determine whether they were arousable. All the *w;Tdc2–Gal4/NaChBac;*+ flies were more arousable at each stimulation level, and there was a much lower percentage of animals that did not respond at all (*w*;*Tdc2–Gal4*/*NaChBac*;+, $n = 33$; *Iso31*, $n = 58$).

Figure 2-5

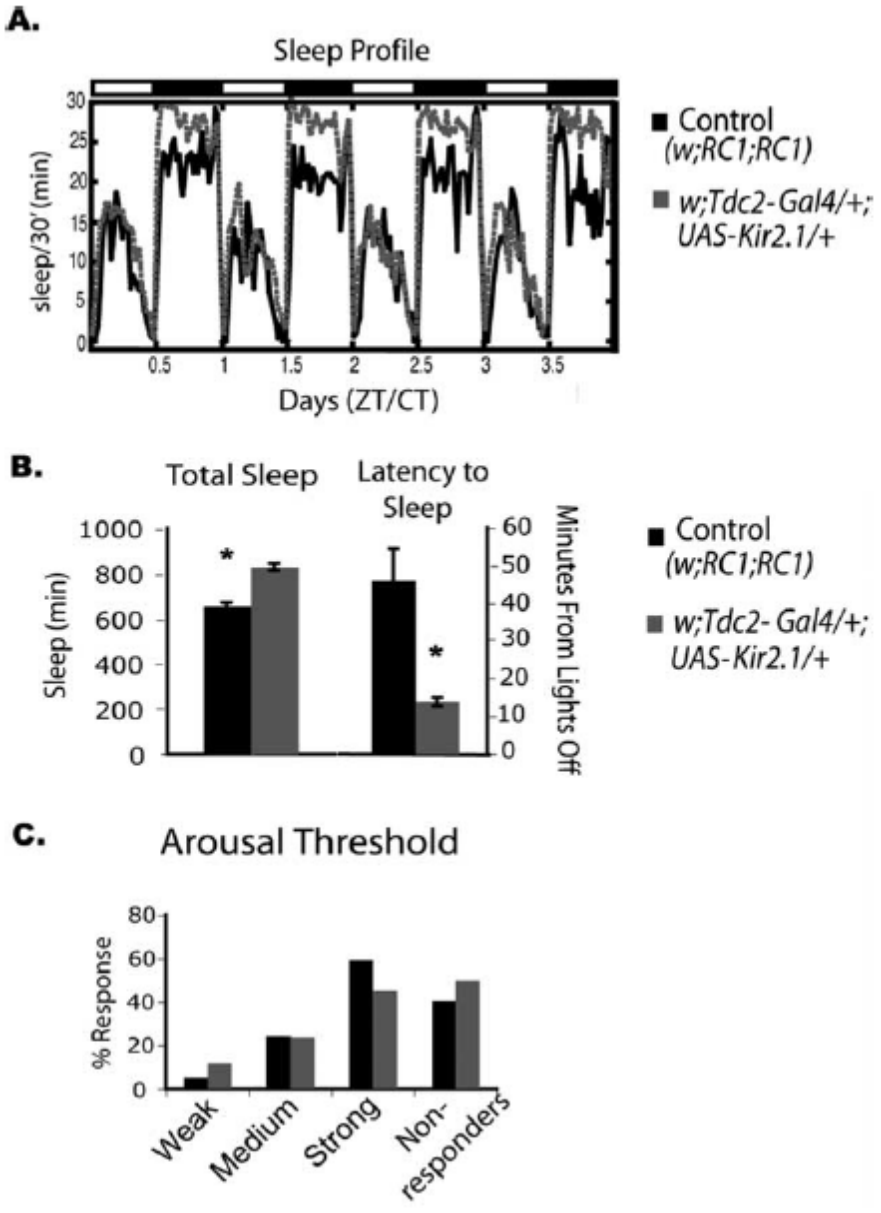

A.
Sleep Profile
sleep/30' (min)
Days (ZT/CT)
Control (w;RC1;RC1)
w;Tdc2-Gal4/+; UAS-Kir2.1/+
B.
Total Sleep
Latency to Sleep
Sleep (min)
Minutes From Lights Off
Control (w;RC1;RC1)
w;Tdc2- Gal4/+; UAS-Kir2.1/+
C.
Arousal Threshold
% Response
Weak
Medium
Strong
Non-responders

Figure 2- 5. **Baseline sleep phenotype produced by hyperpolarizing *Tdc2*-positive neurons.** Female flies carrying a *UAS-Kir2.1* transgene under the control of *Tdc2-Gal4* flies were assayed for sleep. ***A***, Four days of baseline sleep recording of 16 animals for each group. *w*;*Tdc2–Gal4/Kir2.1;RC1*flies (gray dashed line) show significantly more sleep then their controls, *w;RC1;RC1* (black solid line). Dark bars and white bars indicate nighttime and daytime, respectively. CT, Circadian time; ZT, Zeitgeber time. ***B***, Total sleep is significantly increased in *w*;*Tdc2–Gal4/Kir2.1;RC1*flies (mean ± SEM;*w*;*Tdc2–Gal4/Kir2.1;RC1*, 829.19 ± 14.14, *n* = 47; *W*+;*RC1*;*RC1*, 655.35 ± 15.30, *n* = 47; $p \leq 0.0001$, two-way ANOVA). The latency to sleep is significantly lower in *Tdc2* x *Kir2.1* flies (mean ± SEM; *w*;*Tdc2–Gal4/Kir2.1;RC1*, 13.86 ± 1.19, *n* = 47; *w;RC1;RC1*, 45.68 ± 8.40, *n* = 47; $p \leq 0.001$, two-way ANOVA). ***C***, Arousal threshold in *w*;*Tdc2–Gal4/Kir2.1;RC1* flies. The animals were given three levels of stimulation to determine whether they were arousable. All animals that responded to the first stimulation were aroused on stronger stimulation. Compared with controls, an increased percentage of *w*;*Tdc2–Gal4/Kir2.1;RC1* flies did not respond to any of the three levels of stimulation. At the strongest stimulation, more control flies responded than *w*;*Tdc2–Gal4/Kir2.1;RC1* flies (*w*;*Tdc2–Gal4/Kir2.1;RC1*, *n* = 47; *w;RC1;RC1*, *n* = 47).

Figure 2-6:

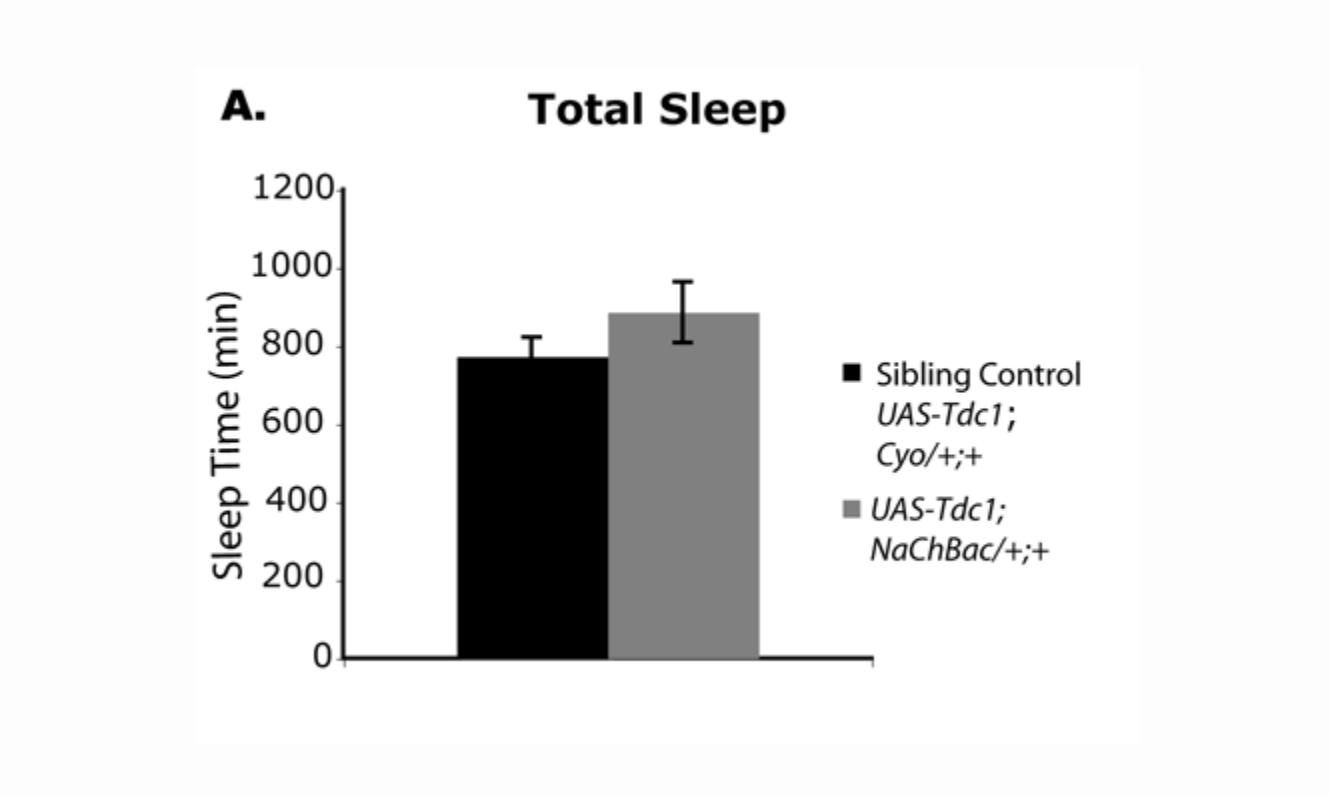


Figure 2-6 (Supplemental Figure 1). Total sleep in *Tdc1-Gal4* female flies expressing *UAS-NaChBac*. A. Total sleep is not significantly different between *Tdc1-Gal4;NaChBac/+;+* flies and sibling controls. (*Tdc1-Gal4;NaChBac/+;+*= 774 ± 48 S.E.M. N=32 *Tdc1-Gal4;Cyo*/+;+ (sibling controls) = 886 ± 78 S.E.M. N=32 2-way ANOVA)

Figure 2-7

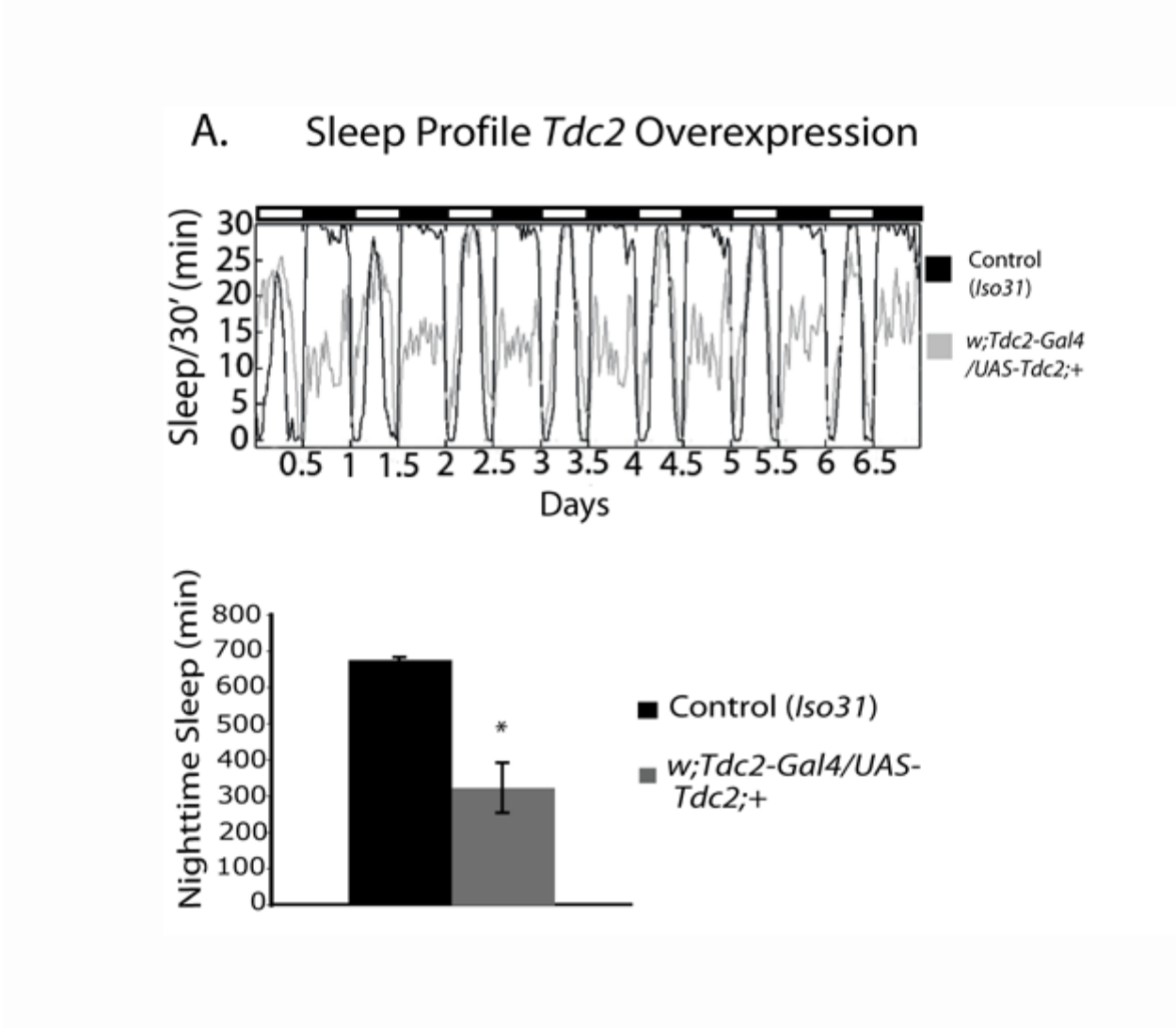
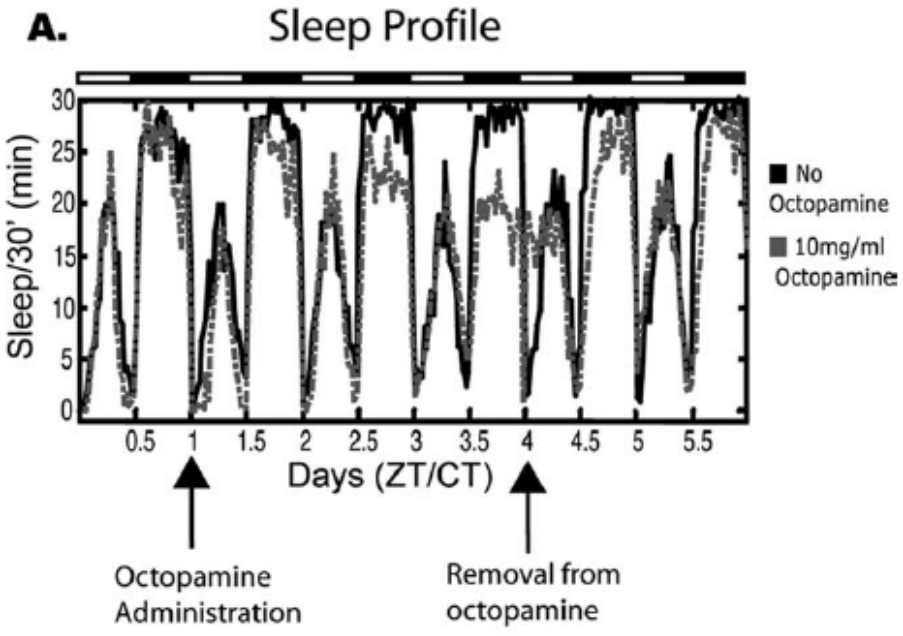
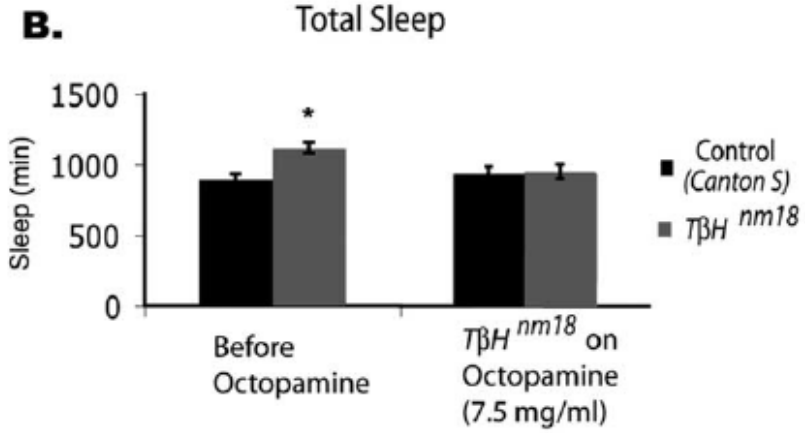


Figure 2-7 (Supplemental Figure 2). Overexpression of *Tdc2* causes decreases in sleep. A. Female flies carrying a *UAS-Tdc2* transgene under the control of *Tdc2-Gal4* were assayed for sleep. Baseline sleep was measured for six days in 16 animals of each genotype and total nighttime sleep was quantified. *Tdc2 x Tdc2* flies (grey line) show significantly less sleep than controls (black solid line) (w+;*UAS-Tdc2/ Tdc2-Gal4;*+ = 322 ± 69 S.E.M. N=16 *w+;Tdc2-Gal4/+;*+ = 676 ± 6 S.E.M. N=16 p≤.0001 2-way ANOVA)


A.
Sleep Profile
Sleep/30' (min)
30
25
20
15
10
5
0
0.5
1
1.5
2
2.5
3
3.5
4
4.5
5
5.5
Days (ZT/CT)
No Octopamine
10mg/ml Octopamine
Octopamine Administration
Removal from octopamine


B.
Total Sleep
Sleep (min)
1500
1000
500
0
*
Before Octopamine
TβH nm18 on Octopamine (7.5 mg/ml)
Control (Canton S)
TβH nm18

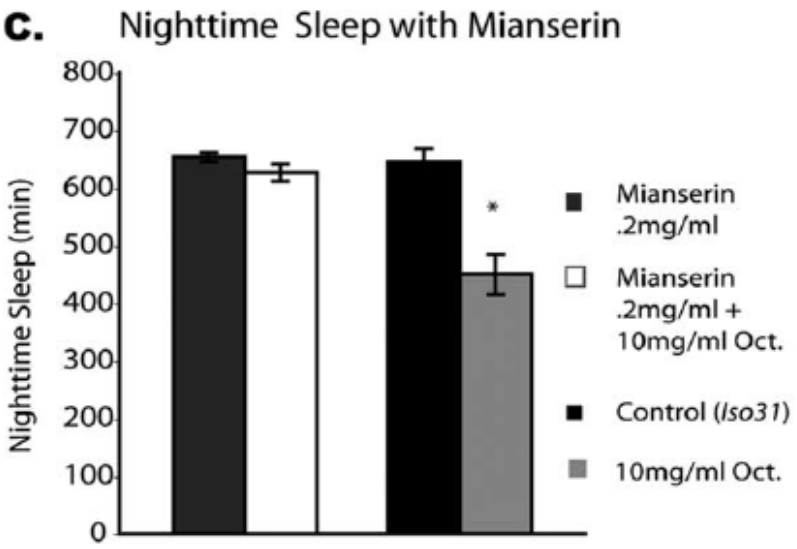

C.
Nighttime Sleep with Mianserin
Nighttime Sleep (min)
800
700
600
500
400
300
200
100
0
*
Mianserin .2mg/ml
Mianserin .2mg/ml + 10mg/ml Oct.
Control (Iso31)
10mg/ml Oct.

Figure 2-8 Oral administration of octopamine decreases sleep in *Iso31* flies and *TβH* ^{nm18} mutant flies. Female flies of the *Iso31* strain were treated with octopamine and assayed for sleep ***A***, The sleep profile shows 1 d of baseline sleep, followed by administration of 10 mg/ml octopamine (gray dashed line) for 3 d. Octopamine was then removed and sleep was assayed for 2 more days. Control flies (black line) were fed normal food during this time. Arrows indicate the time when food was changed. The data are from 16 animals for each group. There was a significant decrease in nighttime sleep for the *Iso31* flies on 10 mg/ml octopamine (mean ± SEM; control, 612.04 ± 8.47, $n = 40$; 10 mg/ml octopamine, 409.33 ± 19.77, $n = 40$; $p \leq 0.0001$, two-way ANOVA). CT, Circadian time; ZT, Zeitgeber time. ***B***, Sleep in *TbH*^{nm18} mutant flies is restored to control levels through the administration of octopamine. Before octopamine administration, *T*β*H*^{nm18} flies showed a significant increase in total sleep time; after administration of octopamine, sleep in *T*β*H*^{nm18} flies was not significantly different from that of control (*Canton S*) flies not on octopamine (*Canton S*, 796 ± 55 min; *Canton S* plus 7.5 mg/ml octopamine, 792 ± 54 min; $n = 16$ for each group). ***C***, Coadministration of mianserin blocks the effect of octopamine on nighttime sleep. When 10 mg/ml octopamine was administered along with 0.2 mg/ml mianserin, there was no longer a significant drop in nighttime sleep (compare with columns on the right). Sleep in flies treated with mianserin alone was not significantly different from that of controls [mean ± SEM; mianserin, 654 ± 8, $n = 32$; mianserin plus 10 mg/ml octopamine, 627 ± 15, $n = 32$; Iso31

(Control), 647 ± 22, n = 32; 10 mg/ml octopamine, 450 ± 35, n = 32; $p \leq 0.001$, two-way ANOVA].

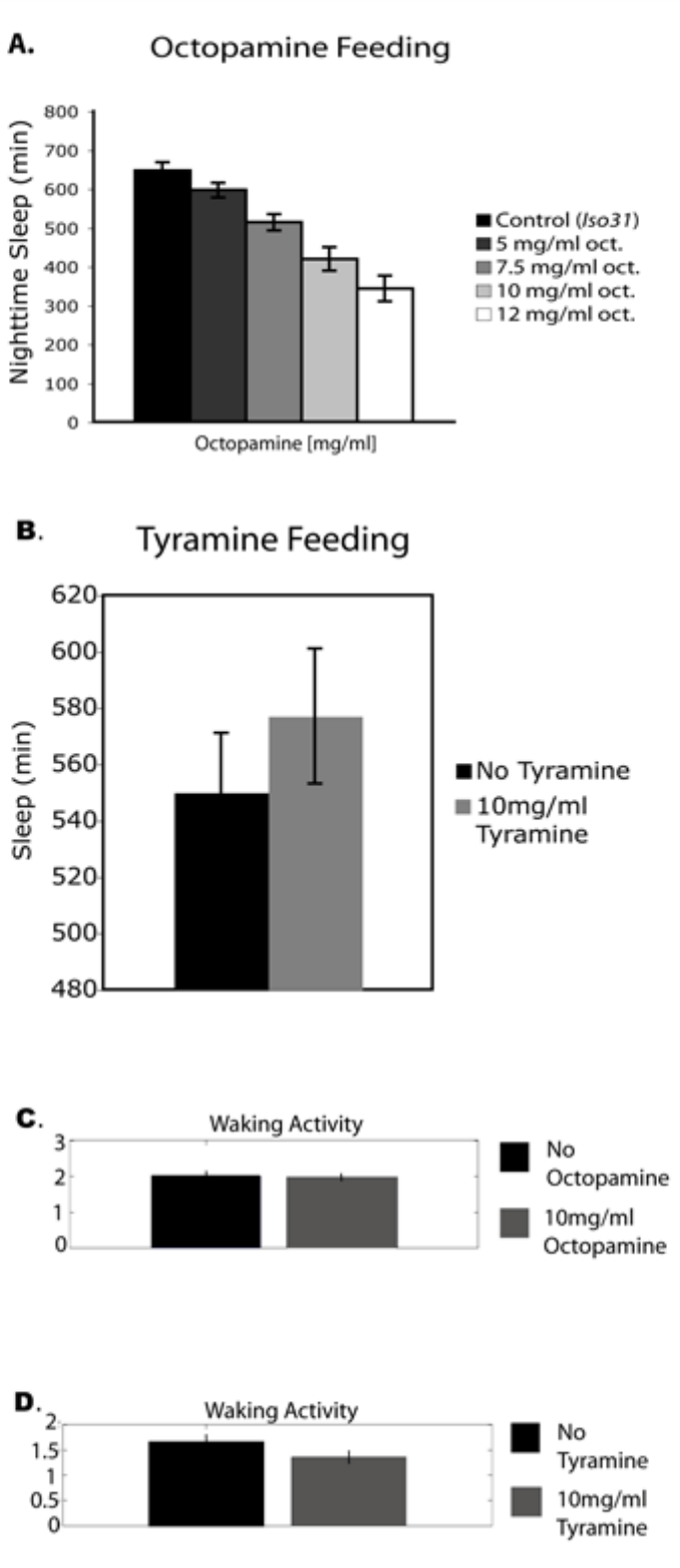

A.
Octopamine Feeding
Nighttime Sleep (min)
800
700
600
500
400
300
200
100
0
Octopamine [mg/ml]
Control (Iso31)
5 mg/ml oct.
7.5 mg/ml oct.
10 mg/ml oct.
12 mg/ml oct.
B.
Tyramine Feeding
Sleep (min)
620
600
580
560
540
520
500
480
No Tyramine
10mg/ml Tyramine
C.
Waking Activity
3
2
1
0
No Octopamine
10mg/ml Octopamine
D.
Waking Activity
2
1.5
1
0.5
0
No Tyramine
10mg/ml Tyramine

Figure 2-9 (Supplemental Figure 3). A. Dose response curve for effects of octopamine on sleep. As the concentration of octopamine increases there is a corresponding decrease in nighttime sleep. (0 oct. 647 ± 22 S.E.M. n=18, 5mg/ml oct. 597 ± 19 S.E.M. n=8, 7.5 mg/ml oct. 514 ± 21 S.E.M. n=24, 10mg/ml oct. 420 ± 31 S.E.M. n=30, 12 mg/ml oct. 343 ± 33 S.E.M. n=21) **B**. **Effect of tyramine on nighttime sleep**. 10 mg/ml tyramine did not significantly alter nighttime sleep amounts in Iso31 flies. (Iso31 n=32, Iso31 + Tyramine n=32) **C and D.** Waking activity was not significantly different in flies fed either 10mg/ml octopamine or 10mg/ml tyramine.

Figure 2-10

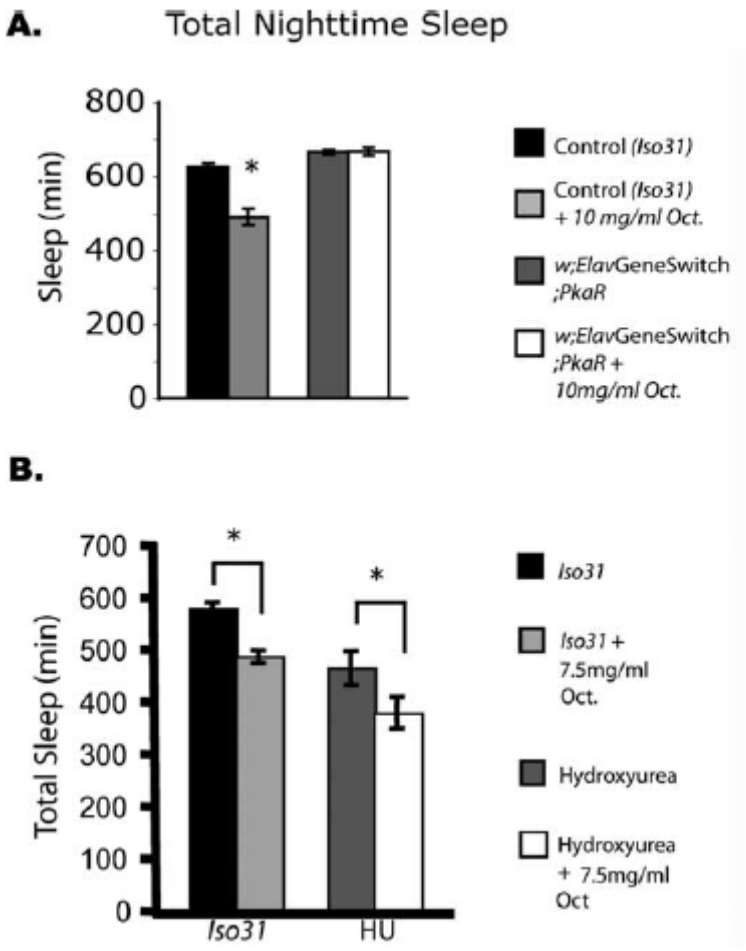


Figure 2-10 Effects of octopamine are mediated by PKA and are independent of the Mushroom Body. ***A***, Total nighttime sleep in control (*Iso31*) and*Elav*GeneSwitch flies crossed with *UAS–BDK33* (*PKAr*). The control on 7.5 mg/ml octopamine shows a significant decrease in nighttime sleep, whereas flies expressing *PKAr* under the control of *Elav*GeneSwitch do not show a decrease in sleep in response to octopamine [mean ± SEM; Control (*Iso31*), 623 ± 11, $n = 24$; control plus octopamine, 489 ± 22, $n = 24$; $p \leq 0.001$, two-way

ANOVA; *Elav*GeneSwitch, 665 ± 7, *n* = 51;*Elav*GeneSwitch plus octopamine, 667 ± 11, *n* = 51]. ***B***, *Iso31* flies subjected to hydroxyurea treatment to ablate the mushroom body still show sensitivity to the sleep-reducing effects of octopamine. Hydroxyurea-treated flies show a significant decrease in sleep compared with controls; they also show an additional decrease in sleep when placed on 7.5 mg/ml octopamine (mean ± SEM; control, 583 ± 11, *n* = 24; control plus 7.5 mg/ml octopamine, 491 ± 12, *n* = 24; $p \leq 0.01$, two-way ANOVA; HU, 469 ± 32, *n* = 24; HU plus 7.5 mg/ml octopamine, 382 ± 30, *n* = 22; $p \leq 0.01$, two-way ANOVA).

Table 2-1

Line	Sleep	Sleep-Day	Sleep-Night	Latency	Peak Activity	Activity per Waking Minute	Number
Tdc2^{RO54} F	1080 ± 19	497 ± 16***	617 ± 17	20.6 ± 4.6*	72.4 ± 5.6	1.2 ± 0.09*	23
Tdc2^{RO54} M	1214 ± 38***	610 ± 17***	604 ± 29	18.8 ± 8.7*	73.8 ± 6.6	1.1 ± 0.1*	24
w^{1118} F (Control for *Tdc2*^{RO54})	927 ± 33	280 ± 30	616 ± 11	47.5 ± 8.7	102.2 ± 7.6	1.5 ± 0.08	32
w^{1118} M (Control for *Tdc2*^{RO54})	985 ± 52	378 ± 36	607 ± 27	34.2 ± 7.1	80.9 ± 5.5	1.7 ± 0.1	32
TβH ^{nm18} F	1176 ± 17***	506 ± 14***	659 ± 8	19.6 ± 3.1	107.6 ± 5.7	1.84 ± .13*	46
TβH ^{nm18} M	1202 ± 13***	571 ± 7***	632 ± 9	26.8 ± 3.1	111.3 ± 5.4	1.86 ± .05*	62
Canton S F (Control for *TβH* ^{nm18})	883 ± 20	231 ± 16	651 ± 9	14.9 ± 1.3	100.7 ± 3.8	1.57 ± .05	52
Canton S M (Control for *TβH* ^{nm18})	1052 ± 19	405 ± 13	648 ± 10	28.2 ± 3.3	127.4 ± 5.1	1.7 ± .06	52
w;Tdc2-Gal4/ NaChBac:+ F	575 ± 45***	306 ± 22**	267 ± 31***	107.4 ± 20.3*	119.6 ± 5*	1.7 ± .2*	33
w;Tdc2-Gal4/ NaChBac:+ M	670 ± 55***	418 ± 20	253 ± 41***	124.4 ± 22.4**	139 ± 8.4	2.09 ± .06	27
Control F (*Iso31*)	846 ± 25	233 ± 19	613 ± 9	33.9 ± 3.2	140.2 ± 6.8	2.15 ± .07	58
Control M (*Iso31*)	1067 ± 17	448 ± 12	619 ± 9	24.1 ± 3	139.8 ± 8	2.38 ± .10	32
w;Tdc2-Gal4/Kir2.1;RC1 F	829 ± 14***	192 ± 12***	652 ± 5***	13.9 ± 1.2**	182.7 ± 7	2.14 ± .06***	47
w;Tdc2-Gal4/Kir2.1;RC1 M	872 ± 18***	300 ± 15***	598 ± 9***	13.6 ± 1.2***	205.4 ± 9.6**	2.29 ± .07***	41
Control F *w;RC1;RC1*	655 ± 15	76 ± 8	587 ± 8	45.7 ± 8.4	186.3 ± 4.6	3.87 ± .97	47
Control M *w;RC1;RC1*	709 ± 22	184 ± 13	522 ± 13	37.4 ± 3.9	227.3 ± 6.6	2.97 ± .08	46

Table 2-1. Sleep and activity in flies mutant for the octopamine pathway.

Table 2-2

Line	Sleep Bout Length-Day	Sleep Bout Length-Night	Sleep Bout Number-Day	Sleep Bout Number-Night
Tdc2^{RO54} **F**	26.4 ± 1.9	93.2 ± 12.5	21.6 ± 1.0**	10.7 ± 1.3**
Tdc2^{RO54} **M**	37.1 ± 5.4	69.9 ± 28.9	21.6 ± 2.3*	19.2 ± 2.9*
w^{1118} **F** (Control for *Tdc2*^{RO54})	22.3 ± 2.9	115.1 ± 14.7	14.2 ± 1.5	8.1 ± 1
w^{1118} **M** (Control for *Tdc2*^{RO54})	32.3 ± 4.7	106 ± 21.7	15.4 ± 1.9	11.1 ± 1.9
TβH ^{nm18} **F**	33.8 ± 2.5***	202.2 ± 16.7*	18.8 ± 0.8*	6.9 ± 0.7**
TβH ^{nm18} **M**	62.9 ± 5.2	183.4 ± 9.9***	13.2 ± 0.7*	6.9 ± 0.7*
Canton S **F** (Control for *TβH* ^{nm18})	19.9 ± 1.7	141.4 ± 14.3	14.1 ± 1.1	9.2 ± 0.9
Canton S **M** (Control for *TβH* ^{nm18})	56 ± 5.2	117.5 ± 16.1	10.7 ± 0.7	9.3 ± 0.7
w;Tdc2-Gal4/ NaChBac:+ **F**	17.3 ± 2.0**	18.9 ± 3.5***	21.4 ± 1.4***	18.3 ± 1.6***
w;Tdc2-Gal4/ NaChBac:+ **M**	38.4 ± 7.39***	32.2 ± 7.5***	20.9 ± 1.5**	81.0 ± 4.3***
Control F (*Iso31*)	25.5 ± 2.0	102.8 ± 7.9	10 ± 0.8	8.6 ± 0.5
Control M (*Iso31*)	66.9± 4.7	146.2 ± 11.5	8.7 ± 0.6	108.2 ± 17.3
w;Tdc2-Gal4/Kir2.1;RC1 **F**	12.5 ± 0.6	109.4 ± 8.2	15.3 ± 0.8***	9.9 ± 0.6
w;Tdc2-Gal4/Kir2.1;RC1 **M**	18.8 ± 1.0	88.9 ± 7.4*	16.6 ± 0.6***	11.8 ± 0.7
Control F *w;RC1;RC1*	10.9 ± 0.7	101.6 ± 9.3	6.2 ± 0.6	10.5 ± 0.6
Control M *w;RC1;RC1*	20.4 ± 1.8	57.1 ± 4.3	10 ± 0.6	12.4 ± 0.6

Table 2-2. Sleep bout analysis in flies mutant for the octopamine pathway

Chapter III:

Novel neural circuitry mediates effects of octopamine on sleep in *Drosophila*

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Abstract:

An understanding of sleep requires the identification of distinct cellular circuits that mediate the action of specific sleep:wake-regulating molecules, but such analysis has been very limited. We identify here a circuit that underlies the wake-promoting effects of octopamine in *Drosophila*. Using MARCM, we identified the ASM cells in the medial protocerebrum as the wake-promoting octopaminergic cells. We then blocked octopamine signaling in random areas of the fly brain and mapped the post-synaptic effect to insulin-secreting neurons of the pars intercerebralis (PI). These PI neurons show altered potassium channel function as well as an increase in cAMP in response to octopamine, and genetic manipulation of their electrical excitability alters sleep:wake behavior. Effects of octopamine on sleep:wake are mediated by the cAMP-dependent isoform of the OAMB receptor. These studies define the cellular and molecular basis of octopamine action and suggest that the PI is a sleep:wake-regulating neuroendocrine structure like the mammalian hypothalamus.

Introduction:

Although sleep occupies a large part of daily life for many organisms, it remains a poorly understood phenomenon. Analysis of sleep regulation would be facilitated by a focus on specific cellular loci, but knowledge of such loci is limited (Kryger et al., 2005). Molecules known to affect sleep are expressed widely and function in many different processes (Kryger et al., 2005; Saper et al., 2005). Delineating the cellular basis of their effect on sleep is complicated in

mammals because of the complexity of the brain structure (Hendricks et al., 2000b). The fruit fly, *Drosophila melanogaster*, on the other hand, has a relatively simple brain, making it a better model to address the cellular circuitry underlying sleep (Hendricks and Sehgal, 2004). Similarities between mammalian and fly sleep have been established and extend to the neurotransmitters underlying the regulation of sleep (Agosto et al., 2008; Andretic et al., 2005; Hendricks et al., 2000a; Shaw et al., 2000; Yuan et al., 2006).

We demonstrated recently that octopamine, the *Drosophila* equivalent of norepinephrine, is a potent wake-promoting signal (Crocker and Sehgal, 2008) . Octopamine is synthesized from tyramine by the action of *tyramine β-hydroxylase* (*Tβh*), and tyramine is synthesized from tyrosine by *tyrosine decarboxylase* (*dTdc*) (Roeder, 2005). Mutations in both *Tβh* and *Tdc* result in an increased amount of sleep, and electrical manipulation of *Tdc*-expressing neurons also has predictable effects on sleep (Crocker and Sehgal, 2008). Likewise in mammals, norepinephrine promotes wakefulness. However, the many targets of norepinephrine-producing cells and the existence of multiple receptors have made it difficult to dissect the function of this pathway in sleep (Insel, 1996; Sara, 2009). There are five different classes of adrenergic receptors, with a total of nine receptors, both α and β, and conflicting data on the roles these different receptors may play in sleep (Insel, 1996). Octopamine-producing neurons in *Drosophila* also project all over the brain and octopamine can signal through several different receptors (Evans and Robb, 1993). Nevertheless, the system is still simpler than in mammals with a total of 4 known

genes for octopamine receptors: OAMB, Octβ1R (OA2), Octβ2R and Octβ3R (Evans and Robb, 1993; Han et al., 1998). In addition, the gene for OAMB encodes two isoforms, AS and K3, resulting in five possible receptor types (Han et al., 1998; Lee et al., 2009).

Other neurotransmitters shown to affect *Drosophila* sleep are dopamine, serotonin, and GABA (Agosto et al., 2008; Andretic et al., 2005; Kume et al., 2005; Yuan et al., 2006). However, each of these is produced by many neurons and the specific sub-group of neurons responsible for the effect on sleep is not known for any neurotransmitter. Dopamine and serotonin exert at least some of their effects through receptors expressed in the mushroom bodies (MBs), a region previously implicated in the regulation of sleep (Andretic et al., 2005; Joiner et al., 2006; Pitman et al., 2006; Yuan et al., 2006). However, the MBs are a complex structure (Tanaka et al., 2008) and the specific neurons relevant for the serotonin/dopamine phenotype were not identified The circadian large ventral lateral neurons mediate effects of light and GABA on sleep, but the source of the GABA is not known (Chung et al., 2009; Parisky et al., 2008; Sheeba et al., 2008a). Finally, neurons in the pars intercerebralis (PI) region promote sleep, but their neurochemical identity remains to be determined (Foltenyi et al., 2007). Thus, little is known about the cellular basis of sleep in *Drosophila* and there has been no concerted effort to dissect the cellular and molecular circuit underlying the effect of any particular neurotransmitter.

In this study, we set out to map the octopamine pathway important for sleep. Using a technique known as Mosaic Analysis with a Repressible Cell

Marker (MARCM), we were able to map the wake-relevant octopamine-producing cells to specific cells in the medial protocerebrum (Wu and Luo, 2006). We also used an unbiased approach to map the octopamine target neurons that mediate the effects on sleep:wake. Building upon the observation that the effects of octopamine on sleep are protein kinase A (PKA)-dependent, we blocked PKA signaling in various parts of the fly brain and assayed the behavioral response to orally ingested octopamine. We find that a subset of neurons in the pars intercerebralis (PI) mediates effects of octopamine on sleep. Consistent with their identification as octopamine-responsive cells, these neurons in the PI demonstrate changes in electrical activity, as well as, in cAMP levels, in response to exogenous octopamine. Acting through the OAMB receptor on these PI neurons, octopamine signals through cAMP and PKA to promote wakefulness. Our data provide a molecular pathway as well as the cellular circuitry that mediates the wake-promoting effects of octopamine. Because the PI neurons implicated here are an integral part of the neuroendocrine PI, we believe these findings highlight the similarity between the PI and the mammalian hypothalamus. These findings may also provide insight into how the adrenergic system works to influence sleep in mammals.

Methods:

Fly Stocks:

The following lines were ordered from the Bloomington Stock Center: *Tdc2*–GAL4 (9313), *UAS–NaChBac* (9466), *UAS–GFP syt*(6925), *UAS-*

mCD8::GFP (5137), *UAS–GFPnls* (7032), *P{neoFRT}19A, P{tubP-GAL80}LL1, P{hsFLP}1, w[*]; Pin[Yt]/CyO* (5133), *y[1] w[1118] P{neoFRT}19A* (1744), 18896 (OctB2R),and Iso31 (5905). MBGS, H24-GAL4, 17d-GAL4, c747-GAL4, c309-GAL4, 1366-GAL4, MJ63-GAL4, c507-GAL4, 104y-GAL4, D42-GAL4, mai301-GAL4 , *Elav*GeneSwitch, 30y-GAL4, Sep54-GAL4, 201y-GAL4, *Dilp2*-GAL4, *UAS-CD8-GFP* and *UAS-BDK33(PkaR)* were used previously in the laboratory (Crocker and Sehgal, 2008; Joiner et al., 2006; Zheng et al., 2007). OAMB 286, OAMB 584 and UAS-K3;OAMB286 were a gift from Dr. K. Han, 50y-GAL4 was a gift from Dr. R. Greenspan and Kurs58-GAL4 was a gift from Dr. U. Heberlein. *UAS-Epac1-cAMP*50 was a gift from Dr. P. Taghert.

Generating flies for MARCM:

The MARCM method was used to generate flies in which different, random subsets of *Tdc2*-expressing neurons expressed the sodium channel, *NaChBac* (Lee and Luo, 1999). The crosses that created the MARCM flies are shown in Fig 3-1. The original lines are: *P{neoFRT}19A, P{tubP-GAL80}LL1, P{hsFLP}1, w[*]; Pin[Yt]/CyO* (5133)of the Bloomington stock Center (donated by L. Luo) *UAS–NaChBac*(9466) line of the Bloomington stock Center (donated by B. White) *y[1] w[1118] P{neoFRT}19A* (1744) line Bloomington Stock Center (donated by L. Luo) *Tdc2*–GAL4 (9313) line Bloomington Stock Center (donated by J. Hirsh) The final MARCM flies contain a *Tdc2*-Gal4, a UAS-*NachBac* (B16B) a tubulin-Gal80 and a heat shock promoter-FLP recombinase transgene. In the presence of the Gal80, Gal4 activity is suppressed and so *NachBac* is not

expressed in any cell. However, the Gal80 can be randomly excised from subsets of cells by the heat shock activated FLP recombinase. Crosses were raised at 18°C and the parental generations were transferred to new vials following egg laying for the times indicated in Fig. 3-1. To remove the GAL80, animals received a heat shock at 37°C for 20-30 minutes between 1 and 2 days of development.. *P{neoFRT}19A, P{tubP-GAL80}LL1, P{hsFLP}1, w[*]/P{neoFRT}19A ; UAS-NaChBac-EGFP/TDC2-Gal4* female flies were selected for sleep and mapping analysis.

Sleep Analysis:

Sleep analysis was performed as described previously (Gilestro and Cirelli, 2009; Joiner et al., 2006). All flies were kept on a 12 h light/dark (LD) cycle at 25°C schedule. Female MARCM flies, 6–8 d old, were placed in 65 x 5 mm tubes containing 5% sucrose and 2% agar and entrained for 24–36 h before the sleep recording. Baseline sleep was determined by monitoring activity for at least 3 d with no disruptions in an LD cycle. Locomotor activity was monitored using the DAMS/Trikinetics system as described previously (Gilestro and Cirelli, 2009; Joiner et al., 2006). Sleep was defined as a 5 min bout of inactivity as described previously (Shaw, 2003). Latency to sleep was determined for the octopamine receptor mutants and is defined as the time in minutes from the moment lights are turned off to the first bout of sleep.

Immunohistochemistry:

Brains of adult female flies were dissected in cold PBS and fixed in 4% paraformaldehyde in PBS at 4°C overnight. After four 10-min washes in 0.1% Triton X-100 in PBS samples were permeabilized with 3% normal goat serum (NGS) 2 h at room temperature. Samples were then incubated in a primary antibody solution containing mouse anti-nc82 (1:50; Developmental Studies Hybridoma Bank) and rabbit anti-GFP IgG (1:1000; no. A11122, Invitrogen) in 3% NGS at 4°C overnight. After four 10-min washes in 0.1% Triton X-100 in PBS, brains were incubated at 4°C overnight in a secondary antibody solution containing Alexa Fluor 568 goat anti-mouse IgG (1:1000, Invitrogen) and Alexa Fluor 488 goat anti-rabbit IgG (1:1000, Invitrogen) in 3% NGS. After 4 10-minute washes in PBS brains were mounted using Vectashield mounting medium (Vector Labs) and covered with a no. 1 glass coverslip before imaging. Immunolabeled adult brains were imaged with a 488 and 510 laser-scanning confocal microscope (Leica) under 20x magnification. *z* stack images were scanned at 1µm section intervals with a resolution of 1024 x 1024 pixels.

Analysis of MARCM data:

Following sleep analysis, and immunohistochemistry to detect GFP, individual brains were analyzed to correlate the sleep phenotype with the cellular expression of *NaChBac*-GFP. All mapping analysis was performed blind to the sleep analysis. Each group contains flies with expression only in that subgroup, with the exception of the ASM flies and PSM flies. Due to the low numbers of flies that expressed *NaChBac*-GFP in these regions alone we included flies that

also expressed in other areas. We had four flies that had expression only in ASM neurons and an additional 8 flies which had expression in other areas in addition to ASM, bringing the total number to 12. These 8 flies were not counted in any other category. In the group showing expression in only a single ASM neuron, we had 3 flies with only one such neuron labeled and another 8 lines which had expression elsewhere, in addition to the single ASM neuron, to bring the number to 11. These lines were not counted in any other category. The number of flies indicated in the "no cells" category is probably an underestimate since we stopped counting these flies at some point. Over 1,000 flies were screened in this study, similar to the number Busch and colleagues screened to map octopamine neurons (Busch et al., 2009).

Mapping the site of PKA action:

To map the site of PKA action, we crossed each of the following drivers- MBGS, H24-GAL4, 17d-GAL4, c747-GAL4, c309-GAL4, 1366-GAL4, MJ63-GAL4, c507-GAL4, 104y-GAL4, D42-GAL4, mai301-GAL4, 50y-GAL4, ElavGS, 30y-GAL4, Sep54-GAl4, 201y-GAL4, *Dilp2*-GAL4, Kurs58-GAl4- into *UAS–BDK33* flies (Rodan et al., 2002). Female flies were used from these crosses for all sleep and octopamine analysis. Octopamine at 10 mg/ml as previously described was fed to each fly for 3 days following a 3 day baseline(Crocker and Sehgal, 2008). The GeneSwitch construct in elavGS and MBGS can be turned on during adulthood using the drug RU486 (11β-(4-dimethylamino)phenyl-17β-hydroxy-17-(1-propynyl)estra-4,9-dien-3-one). We placed the animals on 5%

sucrose/2% agar tubes containing either 500 µM RU486 dissolved in ethanol or ethanol alone (1%) for 3 days. Half of each group was then transferred to octopamine-containing food with either 5% sucrose/2% agar plus 500 µM RU486 plus octopamine or 5% sucrose/2% agar plus ethanol (1%) plus octopamine. Both groups were also simultaneously fed 10 mg/ml octopamine for 3 days. At the lights-on transition at the end of this period, animals were transferred off of octopamine onto 5% sucrose/2% agar containing either 500 µM RU486 or ethanol (1%). Sleep analysis was performed as described above.

Electrophysiology:

For *in vivo* patch recording from PI neurons, flies were anesthetized with CO_2 and glued ventral side down to a glass cover slip. The cover slip was placed in a chamber containing extracellular solution (101mM NaCl, 3mM KCl ,4mM $MgCl_2$, 1mM $CaCl_2$, 1.25mM NaH_2PO_4, 20.7mM $NaHCO_3$, 5mM Glucose with pH 7.2) and then the cuticle was peeled off using fine forceps to expose the surface of the brain. The chamber was placed on the stage of an Olympus BX51 fluorescent microscope, and PI neurons were identified by their location and bright green fluorescence. Patch recording electrodes (WPI, Inc) were fire-polished, and had resistances from 3 – 4 MΩ when filled with intracellular solution (102 mM K-gluconate, 17mM NaCl, 0.085mM $CaCl_2$, 4mM Mg-ATP, 0.5mM Na-GTP, 0.94mM EGTA, 8.5mM HEPES with pH 7.2). Standard technique was used to record macroscopic currents in the whole-cell voltage-clamp mode with an Axopatch 200A amplifier (Molecular Devices, Union City,

CA). Data were digitized with a Digidata 1322A interface (Molecular Devices, Union City, CA) and stored on a PC hard drive for further analysis with pClamp9 software (Molecular Devices, Union City, CA).

FRET Imaging:

The *TDC2-GAL4* flies were crossed to flies carrying a *UAS-Epac1-cAMPs(50A)* transgene (Shafer et al., 2008). Brains were dissected under ice-cold calcium-free fly hemolymph-like saline (HL3) containing 70 mM NaCl, 5 mM KCl, 20 mM $MgCl_2$, 10 mM $NaHCO_3$, 5 mM trehalose, 115 mM sucrose, and 5 mM HEPES (pH 7.1). The brains were then laid at the bottom of a 35 × 10 mm plastic FALCON Petri dish (Becton Dickenson Labware), given a few seconds to adhere and covered with 1.6 ml HL-3 containing 1.5 mM $CaCl_2$ (Shafer et al., 2008).

Time course FRET imaging of Epac1-camps was performed on individual brains using the Leica TCS SP5 confocal microscope using a HCX APO L 40x/0.80 dipping objective. We performed octopamine experiments by administering 140ul of 125mg/ml of octopamine in dH2O into the dish making the final concentration of octopamine 10mg/ml octopamine. In the water control, 140 ul of water was added to the well. For the mianserin experiments, initially mianserin alone was added at a concentration of 0.25mM, followed by a second application of 0.25mM mianserin along with 140 ul of the 125 mg/ml stock of octopamine. Analysis of FRET was done using the Sensitized Emission FRET wizard built into the Leica Application Suite for the TCS SP5 using Method 1 for

FRET analysis. Images were taken every 5 seconds at 512 x 512 and line averaged 3 times. A time series was taken for 5 minutes of baseline, followed by octopamine administration and 15 minutes post octopamine administration. For the mianserin experiments, the 5 minutes of baseline preceded the first application of mianserin alone; this was followed 1 minute later by mianserin plus octopamine. We then outlined the PI neurons and selected them as our region of interest for analysis. Results are plotted as the minute prior to octopamine administration followed by 240 seconds post octopamine administration. Longer periods of time did not change the FRET levels. 12 brains were plotted for octopamine administration, 8 for the water control and 9 brains for the mianserin treatment.

Receptor Characterization:

RT-PCR was run according to previously published protocols (Ousley et al., 1998). RT-PCR primers: OAMB primers were previously described (Lee et al., 2009); we also generated new primers for the K3 and AS isoforms. New primers are for K3: forward 5'-CTGCCGTGAGAACGACGAG-3'; reverse 5'-GCGCAATATGAGCTGGGACT-3'. Primers for AS were: forward 5'-CTGCCGTGAGAACGACGAG-3'; reverse 5'- ATGTATGCGCAATGTGAGGC-3'. OctB2R receptor primers were: foraward 5'-ATGCTGATGCACCGACCAT-3'; reverse 5' AAGGCAGCCAGCAGAGGAT-3'.

Single cell RT-PCR:

Whole-cell extracts were taken from *Dilp2* neurons following electrical recording. Ten cells were pooled and then RT-PCR was run on them with the following modifications:10ul of buffer (150 mM sodium acetate, 50 mM Tris, pH 9.0, 5 mM EDTA, pH 8.0, 1% SDS containing 1/100th volume diethyl pyrocarbonate) was used to place each recording pipette tip into, than those were pooled to equal 100ul. Following PCR with K3 and AS primers, the K3 band was cut out of the gel and sent for sequencing to verify K3 band. We did not see an AS band so that was not sequenced.

Results

Identifying the Octopaminergic Cells that Regulate Sleep

In mammals, the release of norepinephrine from a specific group of cells in the Locus Coeruleus (LC) is known to regulate sleep (Sara, 2009). We set out to determine whether a specific cell group in the fly is responsible for the effect of octopamine on sleep. To address, this question we employed the MARCM technique which allows one to restrict the expression of a transgene to a subset of its normal pattern (Wu and Luo, 2006); (Agosto et al., 2008). We showed previously that expression of a sodium channel (*NaChBac*) by a *tyrosine decarboxylase 2* (*Tdc2*) GAL4 driver, which expresses in tyramine- and octopamine-producing cells, decreases sleep (Cole et al., 2005; Crocker and Sehgal, 2008). The *NaChBac* transgene is derived from a gene encoding a bacterial Na^+ channel which has the characteristics of high open probability and low inactivation, thus driving membrane voltage to a more depolarized and easily

excited state (Luan et al., 2006; Nitabach et al., 2006). Thus, excitation of *Tdc2*-positive neurons results in decreased sleep. However, *Tdc2*-GAL4 is expressed in a number of cells in the fly brain (Figure 3-2a), any of which could be responsible for the decrease in sleep. In the MARCM technique, a repressor of GAL4 activity, GAL80, is co-expressed and then is excised in a random fashion from groups of cells (see Figure 3-1). By driving this excision in populations of developing *Drosophila*, one can generate large numbers of flies, each of which expresses GAL4 in a different subset of the original pattern. Using MARCM, we were able to reduce the number of octopamine cells expressing the Na^+ channel down to a single cell in some cases. We chose the Na^+ channel in part because it has a robust effect on sleep and also because it is tagged with green fluorescent protein (GFP). The GFP marker allowed us to identify the cells expressing the sodium channel.

Because each fly generated through MARCM expresses the relevant transgene in a distinct pattern, we assayed each individual fly for sleep and then for expression of GFP. Figure 3-2b shows the different cell populations labeled by *Tdc2*-GAL4 in a schematic of the anterior and posterior sections of the fly brain. The schematic is derived from the anatomical map generated by Sinakevitch and Strausfeld (Sinakevitch and Strausfeld, 2006), whereas the nomenclature is based upon the studies of Busch et al., 2009. We were unable to discriminate some cell clusters as anterior or posterior and so grouped them together, such as the ASM cells. We found that expression of the Na^+ channel in the ASM group of cells produced a decrease in sleep similar to that seen with the

Tdc2-GAL4 driver expressing the Na^+ channel in all neurons (Figure 3-2c,d, Table 3-1). The sleep loss was approximately 320 min when the Na^+ channel was expressed in multiple cells of the ASM group, but was less when it was expressed in only one cell in this group. Thus, the effect of this neuronal group on sleep appears to reflect the cumulative contribution of multiple cells. Expression of the sodium channel in other areas of the brain and thorax did not result in a significant drop in sleep (Figure 3-3c).

Mapping Brain Regions that Mediate Post Synaptic Effects of Octopamine

We showed previously that PKA is necessary for the relay of the wake-promoting octopamine signal (Crocker and Sehgal, 2008). In this study, we attempted to localize the areas of the fly brain that respond to this signal. We overexpressed the regulatory subunit of PKA (PkaR), which acts to block PKA signaling, using a number of different GAL4 drivers (Figure 3-3a)(Rodan et al., 2002). We found that many of the resulting lines showed increases in total sleep as compared to the control line Iso31 (Isogenic w^{1118} line). This effect is consistent with our previous work showing that an increase in PKA signaling reduces sleep (Hendricks et al., 2001). In addition, a subset of the GAL4 lines expressing PkaR were no longer sensitive to the decrease in sleep caused by feeding octopamine (Figure 3-3a, Table 3-2). Although some of these lines express GAL4 in many cell types, the one region of the brain where the expression overlaps is the PI (Figure 3-3b). In fact, the *Dilp2* GAL4 driver, which

expresses GAL4 in cells producing *Drosophila insulin like peptide 2* (*Dilp2*), is expressed in only a subset of PI neurons (Rulifson et al., 2002) and expression of PkaR by this driver has as robust an effect as any other. Although this line did not show a significant increase in nighttime sleep relative to wild-type, it did show a significant increase in sleep consolidation— decreased number of sleep bouts with a corresponding increase in sleep bout length (Fig. 3-4). Interestingly, expression of PkaR in the mushroom body, a region previously implicated in the regulation of sleep, does not affect the response to octopamine.

We were interested in determining whether wake-promoting octopaminergic cells project to the PI. To this end, we expressed a synaptically tagged GFP under the control of the *Tdc2*-GAL4 and co-labeled the brains with antibodies specific for the *Dilp2* neurons. As shown in Figure 3c, synaptic boutons of *Tdc2* neurons are found in the vicinity of the *Dilp2* neurons. In addition, expression of a membrane bound GFP (CD8-GFP) in *Tdc2*-producing cells indicates dorsal as well as lateral projections of ASM neurons. This is similar to the findings of Busch et al, who examined whole-mount projections of the octopamine-producing ASM neurons (Busch et al., 2009) . Together, these data indicate that the *Dilp2* neurons likely receive octopaminergic signals and mediate wake-promoting effects of octopamine.

Modulation of *Dilp2*-Producing Neurons Alters Sleep

If octopamine signals through the PI to regulate sleep, then modulating the firing of the relevant PI neurons should also affect sleep. Thus, we used the

Dilp2–GAL4 driver to express either a depolarizing or a hyperpolarizing ion channel (*UAS–NaChBac* or *UAS–EkoIII*) (Luan et al., 2006; White et al., 2001) and examined effects on sleep. Expression of the Na^+ channel in *Dilp2*-positive cells resulted in an average decrease in daily sleep of 172 min (Figure 3-5) (Table 3-5). The loss of sleep was more specific to the nighttime, similar to what we see when *Tdc2*-producing cells are depolarized (Crocker and Sehgal, 2008). A similar decrease in sleep was seen in male flies expressing the Na^+ channel in the *Dilp2* neurons (Figure 3-5)(Table 3-3).

When *UAS-EkoIII*, a modified Shaker K^+ channel, was expressed in *Dilp2* neurons, there was an increase in sleep during both the night and the day (Figure 3-5a, b) (Table 3-3). These data support the idea that excitation of the *Dilp2* neurons promotes wakefulness whereas silencing these neurons increases sleep.

Octopamine Modulates a Potassium Current in PI Neurons

Because PI neurons are implicated in the wake-promoting effects of octopamine, we asked whether octopamine application could influence their electrical excitability. Accordingly, we generated flies in which the PI neurons were labeled with green fluorescent protein (GFP) under the control of the *Dilp2 GAL4* driver. The PI neurons are large and located close to the surface of the brain, and so we were able to measure whole-cell currents by patch clamp analysis, in the brains of living flies (Shahidullah et al., 2009). Voltage-dependent outward currents were evoked by depolarizing voltage steps in the whole-cell

recording mode (Figure 3-6a). In order to examine the outward current in the presence of octopamine, we evoked currents by applying a repetitive depolarizing pulse to +60 mV from a holding potential of -70 mV, and then added 1 mM octopamine to the bath solution. As shown in Figure 3-6b, 1 mM octopamine reduces the total outward current by approximately 30%. Because mianserin antagonizes effects of ingested octopamine on sleep:wake (Crocker and Sehgal, 2008), we determined whether this antagonist (Maqueira et al., 2005) could block the effects of octopamine on the whole cell outward current. We applied the same protocol described above to evoke outward current, and then added 0.25 mM mianserin to the bath solution followed by 1mM octopamine (Figure 3-6c). Indeed, mianserin greatly reduces the effect of octopamine on the outward current.

We next asked which component of the outward potassium current is modulated by octopamine application. At low concentrations, the potassium channel blocker tetraethylammonium (TEA) is highly selective for the calcium-dependent potassium current carried by Slowpoke channels (Shen et al., 1994), and we have demonstrated that 1 mM TEA selectively blocks Slowpoke current in the PI neurons *in vivo* (Shahidullah et al., 2009). As shown in Figure 3-6d, 1 mM TEA has little effect on the outward current following octopamine application, suggesting that octopamine inhibits the TEA-sensitive component of outward current attributable to Slowpoke. Following washout of octopamine, the TEA-sensitive current returns. These data demonstrate that octopamine selectively

inhibits the Slowpoke calcium-dependent potassium current in the *Dilp2* neurons, thereby increasing their excitability.

Octopamine Affects cAMP Signaling in PI Neurons

As mentioned above, we found that altering PKA levels in the PI neurons blocks the flies' response to octopamine, suggesting that octopamine increases cAMP. Thus, we asked whether there is a change in cAMP signaling in *Dilp2* neurons following application of octopamine. To monitor cAMP activity, we used a recently described *Epac*-cAMP construct which reports the activation of *Epac* by cAMP through a FRET-based assay (Shafer et al., 2008). An increase in cAMP produces a decrease in the FRET associated with this construct. We drove the *UAS-Epac* construct with a *Dilp2* driver, dissected brains from the resulting flies and measured FRET in response to octopamine. Figure 3-7a shows that the FRET signal decreases following application of 10mg/ml octopamine in the bath. The response is fairly rapid, similar to that seen when Gs coupled receptors in other parts of the brain are stimulated (Shafer et al., 2008). As one might expect, most of the changes in FRET are intracellular (Figure 3-7b). We also determined if 0.25mM mianserin, a blocker of octopamine receptors, could block the effects of octopamine application. The addition of mianserin completely blocks the increase in cAMP activity seen with octopamine administration alone (Figure 3-7d). Interestingly mianserin alone produces a small decrease in cAMP activity. Together, these studies show that octopamine

both depolarizes *Dilp2* neurons and activates cAMP signaling in them, with a similar time course.

The OAMB Receptor Acts in PI Neurons to Regulate Sleep

Mammals express many different norepinephrine receptors, and it has been difficult to pinpoint those relevant for sleep. Because our previous work indicated that effects of octopamine are mediated by PKA, we focused on the octopamine β and OAMB receptors which signal through cAMP and are homologous to the β adrenergic receptors. As noted above, the OAMB receptor has two isoforms, K3 and AS. Activation of the K3 isoform results in increased cAMP signaling and Ca^{2+} signaling whereas activation of the AS isoform only increases Ca^{2+} (Lee et al., 2009).

OctB2R is an octopamine β receptor that is known to signal through cAMP (Evans and Robb, 1993), but has not been characterized genetically in the fly. We obtained flies carrying a P-element insertion in the 5th exon of the OctB2R gene and analyzed them on molecular and behavioral levels. This P-element disrupts the conserved 7^{th} transmembrane domain of the predicted protein and results in a loss of mRNA signal, as seen in Figure 3-8. To assay the effect of the P-induced mutation on sleep, we first outcrossed it into an Iso31 background. We found that disruption of the OctB2R gene did not affect nighttime sleep, but produced a significant decrease in daytime sleep, resulting in an overall decrease in sleep (Table 3-3). Since this P-element disrupts expression of the OctB2R receptor and yet affects sleep in the opposite direction from octopamine,

we did not consider it further as a candidate for mediating effects of octopamine (Figure 3-9b)

We examined two previously characterized OAMB mutants, the OAMB 286 mutant and the OAMB 584 mutant (Lee et al., 2003), for effects on sleep:wake. The OAMB 286 mutant is a known null (Figure 3-8) and the OAMB 584 mutant deletes the first 3 exons of the OAMB gene. Both OAMB 286 and OAMB 584 disrupt the K3 and AS isoforms. Both mutants show increases in sleep (Figure 3-9a), which manifest as longer sleep bouts at night and greater numbers of sleep bouts during the day (Table 3-3). An effect on number and duration of sleep bouts suggests that the mutants affect the initiation as well as the maintenance of sleep. For the rest of our studies we focused on the null OAMB 286.

OAMB is expressed in the mushroom body, thereby its name (octopamine mushroom body receptor), but was not known to express in the PI neurons (Han et al., 1998). To determine if OAMB is expressed in the PI, we collected extracts from single *Dilp2* neurons via the whole-cell recording electrode, prepared RNA and ran RT-PCR experiments. We found that the K3 isoform of OAMB, which couples to both cAMP and calcium, is expressed in the *Dilp2* neurons. Indeed, sequence analysis of the single band observed on the gel confirmed its identity as the K3 isoform (data not shown). We did not detect the Ca^{2+} -only isoform, A3 (Fig. 3-10). To confirm the specificity of the RT-PCR experiments, we also ran them on OAMB mutants. The *Dilp2* neurons of these mutants did not express the K3 isoform of OAMB (Fig. 3-10).

We then asked whether restoring OAMB to the PI neurons would rescue the sleep phenotype of the OAMB mutants. To address this question, we drove expression of the K3 isoform in the *Dilp2* neurons of OAMB 286 mutants. Expression of K3, under the control of the *Dilp2* driver, resulted in a significant reduction in sleep in the 286 background, thus effectively rescuing the phenotype (Figure 3-9a). These data support the conclusion that octopamine acts through OAMB mediated cAMP signaling in *Dilp2* neurons to modulate sleep.

The OAMB Receptor Mutants Act in PI Neurons to Mediate Wake-Promoting Effects of Octopamine

We then asked whether OAMB mutants are responsive to the wake-promoting effects of octopamine. Feeding 10mg/ml octopamine to OAMB 286 flies did not produce the decrease in nighttime sleep seen in wild-type controls (Figure 3-11a). However, consistent with a role for the *Dilp2* neurons in mediating effects of OAMB on sleep:wake, the response of 286 flies to octopamine was rescued by *Dilp2*-GAL4-driven expression of the K3 isoform (Figure 3-11a).

We also recorded *in vivo* from *Dilp2* neurons in the OAMB 286 flies and found they are virtually unresponsive to octopamine (Figure 3-11b). To determine whether the Slowpoke calcium-dependent potassium current is present in *Dilp2* neurons in the OAMB 286 mutant flies, we applied 1 mM TEA after several attempts to modulate the current with octopamine (Figure 3-11b). TEA blocks about 30% of the outward current, as it does in wild-type flies (Figure

3-6), confirming that octopamine receptor deletion does not directly affect the Slowpoke current, but eliminates its modulation by octopamine.

Discussion:

Most animals have evolved mechanisms to maintain alertness as well as sleep states (Tobler, 2005). Here we identify a pathway through which octopamine regulates arousal in the insect brain. This study is the first to our knowledge to map the sleep:wake-regulating effect of a particular neurotransmitter to specific cells in the fly brain. We show that a specific cell group, known as the ASM cell group, comprises the octopamine-producing cells important for the effects of octopamine on arousal. These cells are found in the lateral protocerebrum region and are also known as the G1/G4a cells (Sinakevitch and Strausfeld, 2006). Previously, these cells have only been described in terms of anatomical location. Using MARCM to specifically express a sodium channel in ASM cells, we were able to mimic the sleep phenotype seen with Na^+ channel expression in all octopamine-producing cells. We find that the magnitude of the decrease in sleep depends upon the number of ASM cells activated, although even the activation of a single cell produces a phenotype. Thus, as in mammals, discrete cell populations encoding a specific neurotransmitter regulate sleep and arousal in the fly.

We also demonstrate that the Pars Intercerebralis (PI) is an important region of the fly brain for relaying the octopamine arousal-promoting signal. Inhibiting PKA, a downstream signal of octopamine receptors, in specific PI

neurons blocks the effect of ingested octopamine on sleep. Modulating the electrical activity of these PI neurons also affects sleep. Although we have not demonstrated actual synaptic contact between the ASM neurons discussed above and the relevant PI neurons, there is reason to believe that these neuronal groups are directly connected. Our work, and that of Busch et al, suggests that *Tdc2* neurons, including the ASM group, extend projections towards the dorsal region of the brain (Busch et al., 2009). In fact, we show termini of *Tdc2* projections in the vicinity of the PI (Figure 3-3).

Previous work identified sleep-promoting cells in the region of the PI, but those appear to be distinct from the ones that mediate the effects of octopamine (Foltenyi et al., 2007). When we decreased PKA signaling in cells labeled by 50y-GAL4 (which targets the sleep-promoting cells identified in the other study) we observed a normal response to octopamine (Figure 3-3). Conversely, none of the drivers that were effective in our studies were reported in the study that identified the sleep-promoting neurons (Foltenyi et al., 2007). Also, the sleep-promoting neurons appear to be morphologically distinct from many of the other PI neurons, in that they do not show long projections to the tritocerebrum. It should be noted that the PI is a very heterogeneous structure composed of many different cell types (Siegmund and Korge, 2001). Functionally, given that it contains many neuroendocrine cells, it appears to be most analogous to the mammalian hypothalamus (Toivonen and Partridge, 2009). The fact that both sleep-promoting and arousal-promoting neurons can be found in the PI, as in the hypothalamus, lends credence to this analogy. Interestingly, we have found that

the PI is also important for circadian output, which suggests the possibility that it integrates circadian and homeostatic signals (Jaramillo et al., 2004).

The PI neurons that mediate the effects of octopamine are the major insulin secreting cells in the fly (Rulifson et al., 2002). This may have important implications for the much-hypothesized link between metabolism and sleep (Trenell et al., 2007). Recent work on human sleep indicates that sleep deprivation modulates the insulin signaling pathway (Knutson and Van Cauter, 2008; Spiegel et al., 2009). On the other hand, disruptions in metabolism may also cause changes in sleep (Laposky et al., 2008; Laposky et al., 2006; Trenell et al., 2007). It is reasonable to assume that if sleep is conserved across species then its most basic functions may also be conserved. We find that an arousal pathway in flies includes the major neurosecretory cells in the PI (Toivonen and Partridge, 2009). In mammals, growth hormone regulation more closely follows the sleep wake cycle than the circadian cycle (Sassin et al., 1969; Takahashi et al., 1968). It may turn out that similar hormones released from the PI or PI projections closely match the sleep wake cycle. Indeed, insulin, produced by the *Dilp2* neurons, has growth-promoting effects in *Drosophila* (Brogiolo et al., 2001).

In mammals, it has been difficult to tease apart the adrenergic receptors important for the arousal-promoting effects of norepinephrine. In this study we show that the OAMB receptor coupled to Ca^{2+} and cAMP regulates arousal in response to octopamine. Specifically. we demonstrate that the cAMP-stimulating action of this receptor is required. In mammals, the A-1 receptors are coupled to Ca^{2+} or PKC, the A2 receptors inhibit cAMP and the B receptors stimulate cAMP

(Insel, 1996). Given that cAMP also regulates sleep in mammals (Cirelli et al., 1996; Kanyshkova et al., 2009; Zamboni et al., 1999) and CREB appears to be a wake-promoting signal (Graves et al., 2003b), it is likely that mammalian β receptors are responsible for the wake-promoting effects of norepinephrine. Thus, the prediction is that A2 adrenergic receptors will be found to increase sleep while β receptors decrease sleep. In mammals, the agonists of these receptors, when focally applied to the medial preoptic area, do seem to follow the hypothesis above such that the A2 agonists promote sleep and the β agonist promotes wakefulness (Mallick and Alam, 1992). Finer injections of agonists and antagonists specifically into the ventral lateral preoptic area (the major mammalian sleep-promoting area (Saper et al., 2005), will likely provide stronger support for this idea.

In the PI neurons identified here, the activation of cAMP by octopamine is accompanied by an inhibition of the outward potassium current, measured by whole-cell patch clamp *in vivo*. By using low concentrations of the highly selective potassium channel blocker TEA, we identified the Slowpoke calcium-dependent potassium current as the specific target of octopamine. Because Slowpoke current contributes significantly to action potential repolarization (Shao et al., 1999), a decrease in this current will lead to an increase in action potential duration in the PI neurons (Shahidullah et al., 2009), thereby increasing the activity of neural circuits in which these neurons participate. It is noteworthy that calcium-dependent potassium channels have long been known to be modulated by PKA-dependent phosphorylation and dephosphorylation (Chung et al., 1991;

DePeyer et al., 1982; Ewald et al., 1985; Zhou et al., 2002). The present findings provide an intriguing physiological and behavioral context for these earlier cellular and molecular studies.

To map the octopamine target neurons relevant for sleep:wake, we genetically decreased PKA levels in different brain regions and then monitored the response to food supplemented with octopamine. In many subgroups of neurons, dropping the level of PKA leads to death or very sick flies, making it difficult to address their role in sleep behavior. Thus, there may be areas in addition to the PI that are important for the regulation of sleep by octopamine. Likewise, there are other brain regions that mediate effects of PKA on sleep and wake. For instance, a screen to map the sleep-relevant site of PKA action identified the mushroom body as an important sleep-regulating structure (Joiner et al., 2006; Pitman et al., 2006). We found that over-expressing PKA in *Dilp2* cells results in pupal lethality, which may explain why the PI neurons did not show up in the screen that identified the MBs. In addition, because the octopamine signal did not map to the mushroom body, there are as yet unknown signals that increase PKA in the MB.

Clearly, the regulation of sleep in *Drosophila* is complex and likely involves many sites and signaling molecules. As noted earlier, in addition to the MBs and the PI, the circadian large ventral lateral neurons have a role in the regulation of sleep (Parisky et al., 2008). How these different sites communicate with each other and are ordered in a sleep-regulating circuit will be an important question to address. We propose that the PI lies downstream of these other sites and

integrates circadian and homeostatic signals to control sleep:wake. Consistent with this idea, the PI is known to be a major output of the fly brain (Toivonen and Partridge, 2009). In addition, we know that some projections of the central clock neurons are in the vicinity of the PI (Jaramillo et al., 2004) and that the PI contains wake-and sleep-regulating cells. We hypothesize that the decrease in sleep produced by the ablation of mushroom bodies (Joiner et al., 2006; Pitman et al., 2006) is due to the imbalance of inputs to the PI neurons, resulting in an increase of wake-promoting signals over sleep-promoting signals. This is supported by our data showing that flies lacking MBs are more sensitive to octopamine feeding (Crocker and Sehgal, 2008), implying that the MBs normally exert a moderating influence.

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Figure 3-1

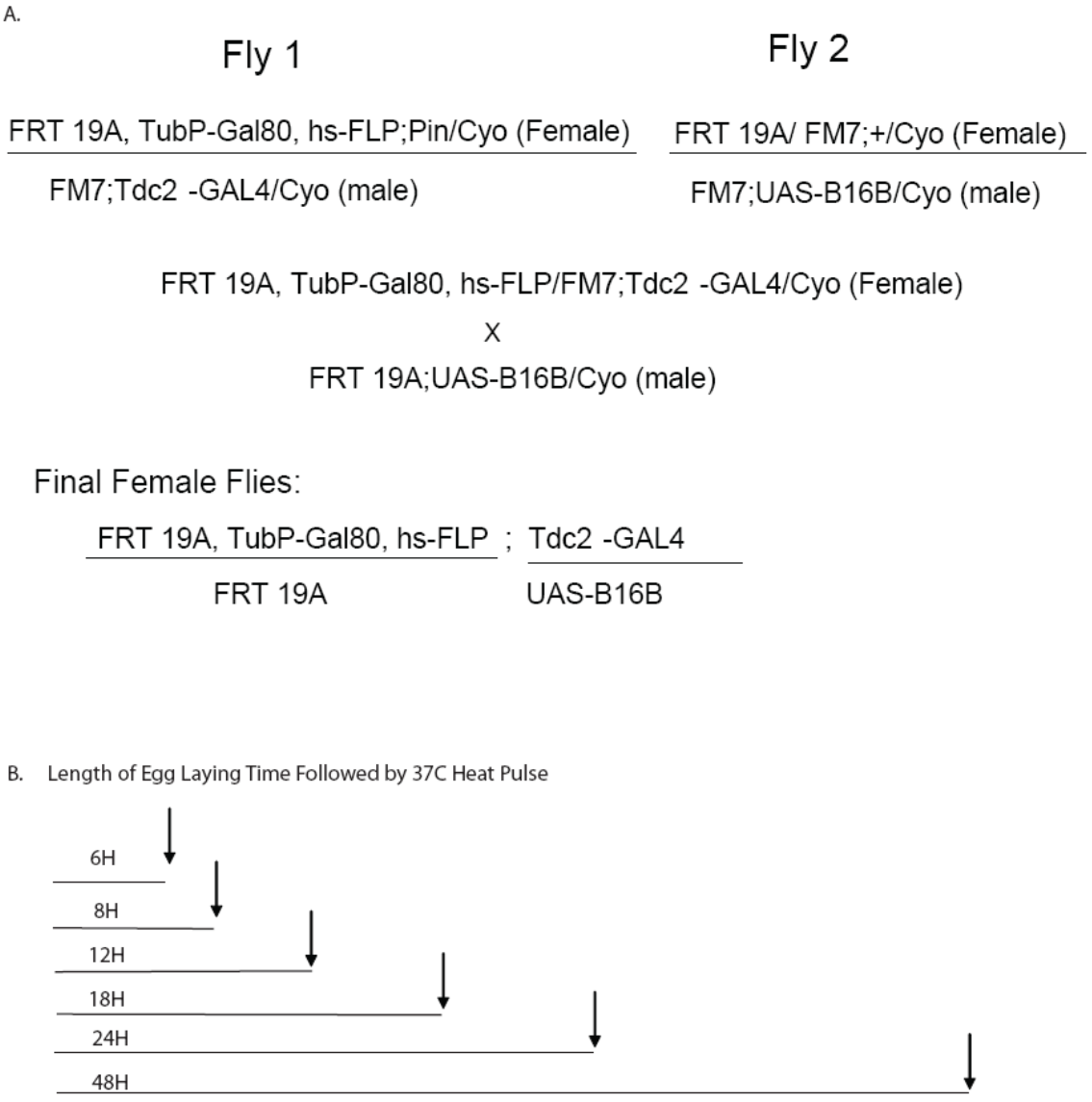


Figure 3-1 (Supplemental Figure 1) : MARCM Methods.

A. Outline of the crosses performed to generate the female flies for MARCM analysis. We used flies with the FRT (Flp recombination target) site as well as the FLP recombinase and the Gal80 driven by a tubulin promoter on the 1st chromosome,. The second chromosome carried the Tdc2-GAL4 and UAS-*NaChBac* transgenes. B. Outline of the heat shock regime to induce expression of FLP recombinase. Parents of the final offspring were allowed to lay eggs for the times indicated before being removed from the vial; eggs/larvae were then heat pulsed at 37 degrees. Note that each population was only subjected to one treatment; induction of the recombinase at different stages in different populations resulted in a wide range of “mosaic” progeny, from those that expressed the sodium channel in many cells to those that showed expression in only one cell.

Figures 3-2

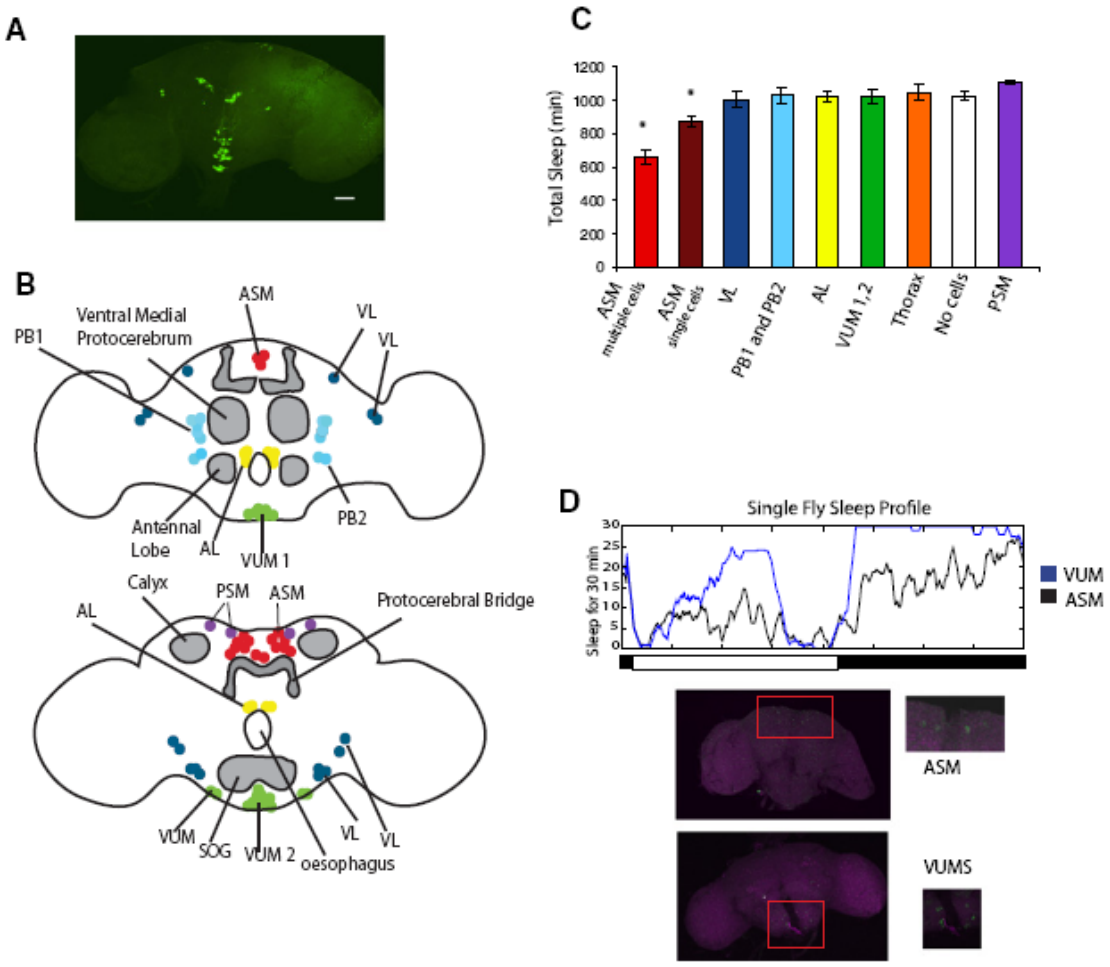


Figure 3-2: Cells in the medial protocerebrum mediate wake-promoting effects of octopamine

A. Expression pattern of the *Tdc2–Gal4* line as visualized with a GFP reporter. Octopamine is produced in a subset of neurons in the brain. Expression was characterized by crossing *Tdc2–Gal4* with *UAS–GFPnls*. B. A schematic of the different octopamine neurons, labeled according to Sinakevitch and

Strausfeld (Sinakevitch and Strausfeld, 2006). Cell groups are clustered according to color, with similar colors indicating cells that could not be distinguished from each other in our analysis. C. Total sleep amounts for each cell population indicated in B. The colors of the graph correspond to clusters of the same color in B. The ASMs were the only cell group that showed a significant decrease in sleep compared to the other cell groups. The graph depicts mean sleep ± S.E. and * is $p \leq 0.01$ using 2 way ANOVA. Values are found in Table 3-1. D. Sleep data for single flies. Sleep over 24 hours is shown for two flies, one expressing Na^+ channel in 4 cells in the ASM region (Black line) and one for a fly expressing the Na^+ channel in about 8 VUMS (Blue line). Day and night are depicted by the black and white lines respectively. Pictures below are of those individual fly brains stained for nc82 (pink) and GFP (green). The generation of the MARCM flies is outlined in Figure 3-8.

Figure 3-3

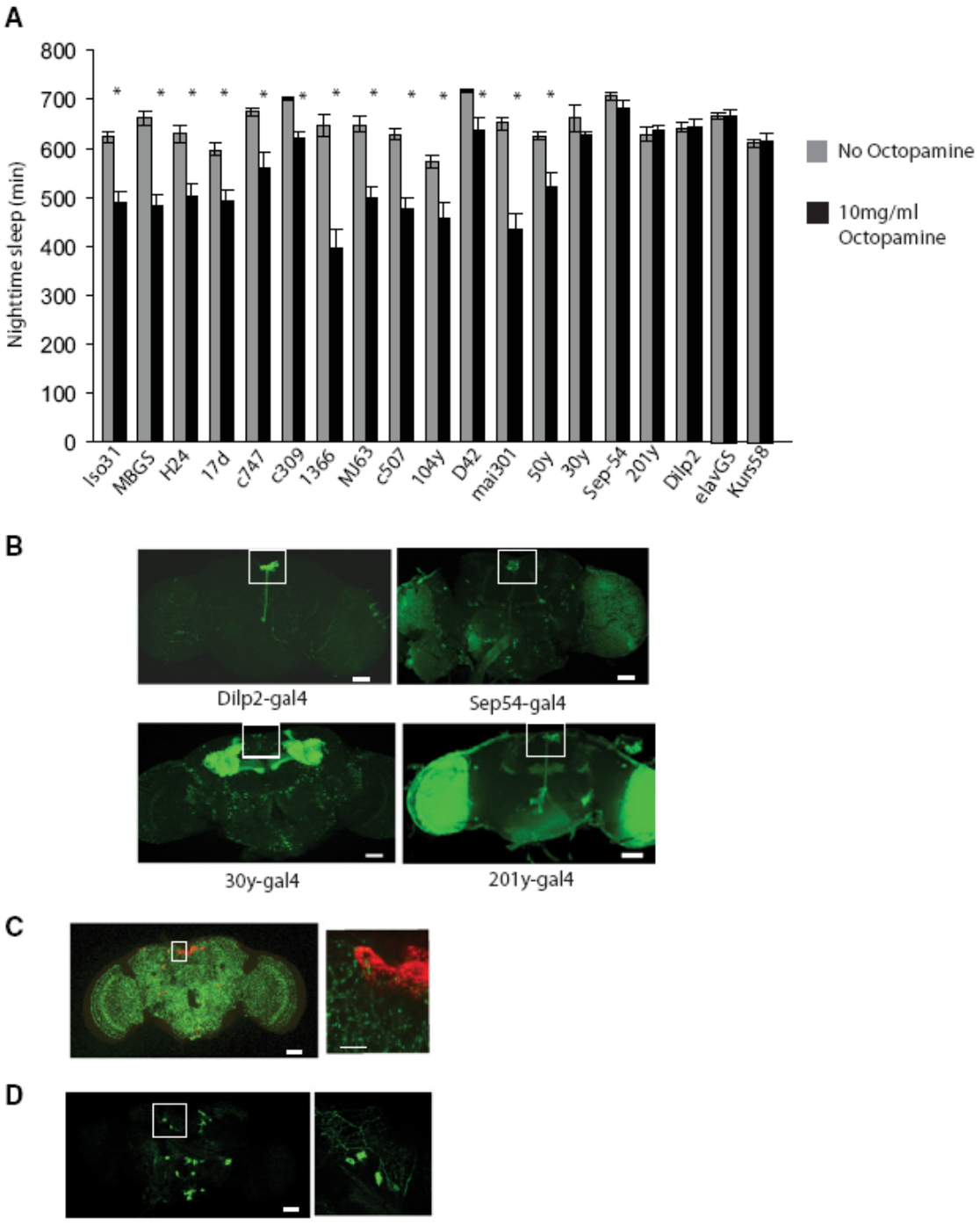


Figure 3-3. Octopamine signals through cells in the pars intercerebralis (PI). A. Sleep in flies expressing the regulatory subunit of protein kinase A

(PkaR) in different regions of the fly brain. Progeny obtained by crossing each of the 18 GAL4 driver lines indicated to w1118;UAS-BDK33 (PkaR) were analyzed for sleep. w1118 (Iso31), the background for all the lines, was used as control line. Sleep values were determined for the night prior to the initiation of 10mg/ml octopamine feeding and then the third night after providing octopamine. Grey bars represent sleep prior to octopamine; black bars, sleep following 10mg/ml octopamine. Sleep values are found in Table 3-2 and are plotted as mean ± s.e.m. Nighttime sleep values for each line is depicted in Table 3-2 and sleep parameters during nighttime sleep for expression of PkaR in *Dilp2*-producing neurons is shown in Figure 3-4. B. Expression of a nuclear-targeted GFP (GFPn) under the control of the following GAL4 drivers; *Dilp2*-GAL4, Sep54-GAL4, 30y-GAL4, 201y-GAL4. The PI region is denoted by the white square in each image. C. Expression of a synaptically targeted GFP (syt-GFP) under the control of Tdc2-GAL4. PI neurons were stained with anti-*Dilp2* and are shown in red. An enlarged image of the PI region is shown in the right hand panel. D. Expression of a membrane bound GFP (CD8-GFP) *Tdc2*-producing cells using the Tdc2-GAL4 driver. A single confocal image through the ASM neurons is shown on the left. On the right is an enlarged image of a 5 □m thick stack through the ASM neurons, which appear to show dorsal as well as lateral projections.

Figure 3-4

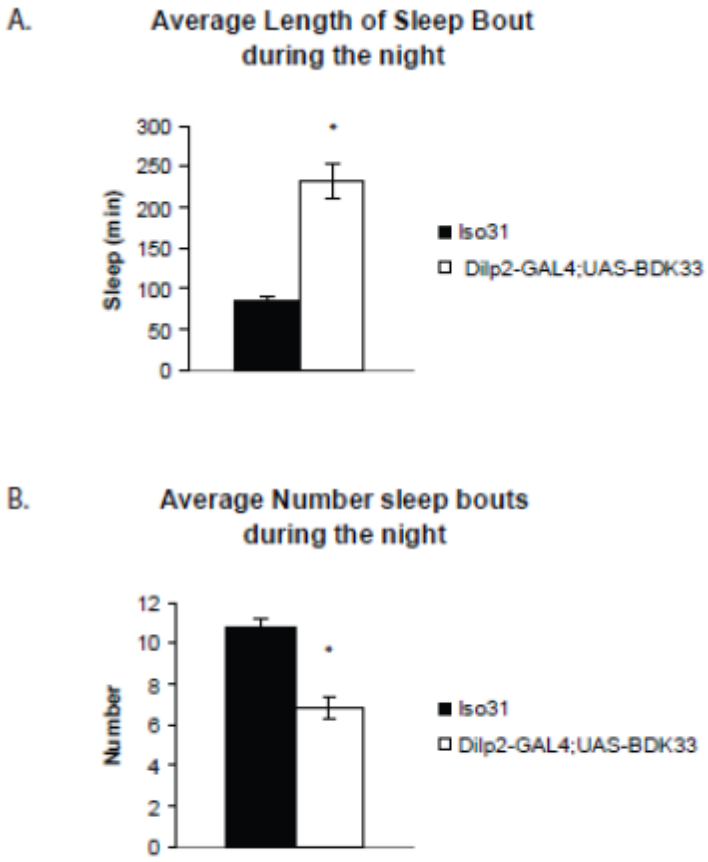


Figure 3-4 (Supplemental Figure 4). Expressing PkaR in *Dilp2* neurons consolidates sleep. A. The average length of sleep bouts is significantly longer in flies expressing UASBDK33 (PkaR) in *Dilp2* neurons using the *Dilp2*-GAL4 driver. Control flies (Iso31) have an average sleep bout length at night of 83.86 ± 7.27 minutes while the *Dilp2*-GAL4;UAS-BDK33 flies have an average night-time bout length of 233.1 ± 21.21 minutes. Sleep bout length is plotted as mean ± s.e.m. Asterisk= $P < 0.01$ by 2-way Anova. B. The average number of sleep bouts is significantly less in flies expressing UASBDK33 (PkaR) in *Dilp2* neurons using the *Dilp2*-GAL4 driver. Control flies (Iso31) have on average 10.6 ± 0.47

sleep bouts at night while the *Dilp2*- GAL4;UAS-BDK33 flies have 6.85 ± 0.56 sleep bouts. Sleep bout number is plotted as mean ± s.e.m. Asterisk= $P < 0.01$ by 2-way Anova.

Figure 3-5

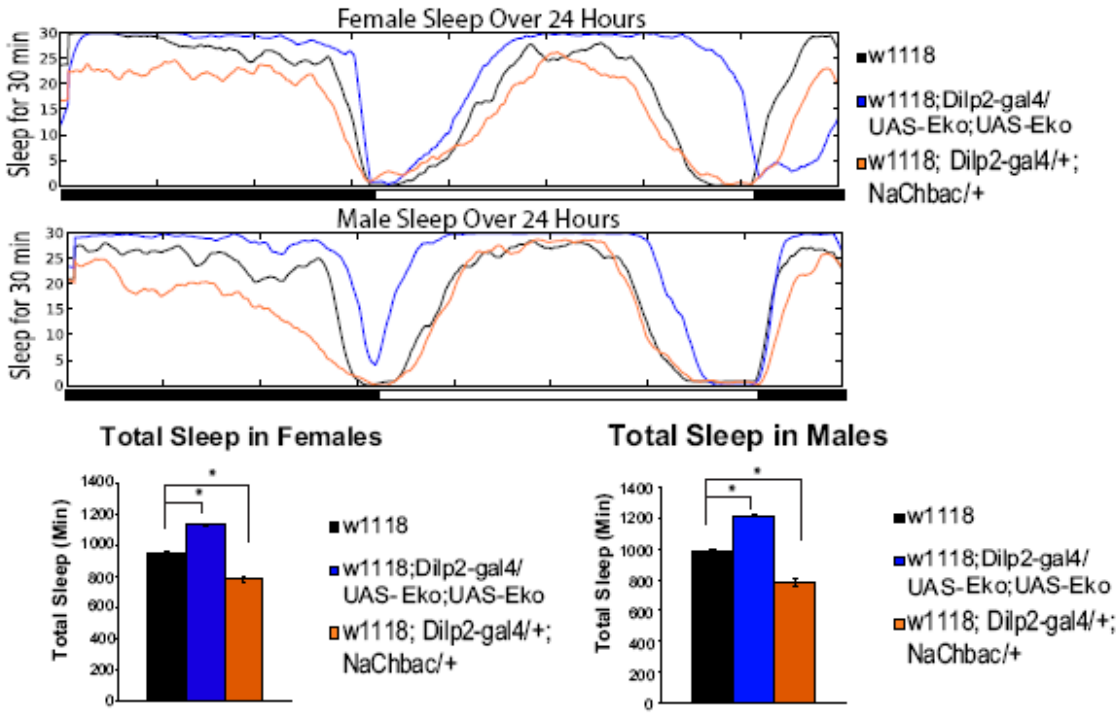


Figure 3-5. Sleep is altered by manipulations of the electrical activity of PI neurons. **A** 24 hour profile of sleep in w1118 flies (black line), and *Dilp2*-GAL4 flies expressing either 3xUAS-*Eko* (Blue line) or UAS-*NaChBac* (orange line). Top panel shows female data and bottom panel male data. Total sleep amount for females and males of each genotype is shown below the profiles. Actual values are in Table 3-3. Total sleep is plotted as mean ± s.e.m. Asterisk= $P <$ 0.01 by 2-way Anova.

Figure 3-6

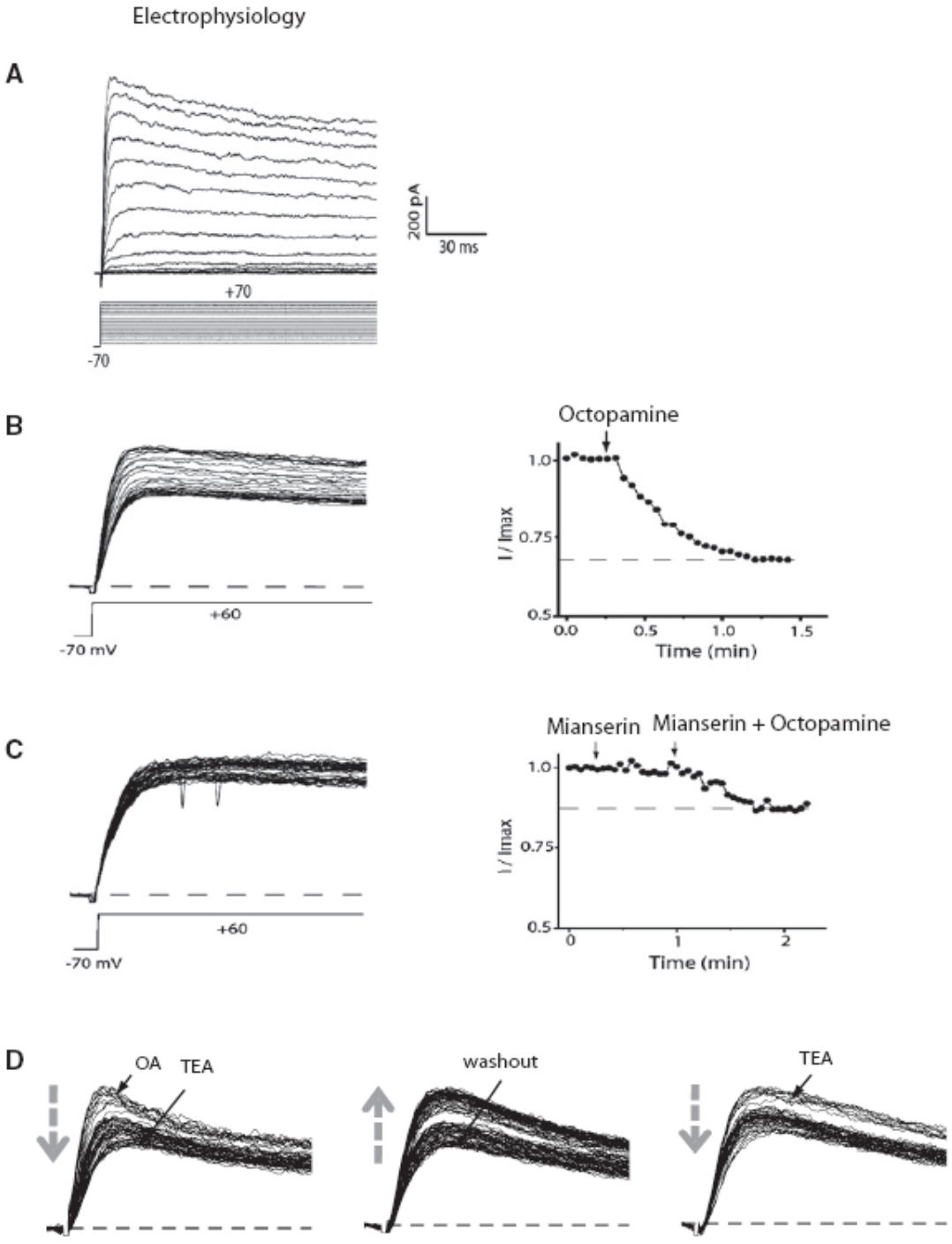

Electrophysiology
A
200 pA
30 ms
+70
-70
B
Octopamine
1.0
0.75
0.5
I / Imax
0.0
0.5
1.0
1.5
Time (min)
+60
-70 mV
C
Mianserin
Mianserin + Octopamine
1.0
0.75
0.5
I / Imax
0
1
2
Time (min)
+60
-70 mV
D
OA
TEA
washout
TEA

Figure 3-6. Octopamine modulates outward potassium current in PI neurons. The *Dilp2* neurons were identified by expressing GFP under the control of a *Dilp2*-Gal4 driver, and voltage-dependent outward current was recorded in the whole cell patch recording mode. A. Whole-cell outward currents evoked by 150 ms depolarizing voltage steps from -60 mV to +70 mV in 10 mV increments, from a holding potential of -70 mV. The scale bars apply to all portions of this figure. B. Repetitive pulses to a single voltage, +60 mV, were used to examine the effects of octopamine (OA) on the outward current. 1 mM OA was added to the bath solution during the recording after which the outward current at +60 mV decreases as a function of time (B, left). Peak current amplitudes are plotted against time and the application of octopamine is shown by an arrow (B, right). C. The same voltage protocol as in B was used to evoke outward current, and 0.5 mM mianserin (MI) was added to the bath solution followed by the application of 1 mM OA (C, left). Peak current amplitudes are plotted against time; the applications of mianserin and OA are shown by arrows (C, right). D. The same voltage protocol was used to evoke outward current, and 1 mM OA was added to the bath solution. After the modulation by octopamine, subsequent addition of TEA does not block the residual outward current (D, left). Following washout of the octopamine and TEA, the current returns to baseline levels (D, middle). Subsequent addition of TEA blocks about 30% of the outward current (D, right), suggesting that octopamine selectively modulates the TEA-sensitive component of the current. The arrows illustrate the direction of current change over time after drug addition or withdrawal.

Figure 3-7

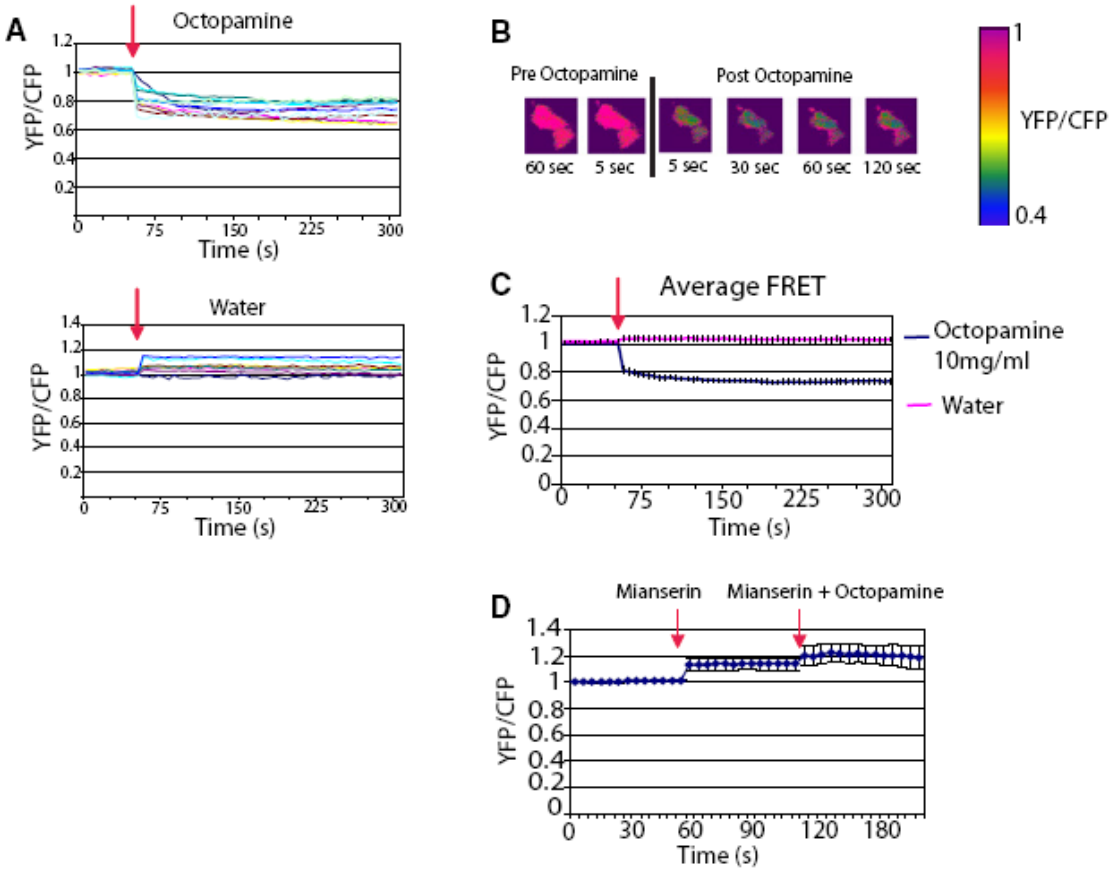


Figure 3-7. Octopamine increases cAMP signaling in PI neurons

A. The response of *Dilp2* neurons to 1 mM Octopamine. A FRET-based cAMP sensor was expressed in *Dilp2* neurons and the FRET signal was measured in dissected brains following the application of octopamine. In all graphs the red arrow indicates the start of bath application of octopamine or vehicle. The lower panel shows the response of *Dilp2* neurons from 8 brains to vehicle (water). B. A pseudocolored time course of Epac1-camps FRET loss in a cluster of 3 *Dilp2* cell bodies in response to 1 mM octopamine. The black bar represents the addition of octopamine. The time course is outlined below the

images. The table values on the right represent raw YFP/CFP ratios. C. Quantification of the data in A showing the average YFP/CFP value for each time point over 300 seconds. Error bars indicate the standard error of the mean. Through one-way repeated measures ANOVA, all time points following octopamine administration were significantly different from vehicle with $p<.001$; prior to octopamine administration there was no difference between vehicle and octopamine groups. D. Effects of mianserin on the response to octopamine. The plot shows the average YFP/CFP value for each time point over 300 seconds following application of 0.25mM mianserin and then 0.25mM mianserin plus 10mg/ml octopamine. Error bars indicate the standard error of the mean. Through one-way repeated measures ANOVA, all time points following octopamine administration were not significantly different from previous time points, despite there being a trend towards an increased ratio.

Figure 3-8

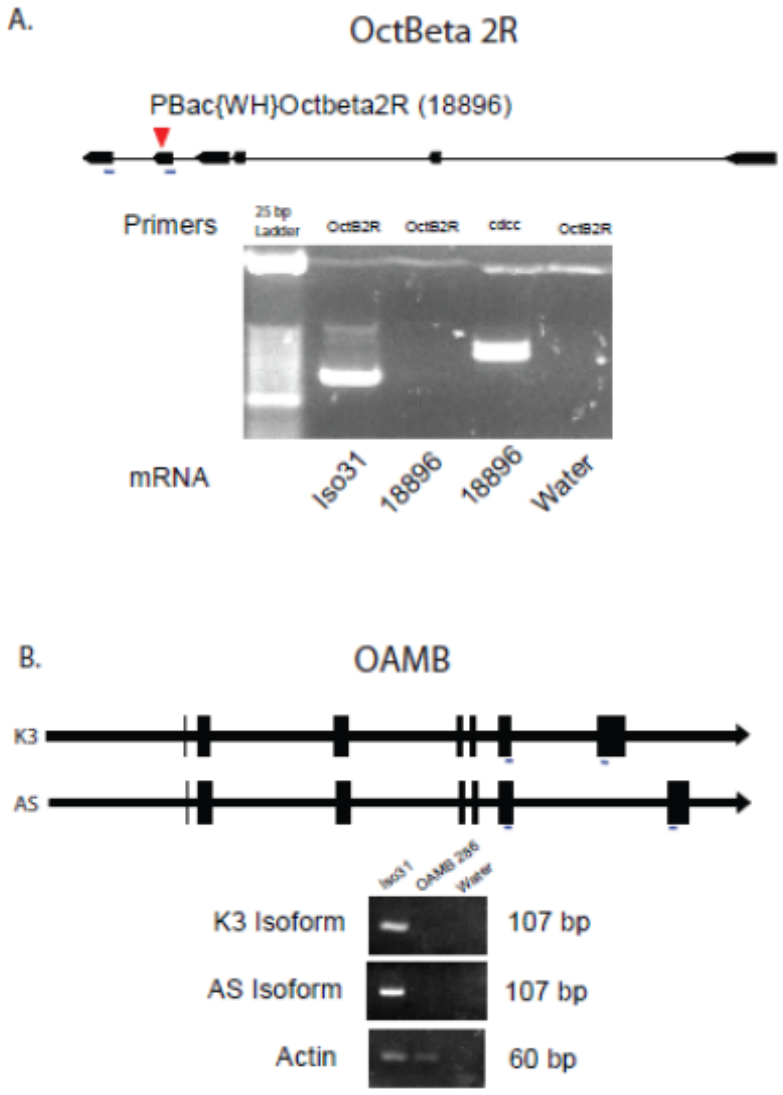


Figure 3-8 (Supplemental Figure 2). Molecular analysis of the OAMB and OctB2 receptors A. Schematic of the OctB2R gene showing the P-element insertion (red arrow). Primers were designed to flank this insert and to span an exon (blue lines under exon) and then RT-PCR was conducted to assay expression of OctB2R. Representative gel showing lack of a product in OctB2R flies as compared to the control lane, implying that these flies are mutant for the OctB2R gene. B.

Verification of the OAMB 286 mutant. RT-PCR was run on control flies and OAMB 286 mutants using the new K3 and AS primers to both verify primer specificity and to verify that OAMB 286 is a true null.

Figure 3-9

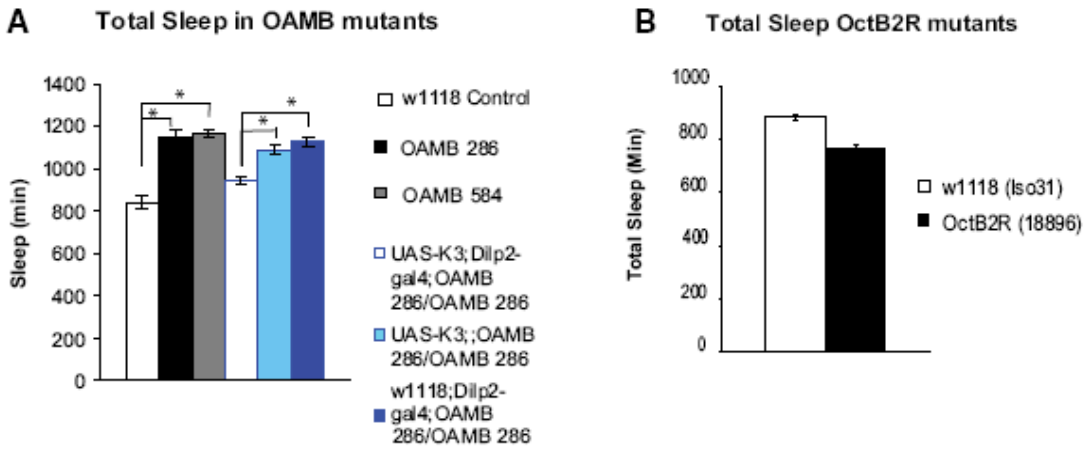


Figure 3-9. Mutations in the OAMB receptor affect sleep levels. A. Sleep amounts for females of the following genotypes- w1118 (white), w1118;;OAMB286/OAMB 286 (black), w1118;;OAMB 584/OAMB 584 (grey), UAS-K3;*Dilp2*-GAL4;OAMB 286/OAMB286 (white), UAS-K3;;OAMB 286/OAMB286 (light blue), and w1118;*Dilp2*-GAL4;OAMB 286/OAMB286 (blue) . Total sleep is plotted as mean ± s.e.m. Asterisk= $P < 0.01$ by 2-way Anova. Actual values and male data are in Table 3-3. B. Sleep amounts for the OctB2R (18896) outcrossed into the w1118 (Iso31) background. Total sleep is shown as in part A for Iso31 (control) in white and 18896 in black. Actual values and male data are in Table 3-3. Verification of loss of OAMB transcript in OAMB 286 mutant and OctB2R transcript in the OctB2R mutant is shown in Fig. 3-8. Expression of OAMB transcript in *Dilp2* -producing neurons is shown in Figure 3-10.

Figure 3-10

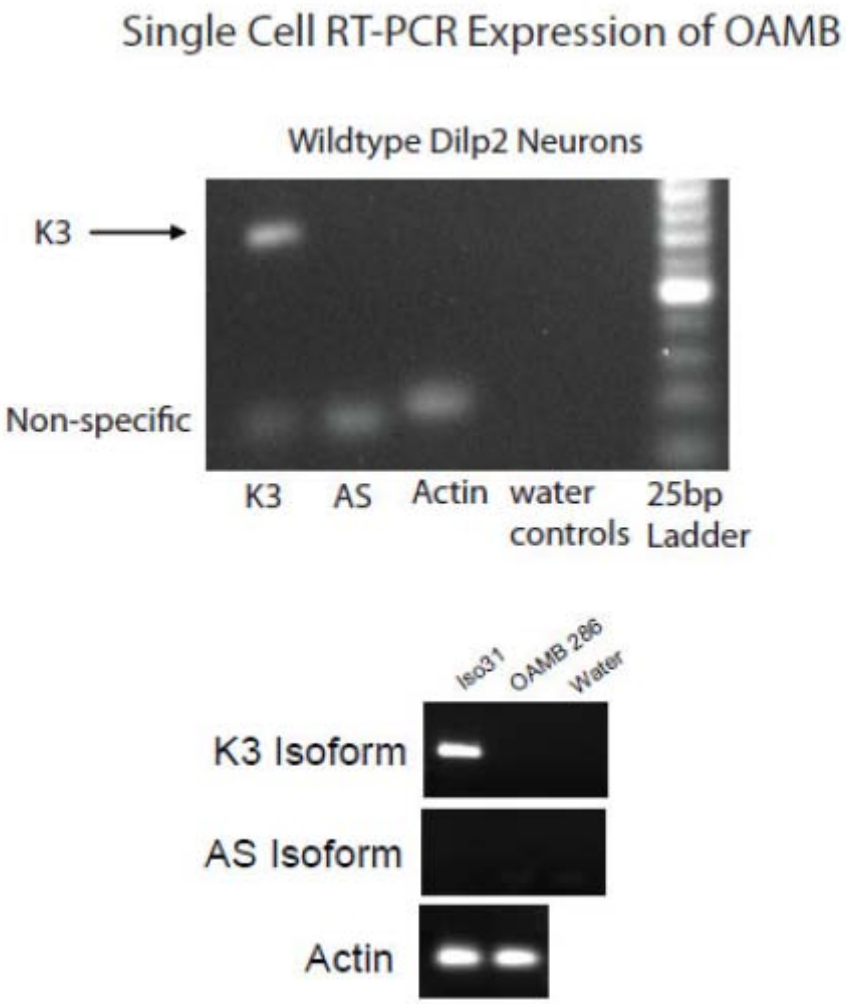


Figure 3-10 (Supplemental Figure 3) . The OAMB receptor is expressed in *Dilp2* neurons Single cell RT-PCR of control flies showing that only the K3 isoform is present in *Dilp2*-producing neurons. B. Representative gel for single cell RT-PCR of control *Dilp2* neurons and OAMB 286 mutant *Dilp2* neurons. In the OAMB 286 mutant the K3 isoform is no longer present.

Figure 3-11

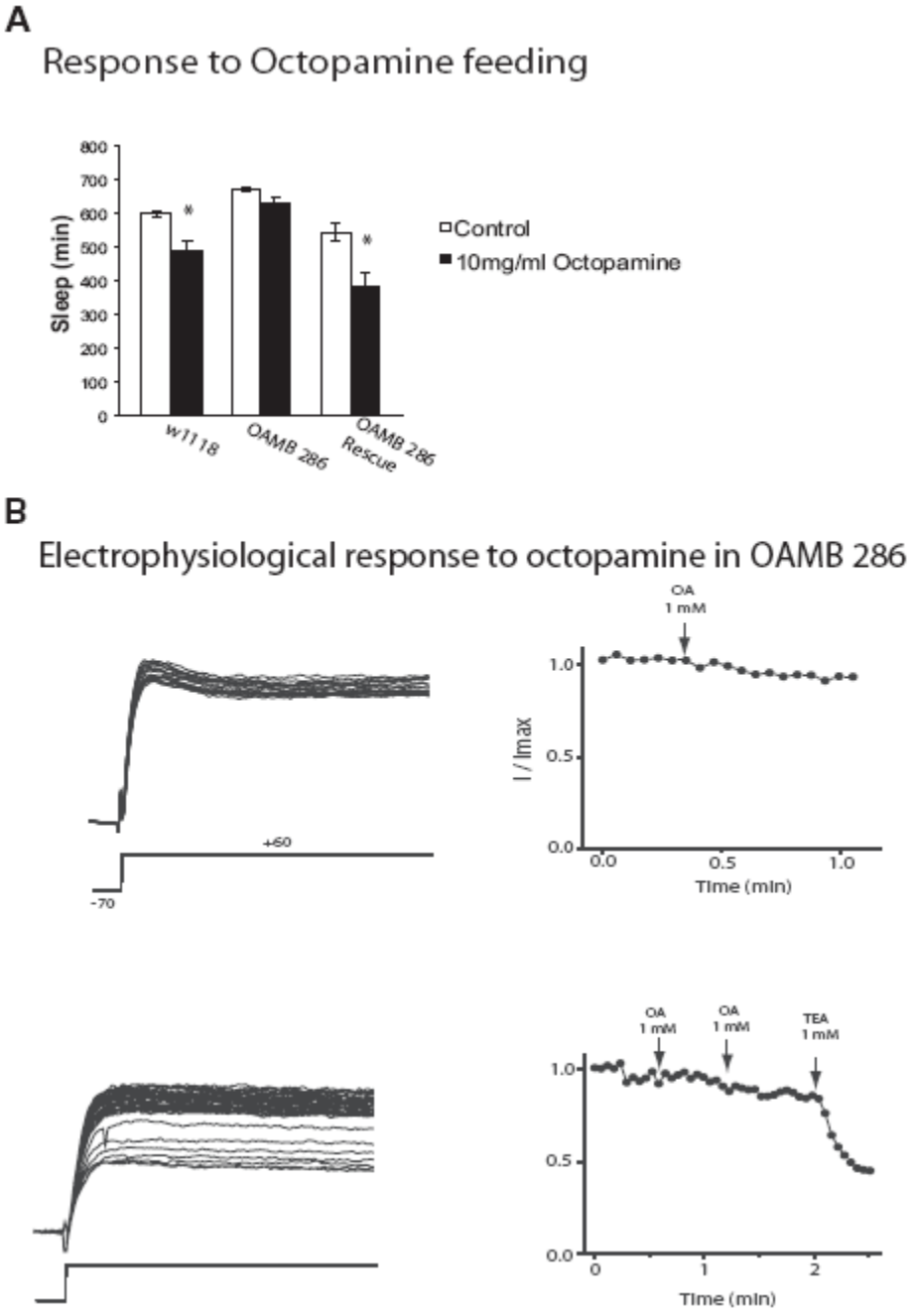

A
Response to Octopamine feeding
Sleep (min)
800
700
600
500
400
300
200
100
0
w1118
OAMB 286
OAMB 286 Rescue
Control
10mg/ml Octopamine
B
Electrophysiological response to octopamine in OAMB 286
+60
-70
OA 1 mM
I / Imax
1.0
0.5
0.0
0.0
0.5
1.0
Time (min)
OA 1 mM
OA 1 mM
TEA 1 mM
0
1
2

Figure 3- 11. The OAMB mutant shows an attenuated response to octopamine. A. Sleep amounts prior to treatment with 10mg/ml octopamine (white bar) and post octopamine (black bar) for females of the genotypes w1118, w1118;;OAMB286/OAMB286, and UAS-K3;*Dilp2*-GAL4;OAMB286/OAMB286. Sleep is plotted as mean ± s.e.m. Asterisk= $P < 0.01$ by 2-way Anova. Nighttime sleep values are shown in Figure 3-9. B. Whole-cell outward current in PI neurons in OAMB 286 flies. Current at +60 mV (as in Figure 4) is modulated only slightly by two successive applications of 1 mM octopamine (OA). Subsequent application of 1 mM TEA blocks about 30% of the outward current (B, left). Peak current amplitudes are plotted against time, and the applications of octopamine and TEA are shown by arrows (B, right).

Table 3-1

MARCM

	Total Sleep	Daytime Sleep	Nighttime Sleep	N
ASM	663.25±36.5*	155.49±25.22*	476.13±39.77*	12
ASM single cell	870.45±25.59*	279.18±33.39*	598.96±17.3	11
VL	1007.18±28.29	372.49±27.97	587.68±21.56	21
PB1 and PB2	1030.21±24.47	393.72±20.47	625.80±11.15	23
AL	1021.13±20.3	381.22±19.66	607.44±20.31	28
VUM 1,2	1016.68±19.91	379.17±16.57	612.61±12.04	47
Thorax	1045.76±23.57	408.24±20.66	606.75±18.74	33
No cells	1025.86±13.45	391.45±11.50	616.27±8.23	84
PSM	1109.92±15.64	458.75±15.76	664.87±15.08	12

Table 3-1 (Supplemental Table 1). Sleep data for flies generated through MARCM Table depicts total sleep, daytime sleep and nighttime sleep for each group of TDC2 positive cells.

Table 3-2

GAL4 Line	Nighttime Sleep	Sleep following 10mg/ml Octopamine		N
Control (Iso31)	623.09 ± 11.27	489.5 ± 22.21	*	64
MBGS	661.21 ± 14.2	483.86 ± 21.45	*	28
H24	629.42 ± 19.64	503.17 ± 24.77	*	24
17d	597.92 ± 11.7	493.71 ± 21.46	*	48
c747	675.35 ± 8.79	559.35 ± 32.51	*	40
c309	699.75 ± 3.46	619.14 ± 15.09	*	56
1366	646.70 ± 21.33	395.00 ± 40.25	*	20
MJ63	648.64 ± 15.51	500.71 ± 19.89	*	32
c507	628.91 ± 11.46	474.91 ± 23.43	*	44
104y	572.96 ± 14.69	459.35 ± 31.38	*	46
D42	717.25 ± 2.75	636.25 ± 24.18	*	30
mai301	650.20 ± 12.63	433.73 ± 32.11	*	30
50Y	624.33 ± 8.75	519.8 ± 33	*	32
30y	663.00 ± 28.00	627.00 ± 8.00		16
Sep-54	705.38 ± 7.78	683.5 ± 11.54		32
201y	629.16 ± 13.97	637.26 ± 11.69		62
Dilp2	642.57 ± 9.18	645.85 ± 12.4		32
elavGS	665.41 ± 7.00	666.77 ± 11.09		44
Kurs58	609.86 ± 9.07	613.96 ± 18.14		32

	Nighttime Sleep	Nighttime sleep following 10mg/ml octopamine		N
w1118	598.5 ± 9.72	490.57 ± 30.35	*	32
OAMB 286	671.96 ± 7.09	635.46 ± 11.85		30
OAMB 286 Rescue	544.43 ± 27.0	387.0 ± 41	*	32

Table 3-2 (Supplemental Table 2) : Effects of octopamine on sleep in different fly lines The first part of the table shows effects of octopamine on sleep in flies expressing PkaR under the control of different GAL4 drivers. The bottom part shows effects on octopamine receptor mutants.

Table 3-3

	Total Sleep	Activity per waking minute	Daytime Sleep	Nighttime Sleep	sleep bout length day	sleep bout length night	sleep bout number day	sleep bout number night
w1118;+;+ F	956.0 ± 14.27	1.85 ± 0.12	342.9 ± 9.22	613.60 ± 6.78	49.41 ± 3.82	141.86 ± 13.53	8.96 ± 0.75	5.6 ± 0.38
w1118; +; + M	977.77 ± 14.62	2.53 ± 0.15	400.44 ± 8.54	577.33 ± 8.35	66.08 ± 8.36	99.51 ± 7.76	8.17 ± 0.50	7.33 ± 0.51
w1118;Dilp2-GAL4; + F	966.19 ± 19.08	1.05 ± 0.03*	349.68 ± 13.90	616.50 ± 8.39	21.16 ± 1.88*	126.51 ± 13.61*	21.03 ± 0.82*	9.24 ± 0.55*
w1118;Dilp2-GAL4; + M	933.34 ± 17.13*	1.74 ± 0.05*	386.43 ± 14.06	546.91 ± 6.81*	48.27 ± 5.78*	87.34 ± 8.22	11.40 ± 0.54*	9.70 ± 0.76*
w1118;; NaChBac F	884.62 ± 29.28*	1.29 ± 0.07*	343.75 ± 30.65	540.88 ± 18.49*	33.61 ± 2.65*	52.87 ± 5.83*	12.02 ± 0.89*	14.41 ± 0.89*
w1118;; NaChBac M	1005.21 ± 9.81	2.8 ± 0.05*	440.80 ± 5.35*	564.41 ± 8.76	169.63 ± 17.06*	121.34 ± 7.36*	3.98 ± 0.35*	5.62 ± 0.38
w1118;Dilp2; NaChBac F	784.33 ± 21.92*†	1.33 ± 0.08*†	273.83 ± 18.32*†	510.50 ± 9.67*†	24.21 ± 2.02*	39.13 ± 2.48*†	12.44 ± 1.02*†	15 ± 0.75*†
w1118;Dilp2; NaChBac M	779.45 ± 22.30*†	2.41 ± 0.2	373.64 ± 7.67	405.81 ± 18.77*†	61.31 ± 4.48	54.83 ± 4.34*†	7.17 ± 0.45	8.5 ± 0.48
w1118; 3xEKOII F	1000.86 ± 12.90*	1.33 ± 0.05*	409.04 ± 12.83*	591.82 ± 7.98	31.49 ± 2.51*	151.14 ± 23.52	15.79 ± 0.98*	7.82 ± 0.89*
w1118; 3xEKOII M	1059.03 ± 10.69*	1.63 ± 0.04*	428.10 ± 8.54	630.92 ± 6.24*	69.17 ± 5.39	262.18 ± 22.83*	8.44 ± 0.59	3.88 ± 0.36*
w1118; Dilp2/3xEKOII F	1135.19 ± 7.99*†	1.04 ± 0.09*	560.73 ± 5.66*†	574.46 ± 5.53*†	71.34 ± 4.62*†	135.39 ± 15.15	9.24 ± 0.59	5.95 ± 0.45
w1118; Dilp2/3xEKOII M	1210.88 ± 8.36*†	1.54 ± 0.07*†	560.31 ± 6.96*†	650.56 ± 3.30*†	115.59 ± 8.44*†	224.88 ± 17.82*†	5.81 ± 0.35*†	3.8 ± 0.29*†
w1118 F	848.31 ± 40.41	1.87 ± 0.04	253.29 ± 30.34	595.02 ± 13.46	30.86 ± 4.36	72.63 ± 11.30	10.4 ± 0.78	11.68 ±1.13
w1118 M	970.68 ± 9.48	2.35 ± 0.05	446.54 ± 6.64	524.13 ± 6.66	55.38 ± 3.25	94.58 ± 6.91	12.53 ± 0.68	9.99 ± 0.63
OAMB 286 F	1169.52 ± 36.20*	2.60 ± 0.27*	490.37 ± 35.34*	679.15 ± 6.59*	24.20 ± 2.45	142.48 ± 27.21*	21.76 ± 1.53*	7.39 ±1.11*
OAMB 286 M	1215.29 ± 20.63*	2.15 ± 0.18	551.38 ± 14.46*	663.81 ± 7.37*	43.25 ± 4.94*	133.39 ± 17.71*	15.61 ± 1.51*	7.54 ± 0.95
OAMB 584 F	1165.13 ± 26.42*	2.37 ± 0.17*	478.33 ± 22.89*	686.79 ± 5.48*	22.74 ± 1.78	126.56 ± 25.16*	22.50 ± 1.16*	9.20 ± 1.21
OAMB 584 M	1068.38 ± 29.40	2.72 ± 0.12	454.62 ± 21.50	613.85 ± 12.48	38.60 ± 4.54*	72.04 ± 12.74	14.41 ± 1.19*	12.10 ± 1.06*
UAS-K3;;OAMB286/OAMB286 F	1088.18 ± 24.45	2.0 ± 0.16	411.23 ± 23.83	676.14 ± 5.12	23.62 ± 1.93	242.07 ± 37.79	19.96 ± 1.38	5.89 ± 0.89
UAS-K3;;OAMB286/OAMB286 M	1103.90 ± 7.05	1.43 ± 0.04	476.89 ± 6.05	627.01 ± 4.99	45.05 ± 3.50	228.47 ± 23.33	13.22 ± 0.60	4.97 ± 0.41
w1118;Dilp2-GAL4;OAMB 286/OAMB286 F	1127.719 ± 25.58	2.10 ± 0.09	479.72 ± 23.65	648.0 ± 56.9	39.01 ± 4.7	174.0 ± 25.0	13.75 ± 1.04	6.11 ± 1.1
w1118;Dilp2-GAL4;OAMB 286/OAMB286 M	1047.62 ± 19.95	2.38 ± 0.21	455.26 ± 13.18	592.35 ± 11.60	25.76 ± 1.69	80.94 ± 9.37	19.74 ± 0.88	10.52 ± 0.93

UAS-K3;Dilp2-GAL4;OAMB286/OAMB286 F	945.0 ± 16.73**	1.5 ± 0.05**	330.13 ± 13.44**	614.53 ± 8.78**	37.14 ± 5.68*	167.02 ± 20.84*	18.37 ± 0.77*	10.63 ± 0.95**
UAS-K3;Dilp2-GAL4;OAMB286/OAMB286 M	953.98 ± 20.54**	2.03 ± 0.08**	426.32 ± 18.06*	527.66 ± 11.41**	49.63 ± 5.69*	142.42 ± 19.99**	11.5 ± 0.68*	5.86 ± 0.44**
Iso31 F	881.17 ± 28.45	1.46 ± 0.03	311.73 ± 21.57	569.43 ± 10.95	31.49 ± 1.70	104.35 ± 7.44	12.8 ± 0.43	8.94 ± 0.40
Iso31 M	952.59 ± 17.60	2.03 ± 0.06	388.72 ± 12.20	563.87 ± 9.03	63.90 ± 5.45	119.89 ± 10.48	8.21± 0.54	6.58 ± 0.48
OctB2R (18896) F	765.58 ± 25.60*	1.35 ± 0.03*	210.91 ± 20.02*	554.67 ± 12.08	22.58 ± 1.61*	68.58 ± 5.59*	12.18 ± 0.52	10.79 ± 0.44*
OctB2R (18896) M	970.84 ± 20.89	2.11± 0.07	435.55 ± 12.36*	535.29 ± 12.17	90.02 ± 4.69*	93.54 ± 7.39	5.96 ± 0.28*	7.52 ± 0.54

Table 3-3 (Supplemental Table 3) : Sleep in octopamine receptor mutants and in flies with altered excitability of *Dilp2* neurons. In the tables above *Dilp2* refers to *Dilp2*-GAL4, *NaChBac* to UAS-*NaChBac* and 3x*Eko* to UAS *EkoII*;UAS-*EkoI*. The w1118 background for OAMB 286 and OAMB 584 is different from the isogenic w1118 strain, termed Iso31, we used in other experiments. All tables depict mean ± SEM *= p<.01 relative to wild-type control line (w1118 or Iso31, as the case may be). All "†" for w1118;*NaChBac*, w1118;3x*Eko*II, w1118;*Dilp2*/*NaChBac* and w1118;*Dilp2*/3x*Eko*II are relative to each respective GAL4 and UAS control. The "**" for rescue experiments indicate the rescue is significantly different from each control- wild type, mutant and mutant with either UAS or GAL4 alone. *Dilp2*/*NaChBac* females are significantly different from all controls for total sleep, daytime sleep, nighttime sleep, sleep bout length night and sleep bout number night. *Dilp2*/*NaChBac* males are significantly different from all controls for total sleep, daytime sleep, nighttime sleep, and sleep bout length night. *Dilp2*/3x*Eko*II females are significantly different from all controls for total sleep, daytime sleep, and daytime sleep bout length. *Dilp2*/3x*Eko*II males are significantly different from all controls for total sleep, daytime sleep, nighttime

sleep, daytime sleep bout length, nighttime sleep bout length, daytime sleep bout number and nighttime sleep bout number.

Chapter IV:

Effects of octopamine on metabolism in *Drosophila*

Amanda Crocker, Justin DiAngelo, Renske Erion, Alexis Gerber, Adam Watson, Amita Sehgal

Abstract

Why we sleep is one of the major unanswered questions in science. While in the last 100 years many people have devoted their careers to this, we still do not understand this phenomenon. Recent work on sleep in humans indicates deficits in normal metabolic function due to loss of sleep (Knutson and Van Cauter, 2008; Spiegel et al., 2009; Van Cauter et al., 2008). Studies show that normal healthy individuals develop early clinical signs of type-3 diabetes when their sleep is disrupted (Knutson and Van Cauter, 2008). In addition, many metabolic syndromes are accompanied by disrupted sleep (Bopparaju and Surani, 2010; Lisk, 2009; Nixon and Brouillette, 2002; Shipley et al., 1992). While this research points to a link between sleep and metabolism, it is difficult in mammals to address whether these reciprocal effects are a consequence of overlapping pathways or actually reflect mutual regulation. Mammals and *Drosophila* (flies) share many signaling pathways, including those involved in sleep and metabolism (Crocker et al., 2010; DiAngelo and Birnbaum, 2009; Rulifson et al., 2002). Of the pathways that affect both processes, octopamine is a good example. Octopamine is known to regulate sleep in *Drosophila* and in other invertebrate species has been implicated in metabolism(Crocker and Sehgal, 2008; Roeder, 2005). Likewise, the mammalian homolog of octopamine, norepinephrine, is implicated in both physiological processes (Berridge, 2008; Harik et al., 1982; Sara, 2009). Here we show that flies with less sleep due to increased neuronal octopamine signaling have increased triglycerides. Conversely, mutants in the octopamine receptor OAMB showed decreased

triglycerides. The increase in triglycerides seen with increased octopamine signaling does not appear to be due to the loss of sleep, since other short-sleeping flies do not show consistent increases in triglyceride levels. To address whether the effect of octopamine on metabolism drives the loss of sleep, we tested whether changes in insulin signaling, a likely cause of altered triglyceride levels, directly affect sleep. We find that alterations in the insulin pathway, independent of manipulations in octopamine, have only minor effects on sleep.

Introduction

While trying to address why we sleep, it is important to address the question of what keeps us awake. In a world where people are driven to maintain wakefulness for longer and longer periods of time and forego the normal 8 hours of sleep, it is important to understand the consequence of this behavior. Many neurotransmitters and neuropeptides that are necessary for normal wakefulness, such as dopamine, norepinephrine and orexin, are also important for proper metabolic functioning (Berger et al., 2009; Charmandari et al., 2005; Ford et al., 2005; Saper et al., 2005). Serotonin and norepinephrine are also important for proper stress regulation (Berger et al., 2009; Charmandari et al., 2005; Goddard et al.; Leonard, 2005). These factors are important, considering that during sleep deprivation these neurotransmitter systems may be more active then normal.

In humans, sleep deprivation leads to early symptoms of type-2 diabetes (Knutson and Van Cauter, 2008). These signs include reduction in the ability to

regulate blood sugar levels during a glucose tolerance test, decreased release of insulin, and increases in cortisol levels (Spiegel et al., 2009). In addition, patients who suffer from sleep disorders that cause severe fragmentation of sleep, such as sleep apnea and severe restless leg syndrome, display impaired glucose tolerance tests (Keckeis et al., 2010). Just as people who suffer sleep disorders have impaired metabolic functioning, people who suffer from metabolic disorders (including patients with diabetes) have abnormal sleep (Bopparaju and Surani, 2010; Lisk, 2009; Nixon and Brouillette, 2002; Shipley et al., 1992). Mice that are resistant to leptin, a hormone that regulates appetite and glucose and lipid metabolism, show a high degree of sleep fragmentation (Laposky et al., 2008). These findings beg the question of whether sleep is required for normal metabolic function and/or normal metabolic function is required for normal sleep.

To best address this question we need a molecule that overlaps in both metabolic and sleep function. In mammals, this molecule might be norepinephrine, which regulates the fight or flight response and is responsible for heightened arousal and the breakdown of fat stores (Berridge, 2008; Harik et al., 1982). Interestingly, a subset of norepinephrine neurons in the locus coeruleus (LC) also make neuropeptide Y (NPY) which promotes fat storage and feeding behavior, although the primary nucleus that produces NPY is the arcuate nucleus (Holets et al., 1988; Leibowitz, 1991). The function of the co-expression of norepinephrine and NPY is unclear. It may be that increased action of norepinephrine underlies both metabolism and disrupted sleep.

Drosophila have many of the same metabolic and sleep pathways as mammals and provide an ideal model for addressing the mutual regulation of metabolic function and sleep. We have also recently found a potential tool with which to ask these questions. Octopamine, which is the mammalian homolog to norepinephrine, appears to be an ideal candidate for linking sleep and metabolism. Through the experiments described in Chapters 2 and 3, I have demonstrated that octopamine is necessary in flies to maintain normal arousal (Crocker and Sehgal, 2008; Crocker et al., 2010). This arousal signal in part comes from the action of octopamine on neurons in the Pars Intercerebralis (PI), the neurosecretory cells that produce the fly homolog of insulin (Rulifson et al., 2002). In addition, there are reports of strong correlations between octopamine signaling and metabolic function; however, most come from other insects, and not flies (Roeder, 2005). Nevertheless, this potential interaction between the octopamine and insulin systems provided us an opportunity to address whether sleep affects metabolism and/or metabolism affects sleep.

We hypothesized that octopamine alters insulin signaling in the PI and that changes in insulin signaling underlie the sleep phenotype. However, this is not what we found. Consistent with the idea that octopamine stimulates the insulin pathway, we found that triglyceride levels (a readout of insulin signaling in the adult fly) are increased in flies with increased octopamine signaling and decreased in flies with reduced octopamine signaling. This is not the case across all short sleeping flies though. While some sleep mutants show alterations in metabolic function, the changes are not consistent across different

sleep mutants/conditions. To address whether insulin is part of the wake-promoting pathway we examined sleep in flies with altered insulin signaling. We found that alterations in the insulin signaling pathway do not produce the dramatic decreases in total sleep seen with octopamine activation. In fact increases in *Dilp2* (*Drosophila* insulin like peptide) showed only a minor increase in total sleep. Other alterations in the insulin pathway also produced only minor changes in sleep. These data do not rule out octopamine action on insulin, but strongly support the idea that insulin is not the octopamine mediated wake signal in flies.

Methods:

The following fly strains were used in this study: UAS-*dInR CA*, UAS-*myrAKT*, UAS –*AKT RNAi* (Vienna stock collection 2907) *yolk*-GAL4, *elav*-GAL4, *elav*-GeneSwitch, MB-GeneSwitch, UAS-*mc** (constitutively active PKA), OAMB ^{286}, *Tdc2*-GAL4, UAS-*B16b* (*NaChBac* channel), UAS-*TrpA1*, sss^{p1}, *Fumin*, and Iso31 (isogenic *w1118* stock).

Flies were grown on standard cornmeal-molasses medium as described previously, and were grown at 25°C unless otherwise noted (Crocker and Sehgal, 2008). To control for background and growing conditions, crosses were performed to compare driver alone, transgene alone and animals carrying both transgene and driver. In some cases, the driver alone flies or transgene alone flies carried balancers, which could have independent effects on growth and nutrient storage; in these cases, we used separate crosses as controls. For

instance, since the OAMB^{286} mutation is maintained over a Tm6,tb, balancer we used background *w1118* flies grown in a separate vial as controls. For *TrpA1* experiments, flies were raised at 21°C, shifted to 28°C when they were 0-3d old and then tested after 4 days at 28°C to activate the channel (Hamada et al., 2008). We verified that expression of *TrpA1* by *Tdc2*-GAL4 caused loss of sleep, as expected from previous work (Crocker and Sehgal, 2008; Crocker et al., 2010). For experiments utilizing the GeneSwitch system, animals were placed on 500 uM RU486 in 1% EtOH in standard medium for 4 days to activate GeneSwitch activity.

Triglyceride assays: Adult female flies aged 4 to 5 days were taken for the following flies: OAMB^{286}, *Tdc2*-GAL4/UAS-*B16b (NaChBac)*, *Tdc2*-GAL4/UAS-*TrpA1*, *sss*P1 *(sss)*, *Fumin* (*Fmn*), MB-GeneSwitch/UAS-*mc** and respective control lines. All triglyceride assays were done as described by DiAngelo et al. (2009).(DiAngelo and Birnbaum, 2009).

Results:

Decreasing sleep in multiple ways does not lead to a similar change in metabolic function.

Since octopamine has actions on metabolism in other invertebrates and its wake-promoting actions are mediated through the insulin-producing neurons of the brain, we hypothesized that octopamine regulates metabolic function in *Drosophila*. Thus, we measured triglycerides in the fat bodies of flies with increased or decreased octopamine signaling. We found that an increase in

neuronal specific octopamine, through the use of the TrpA1 channel or a bacterial Na^+ channel driven by T*dc2*-GAL4 (GAL4 expressed in octopamine and tyramine-producing neurons), increases triglycerides (Fig. 4-1a). Conversely, flies carrying a mutation in the octopamine OAMB receptor have decreased triglycerides (Fig. 4-1a).

Since sleep deprivation is implicated in obesity and increased fat storage and flies with increased neuronal octopamine signaling sleep significantly less, it is possible that sleep loss accounts for the increased triglycerides. In order to address this issue, we examined triglycerides in other short sleeping flies. We looked at 5 different genotypes previously shown to result in decreased sleep: *Tdc2*-GAL4 driving the bacterial Na^+ channel or the *TrpA1* channel (Na^+/Ca^{2+} channel activated by heat), the *sleepless* (*sss*) flies, *Fumin (fmn)* flies, and flies expressing a constitutively active protein kinase A (PKA) transgene in the mushroom body (Crocker and Sehgal, 2008; Joiner et al., 2006; Koh et al., 2008; Kume et al., 2005). Preliminary data show that, similar to *Tdc2*-GAL4 driven expression of either excitatory channel, the *fumin* mutants showed a trends towards increases in triglycerides (Fig. 4-1a,b). However, the *sss* flies have decreased triglyceride levels. A similar trend towards decreased triglycerides was observed in the flies that express constitutively active PKA in the mushroom body. (Fig. 4-1b). Together, these data imply that the simple loss of sleep does not lead to a consistent increase or decrease in metabolic activity. We suggest that many factors that alter sleep also alter metabolism independent of sleep.

Altering insulin signaling does not alter sleep.

In *Drosophila*, as in mammals, fat storage and normal metabolic function and growth are dependent on insulin (DiAngelo and Birnbaum, 2009). In *Drosophila*, there are 7 different insulin like peptides (*dilp*) but only 3 (*dilp*s 2, 3 and 5) are found in the octopamine-sensitive PI neurons (Brogiolo et al., 2001; Broughton et al., 2010; Ikeya et al., 2002). However, there is only one receptor for all the *Dilps* in flies, known as the Drosophila Insulin Receptor (dInR) (Garofalo, 2002; Oldham and Hafen, 2003). Whether this receptor is expressed in the adult brain is controversial, but it is known to be highly expressed in an area of the fly known as the fat body (Garofalo and Rosen, 1988; Hwangbo et al., 2004). The fat body is equivalent to the mammalian liver and adipose tissue in mammals and is the major site of triglyceride storage. In flies, upregulation of the insulin-signaling pathway leads to increases growth throughout development, while the opposite phenotype is observed when the pathway is inhibited. It is thought that the main action of insulin in flies is to regulate growth throughout development, with less action in adulthood. However, insulin does cause some increase in fat cell mass in the adult stage (DiAngelo and Birnbaum, 2009).

Since octopamine has actions through the insulin-producing neurons of the brain and demonstrate increased triglyceride levels, we asked whether alterations in insulin signaling led to the changes in sleep seen with octopamine modulation. Specifically, if the decrease in sleep seen with *Tdc2*-GAL4 driving the Na^+ channel is due to increased *Dilp* protein, then increasing the insulin signaling pathway should lead to decreases in total sleep while downregulation of

this pathway should lead to increases in total sleep. To address this question, we first overexpressed *Dilp2* in *Dilp2* neurons. *Dilp2* was chosen since it has the highest homology to human insulin and its expression can rescue the growth phenotype observed by partially ablating PI neurons (Rulifson et al., 2002). Expression of *Dilp2* in the PI neurons led to a minor, non-statistically significant increase in sleep. (Fig. 4-2a).

Since *Dilp2* is very tightly regulated by nutrient conditions, we thought we might see a greater effect if we altered other parts of the insulin pathway. Thus, we expressed a constitutively active form of *dInR* in either the adult fly fat body (the center for fat storage and a major site of action of insulin in the fly) or in the brain. Previous work has shown that expression of this in the fat body leads to increases in triglyceride levels (DiAngelo and Birnbaum, 2009). In both cases (fat body and brain) we only saw minor effects on total sleep (Figure 4-2b). This was also true when we expressed an RNAi construct to decrease levels of *dAKT*, an enzyme essential for insulin signaling, in either the fat body or the brain (Fig. 4-2b). When insulin signaling is active it leads to myristylation of *AKT*. We also expressed a constitutively active form of *AKT*, that is always myristylated and targeted to the plasma membrane (*myrAKT*), in either the fat body or the brain. In the fat body this active form of *AKT* had little effect on total sleep, but in the brain we were unable to measure any effects because these flies only lived about 5 days and showed severe developmental defects (Fig. 4-2b). However, when this construct was expressed specifically in adulthood using the GeneSwitch system in the brain there was no effect on total sleep (Fig. 4-2b).

Discussion

A state of wakefulness or consciousness is necessary to sustain life, so it is reasonable to assume that many neurotransmitters and redundant anatomical pathways in the brain are devoted to this. This is true from mammals to flies where multiple pathways have been implicated in maintaining arousal. Since the brain has limited space, it is also likely that there is overlap between these pathways and pathways important for other behaviors or functions such as metabolism. Only a small number of neurons are responsible for producing the necessary neurotransmitters and so it is highly likely that when we are looking at one behavior/process we will also see an effect on another. This is important in thinking about the experiments which record activity of norepinephrine-producing neurons in the LC. These data show that during heightened arousal there is increased firing of these cells (Sara, 2009). Since we do not know the entire connectivity of the LC, we have to assume that these cells project to more than just the wake-promoting areas of the brain. In fact, the LC is known to activate the stress pathway and coordinate the brain's response to stress (Goddard et al.). Thus, it is likely that in human sleep deprivation studies there are effects of norepinephrine on the stress pathway in addition to the arousal pathway. Similarly in flies, overlapping circuitry may be responsible for the diverse phenotypes caused by sleep loss. The preliminary and ongoing studies reported

in this chapter on the role of octopamine signaling in the fly may shed new light on the intricate workings of the LC in mammals.

Here we addressed whether sleep and metabolism are dependent or independent when they converge on a similar cell type. We find that the wake-promoting and metabolic roles of the PI neurons are independent of each other. While *Dilp2* neurons are activated by octopamine to promote wakefulness, the effect seems to be independent of Dilp2*,* as manipulating downstream insulin signaling in the brain or the fat body does not have major effects on sleep. In addition it is not the increase in wakefulness that causes changes in triglycerides in these animals since other short sleepers do not show the same increases in triglycerides. This result implies that octopamine has its own independent action on triglycerides through a currently unknown mechanism. The mechanism may still involve *Dilp2*, since we have not shown any data to the contrary. This action is most likely strongest during development, because increasing octopamine signaling in adulthood (with TrpA1) led to less fat storage than if the octopamine pathway was activated throughout development. Ongoing studies are addressing whether the effect of octopamine on triglyceride levels is insulin dependent or not.

Recent work on insulin in the fly suggests that it is a sleep-promoting molecule (Broughton et al., 2010). We find that there is a trend towards increases in total sleep, but these are very minor and most likely not biologically relevant in adult flies. This leaves the question of what in these *dilp*-producing neurons is the wake-promoting signal. We show here that it is not *Dilp2*. We

have not ruled out *dilp*3 and *dilp*5, which are also expressed in these neurons, but these are also unlikely signals as they would act through the same downstream insulin signaling which is not affected by changes in octopamine. A more in depth characterization of these neurons will be needed to address this question. Finally, metabolic signaling has also been implicated in homeostatic sleep rebound (the ability of an animal to recover sleep lost during a period of deprivation) (Chikahisa et al., 2009; Peterfi et al., 2006). This aspect of metabolic control of sleep was not addressed in this chapter and may also be a product of overlapping circuitry.

Figure 4-1

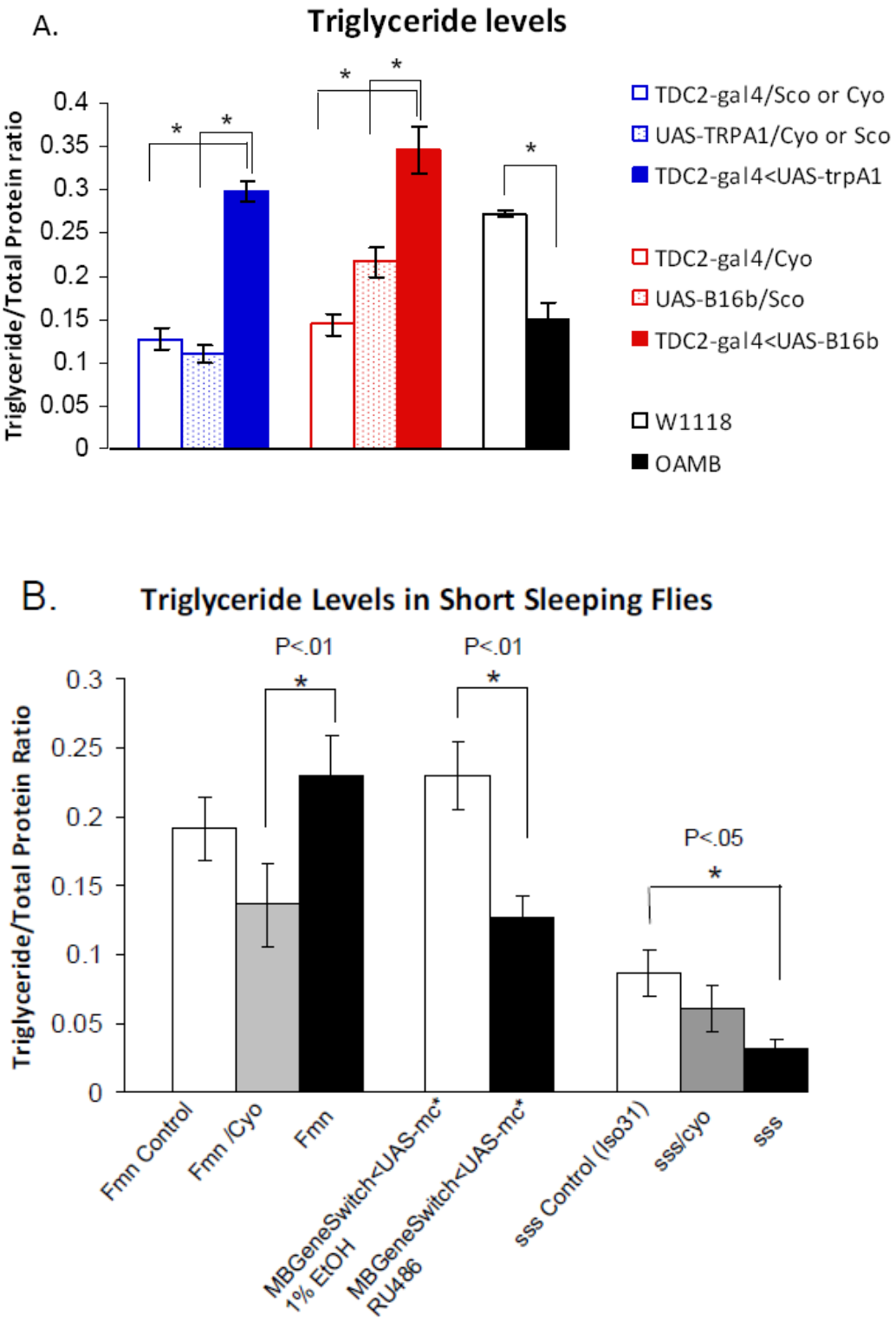

A.
Triglyceride levels
Triglyceride/Total Protein ratio
0.4
0.35
0.3
0.25
0.2
0.15
0.1
0.05
0
TDC2-gal4/Sco or Cyo
UAS-TRPA1/Cyo or Sco
TDC2-gal4<UAS-trpA1
TDC2-gal4/Cyo
UAS-B16b/Sco
TDC2-gal4<UAS-B16b
W1118
OAMB
B.
Triglyceride Levels in Short Sleeping Flies
Triglyceride/Total Protein Ratio
P<.01
P<.01
P<.05
0.3
0.25
0.2
0.15
0.1
0.05
0
Fmn Control
Fmn /Cyo
Fmn
MBGeneSwitch<UAS-mc* 1% EtOH
MBGeneSwitch<UAS-mc* RU486
sss Control (Iso31)
sss/cyo
sss

Figure 4-1: Triglyceride levels in different sleep mutants. A. Triglyceride levels following alterations in octopamine signaling. Triglyceride/ protein ratio of female whole animals 4-5 days old from: *Tdc2*-GAL4<UAS-TrpA1, and its control lines: *Tdc2*-GAL4/Cyo or Sco and UAS-trpA1/ Cyo or Sco; *Tdc2*-GAL4<UAS-*NaChBac*, and its controls: *Tdc2*-GAL4/Cyo and UAS-*NaChBac*/ Sco; and $OAMB^{286}$ mutants and *w1118* background control line. N>12 for each line for total experiment. B. Triglyceride levels in short sleeping flies. Triglyceride/ protein ratio of whole animals from: *Fmn* control lines (*w1118*) (n>16), *Fmn* (n>16) and *Fmn* heterozygous animals over *Cyo* from the same vial (n>16); MBGeneSwitch driving UAS-*mc** (constitutively active PKA) with or without RU486 (n=5) ; *SSS* control lines (*Iso31*) (n=3), *SSS* (n=4) and *SSS* heterozygous animals over *Cyo* from the same vial (n=4). Error bars represent ± SEM. * = p<.01 unless stated otherwise.

Figure 4-2:

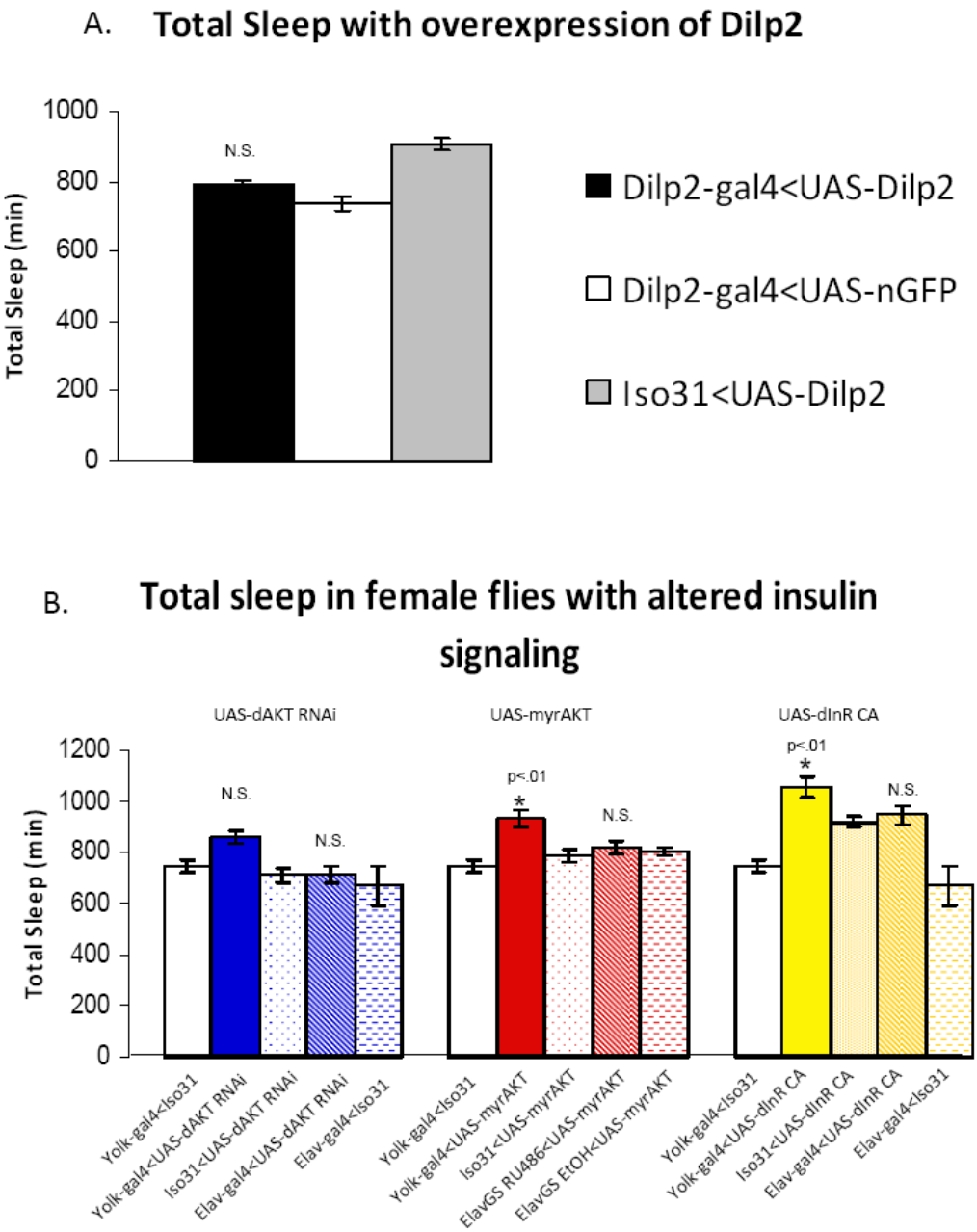


Figure 4-2: Baseline sleep in flies with altered insulin signaling. A. Average total sleep over 24 hr period in female animals overexpressing UAS-*Dilp2* in *Dilp2* neurons, compared to *Dilp2* driver alone (*Dilp2*-GAL4 < UAS-*nGFP*) and UAS-*Dilp2* alone. N=32 for each group. B. Total sleep in female flies expressing UAS-

dAKT RNAi, UAS-*myrAKT* and UAS-*dInRCA*. Total sleep in UAS-*dAKT* RNAi expressed in Fat body (*yolk*-GAL4) and brain (*elav*-GAL4) compared to controls *yolk*-GAL4<Iso31, *elav*-GAL4<Iso31 and Iso31<UAS-*dAKT* RNAi (n=32 for each group). Total sleep in UAS-*myrAKT* expressed in Fat body (*yolk*-GAL4) and brain during adulthood (*Elav*GeneSwitch activated by RU486 in 1%EtOH for 4 days) compared to controls *yolk*-GAL4<Iso31, Iso31<UAS-*myrAKT*, *Elav*GeneSwitch < UAS-*myrAKT* on 1% EtOH. (n=32 for each group). Total sleep in UAS-d*InRCA* expressed in Fat body (*yolk*-GAL4) and brain (*elav*-GAL4) compared to controls *yolk*-GAL4<Iso31, *elav*-GAL4<Iso31 and Iso31<UAS-d*InRACA* (n=32 for each group). Error bars represent ± SEM. * = p<.01 unless stated otherwise.

Appendix:

Other regulators of *Drosophila* sleep

Part 1: Sleep in *Drosophila* is regulated by adult mushroom bodies

William J. Joiner, Amanda Crocker, Benjamin H. White and Amita Sehgal

Joiner, W.J., Crocker, A., White, B.H., and Sehgal, A. (2006). Sleep in Drosophila is regulated by adult mushroom bodies. Nature 441, 757-760

Part 2: Caffeine changes global levels of cAMP

Extracted from Wu, M.N., Ho, K., Crocker, A., Yue, Z., Koh, K., and Sehgal, A. (2009). The effects of caffeine on sleep in Drosophila require PKA activity, but not the adenosine receptor. J Neurosci *29*, 11029-11037.

Part 1: Sleep in *Drosophila* is regulated by adult mushroom bodies

William J. Joiner, Amanda Crocker, Benjamin H. White & Amita Sehgal

Sleep is one of the few major whole-organ phenomena for which no function and no underlying mechanism have been conclusively demonstrated. Sleep could result from global changes in the brain during wakefulness or it could be regulated by specific loci that recruit the rest of the brain into the electrical and metabolic states characteristic of sleep. Here we address this issue by exploiting the genetic tractability of the fruit fly, *Drosophila melanogaster*, which exhibits the hallmarks of vertebrate sleep (Hendricks et al., 2000a; Nitz et al., 2002; Shaw et al., 2000; van Swinderen and Greenspan, 2003). We show that large changes in sleep are achieved by spatial and temporal enhancement of cyclic-AMP-dependent protein kinase (PKA) activity specifically in the adult mushroom bodies of *Drosophila*. Other manipulations of the mushroom bodies, such as electrical silencing, increasing excitation or ablation, also alter sleep. These results link sleep regulation to an anatomical locus known to be involved in learning and memory.

Sleep duration is inversely related to PKA activity in flies (Hendricks et al., 2001). To determine whether specific brain loci regulate sleep, we used the GAL4/UAS (upstream activating sequence) system (Brand and Perrimon, 1993) to express a catalytic subunit of PKA in various regions of the fly brain. We first expressed PKA under the control of the RU486-inducible pan-neuronal driver *elav*GeneSwitch (Osterwalder et al., 2001). Restricting the expression of PKA to adult neurons decreased daily sleep, supporting earlier studies with mutants such as *dunce* (Hendricks et al., 2001) that increase PKA levels, and showing that PKA directly regulates sleep rather than a developmental process that might affect sleep (Fig. A-1a). We next expressed PKA under the control of different GAL4 drivers and examined the changes in total daily sleep in the different driver/transgene combinations relative to driver/background and background/transgene controls (Fig. A-1b). When both controls were taken into account, the expression of PKA by only two drivers led to changes in sleep that exceeded 2 s.d. These were 201Y, which increased sleep by 75 ± 3% and 93 ± 4% respectively, and c309, which decreased sleep by 46 ± 11% and 43 ± 14% per day compared with the two sets of controls. Changes in sleep caused by all other GAL4 drivers remained within 1s.d. of the mean.

We next examined whether activity levels during wake periods were affected by the 201Y and c309 drivers. Many GAL4 driver/UAS–PKA lines were hypoactive, but line 201Y had normal waking activity (Fig. A-1c). Similarly, activity normalized to waking time in c309 was not significantly higher in PKA-driven animals than in either control (Fig. A-1c), indicating that c309 was not

hyperactive. We conclude that the sleep phenotypes of animals expressing PKA under control of the 201Y and c309 drivers are not associated with abnormal waking activity. Interestingly, both these drivers are known to be expressed in the mushroom bodies (MBs) (Joiner and Griffith, 1999), a brain region implicated in associative learning.

Given the strong, yet opposite, effects that 201Y and c309 had on sleep, we further characterized their expression patterns in the fly brain by crossing them into animals bearing a UAS transgene for green fluorescent protein (GFP). We found that 201Y is expressed largely in the γ lobes and the core region of the α/β lobes of the MBs, whereas c309 is expressed in the α/β and γ lobes but not in the core region of the α/β lobes (Fig. A-2a, b, and Fig. A-3). This differential expression pattern within the MBs indicates that PKA might affect the regulation of sleep by the MBs in both a positive and a negative fashion by using anatomically distinct classes of neurons. Consistent with this notion of heterogeneous cell types within the MBs (Armstrong et al., 1998; Crittenden et al., 1998; Zhu et al., 2003), some MB drivers, such as 30Y and 238Y, promoted sleep during the day but inhibited sleep during the night, leading to only marginal overall changes in daily sleep (Fig. A-1, and Fig. A-4). This effect was not observed with any driver that was expressed exclusively outside the MBs (Fig. A-2c and Table A-1). A small increase in daytime sleep was also frequently produced by the pan-neuronal elavGeneSwitch driver, which decreased overall sleep (data not shown, and Fig. A-1a). The expression patterns of 238Y and 30Y overlap those of 201Y and c309, supporting the idea that 238Y and 30Y are

expressed in both sleep-promoting and sleep-inhibiting areas (see Table A-1 and Fig. A-3 for expression patterns of drivers used in this study).

To test the hypothesis that PKA expression in MBs regulates adult sleep, we expressed the PKA transgene under the control of an RU486-activatable MB GAL4 driver, P{MB-Switch} (Mao et al., 2004). We confirmed selective expression of this driver in the MBs by coupling it to a GFP reporter, and found inducible expression in the MBs (Fig. A-2d, e, and Fig. A-3). Sleep was significantly reduced in response to RU486 in MB-Switch/PKA animals (Fig. A-5a, b) but was unaffected by the drug in control animals harboring either the driver or the transgene alone (Fig. A-5c). Thus, PKA overexpressed preferentially in specific neurons of adult MBs is sufficient to reduce sleep.

Next we compared sleep structure in the hyposomnolent animals with that of controls. In both MB-Switch/PKA animals and c309/PKA animals, loss of sleep was caused by a shortened sleep bout duration without a concomitant increase in the sleep bout number (Fig. A-5d, e, and Fig. A-6a, b). The underlying cause of reduced sleep in both sets of animals therefore seems to be impaired sleep need, because the alternative—normal sleep need, but an inability to maintain the sleep state—would be expected to produce an increase in sleep bout number. In contrast, in 201Y sleep bout duration remained unchanged (Fig. A-6c, d).

We then asked whether the reduction of sleep in MB-Switch/PKA animals was due to an impaired accrual of a sleep-inducing signal. If this were so, then a hallmark of sleep, homeostatic rebound—sleep that exceeds baseline to

compensate for lost sleep—should not occur on relief of induced PKA expression. However, when RU486 was withdrawn after about three days of sleep deprivation, an average rebound of 156 ± 38 min was observed (Fig. A-5a, f). This is a robust rebound, comparable to that produced when genetically identical but uninduced flies were submitted to a standard 12h of mechanical deprivation (137 ± 26 min; Fig. A-7). Behavioral rebound was also observed in animals expressing elavGeneSwitch-driven PKA, after withdrawal of RU486 (data not shown), and was accompanied by a decrease in PKA activity in fly heads (Fig. A-5g). Rebound after withdrawal of RU486 indicates that PKA might not prevent the accrual of sleep-promoting signals but might suppress homeostatic output.

To determine whether PKA affects sleep by regulating synaptic output in MB neurons, we inducibly expressed either of two K^+ channels, Kir2.1 (Baines et al., 2001) or *EKO* (White et al., 2001), under the control of the MB-Switch driver. Such transgenic expression should suppress action-potential firing by hyperpolarizing neurons and decreasing membrane resistance, thus leading to reduced synaptic transmission. We found that induction of either Kir2.1 or *EKO* caused a significant increase in sleep (Fig. A-8a, b, and Fig. A-9). Because the opposite was observed with PKA expression in the same neurons, it indicates that PKA might decrease sleep by increasing either excitability or synaptic transmission. To address this issue further, we inducibly expressed a sodium channel (*NaChBac*), which depolarizes neurons and increases excitability (Luan et al., 2006). When expressed under the control of the MB-Switch driver, the

sodium channel caused a decrease in sleep (Fig. A-8c, d), similar to that produced by PKA, confirming that PKA increases the output of these neurons.

The MB-Switch driver is expressed in a subpopulation of MB neurons similar to those labeled by c309 (Fig. A-3), and both drivers had sleep-inhibiting effects. As noted above, this pattern of expression differed from that of other drivers (Fig. A-3), which had sleep-promoting or bidirectional effects on sleep (Fig. A-1b, and Fig. A-4), thus leading us to propose that the MBs contain sleep-inhibiting and sleep-promoting neurons. To determine the overall effect of MBs on sleep, we ablated them with hydroxyurea and examined sleep and activity in adult flies. Consistent with previous reports (Helfrich-Forster et al., 2002; Martin et al., 1998) was our observation of an overall increase in activity (Fig. A-10a). However, normalization of this activity to waking time indicates that the phenotype derives less from hyperactivity than from a reduction in sleep (Fig. A-10b, c). Even so, the reduction in sleep was much less than that seen with other manipulations of the MBs (see above) or in short-sleep mutants such as *minisleep* (Cirelli et al., 2005a). This supports our conclusion that MBs exert both positive and negative influences on sleep that are integrated to produce the overt behavioral state. Our model (Fig. A-11) takes into account our results; notably the integrator downstream of the MBs promotes activity in the default state. Thus, when MBs are ablated the overall effect is increased wakefulness.

Opposing effects of the c309 and 201Y drivers are also observed in a different behavioral model. They parallel MB-dependent changes in brain activity during the sleep/wake cycle that are associated with salience, a behavioral trait

that may correspond to arousal. Consistent with our data was the observation that reducing synaptic transmission using the c309 driver inhibited salience, whereas the 201Y driver in the same type of experiment yielded no change. We would predict increased arousal with 201Y, but in those experiments the animals were already awake (van Swinderen and Greenspan, 2003).

Because MBs receive and transduce considerable sensory, particularly olfactory, input to the fly brain, we speculate that they promote arousal or sleep by allowing or inhibiting the throughput of sensory information. In addition, given the major function that MBs have in regulating plasticity in the fly brain, it is likely that this is linked to their role in sleep. In mammals, sleep deprivation suppresses the performance of learned tasks, and sleep permits memory consolidation (Walker and Stickgold, 2004). Sleep and sleep deprivation also differentially affect cortical synaptic plasticity (Frank et al., 2001). In *Drosophila*, MBs participate in the consolidation or retrieval of memories involving olfactory cues (de Belle and Heisenberg, 1994; Dubnau et al., 2001; McGuire et al., 2001; Zars et al., 2000), courtship conditioning (Joiner and Griffith, 1999; McBride et al., 1999) and context-dependent visual cues (Liu et al., 1999; Tang and Guo, 2001) by mechanisms that include cAMP signaling. Distinct anatomical regions of the MBs have been shown to be important for at least some forms of memory (McGuire et al., 2001; Pascual and Preat, 2001), as we have now also shown for sleep. Thus, memory and sleep may involve similar molecular pathways (cAMP signaling) and anatomical regulatory loci (MBs).

METHODS:

Sleep assays

Female flies 3–7 days old were placed in 65mm × 5mm glass tubes containing 5% sucrose/2% agarose with or without 500µM RU486. Flies were acclimated for about 36h at 25°C in 12h light/12h dark (LD) conditions. Locomotor activity was collected in LD with DAMS monitors (Trikinetics) as described previously (Hendricks et al., 2000a; Hendricks et al., 2001). Sleep was measured as bouts of 5min of inactivity, as described previously (Huber et al., 2004), using a moving window over 30-s intervals. In some experiments RU486 was removed at the time points indicated in figure legends.

Localization of GAL4 expression

Many of the GAL4 lines from Fig. A-1 were crossed to a fly line with a transgene encoding GFP fused to a nuclear localization signal (GFPn). Brains were dissected and fixed for 20–30min in 4% paraformaldehyde in PBS before photography of serial *z*-sections of whole mounts with the use of confocal microscopy. In some cases, images were deconvolved to allow virtual transverse sections to be taken through MB peduncles.

PKA assays

After 1 week in LD, elav-GeneSwitch/UAS-mc* females were switched at ZT0 from drug to drug, from no drug to no drug or from drug to no drug. After 2h, heads were isolated and homogenized on ice in buffer consisting of (in mM): 10 HEPES pH7.5, 100 KCl, 1 EDTA, 5 dithiothreitol, 5 phenylmethylsulphonyl fluoride, 10% glycerol, 0.1% Triton X-100 and one tablet of Complete (Promega). Debris was pelleted for 5min at 4°C in a microcentrifuge and discarded. A protein

assay (Dc kit; Bio-Rad) was performed on supernatant from each sample, and homogenization buffer was added as necessary to make each sample equal in concentration. PKA activity was measured as ATP-dependent quenching of fluorescent kemptide in accordance with the manufacturer's instructions (IQ assay; Pierce) and normalized to values determined for animals maintained on RU486 throughout the experiment.

Hydroxyurea-dependent ablation of MBs

Mated Canton-S flies were placed on standard grape juice/agar plates to induce egg-laying over a 1-h period. On the next day, first-instar larvae were collected within 1h of hatching and placed in 50% yeast paste in the presence or absence of 50mg/ml hydroxyurea for 4h before being transferred to standard food vials, as described previously (de Belle and Heisenberg, 1994). After eclosion, adult male flies were aged for one week and then placed in glass tubes to measure sleep/activity patterns over a three-day period in LD.

Figure A-1

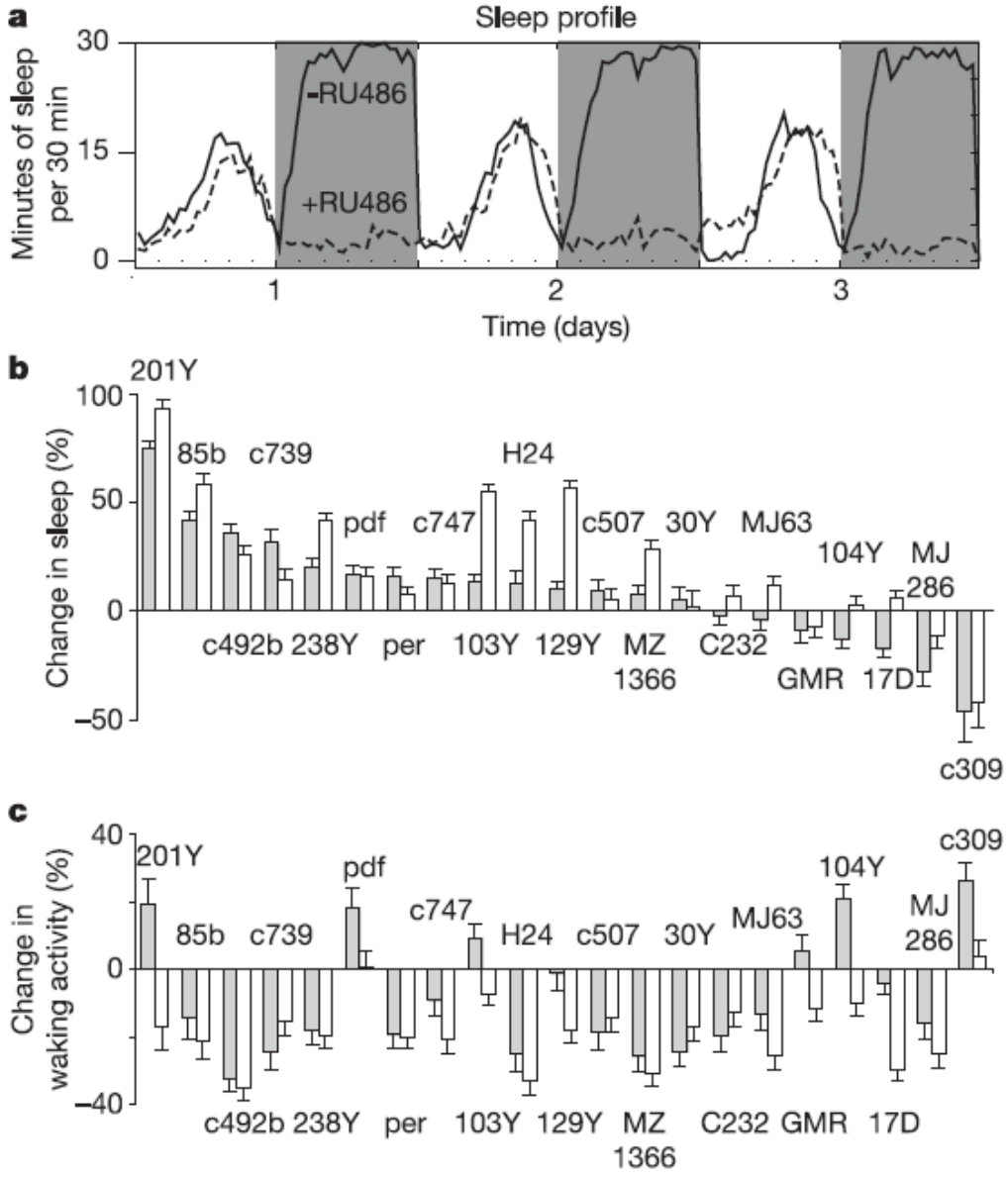


Figure A-1: Expression of PKA in various regions of the fly brain affects sleep differentially. a, The pan-neuronal driver elavGeneSwitch7 was used to express UAS-mc*, which contains a constitutively active subunit of PKA downstream of an upstream activating sequence (UAS)30. Sleep, defined as 5 min of immobility,

was assayed in flies in which expression of PKA was induced with 500 mMRU486 (dashed line) and in uninduced controls (solid line). b, Progeny were collected from crosses between 21 GAL4 drivers and y w;UAS-mc*. For controls, each GAL4 line was crossed to y w flies (the background for the UAS-transgenic line), and yw;UAS-mc* was crossed to w1118 (the background for the GAL4 drivers). Daily sleep was averaged over 4 days. Sleep from control animals was subtracted from the experimental group, then divided by the amount of control sleep to calculate net percentage changes. Grey bars, sleep in w1118/yw flies carrying UAS-mc* and a GAL4 transgene relative to sleep in w1118/y w flies carrying the respective GAL4 transgene alone; white bars, sleep in w1118/y w flies carrying UAS-mc* and a GAL4 transgene relative to sleep in w1118/y w flies carrying only UAS-mc*. c, For each of the crosses in b, average daily activity (beam crossings) was divided by average total wake time. Differences in waking activity between GAL4/UAS-mc* and control flies were calculated as in b. For each group, n = 25. Where errors are shown, they are s.e.m.

Figure A-2

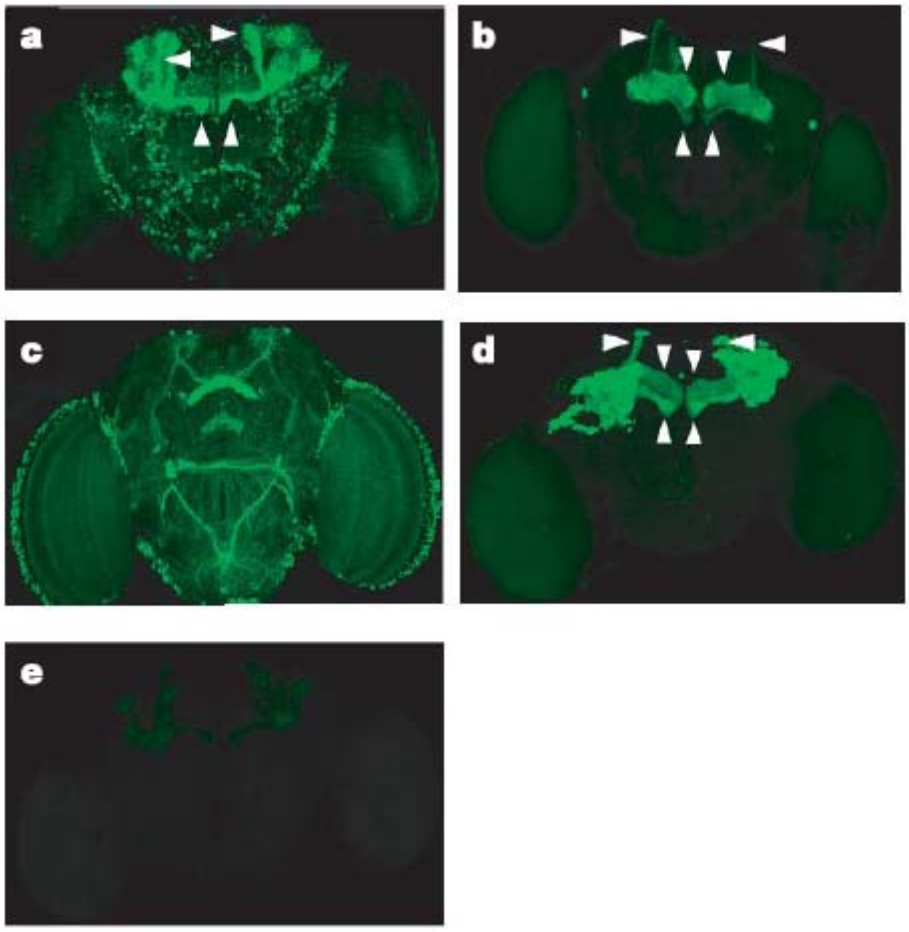
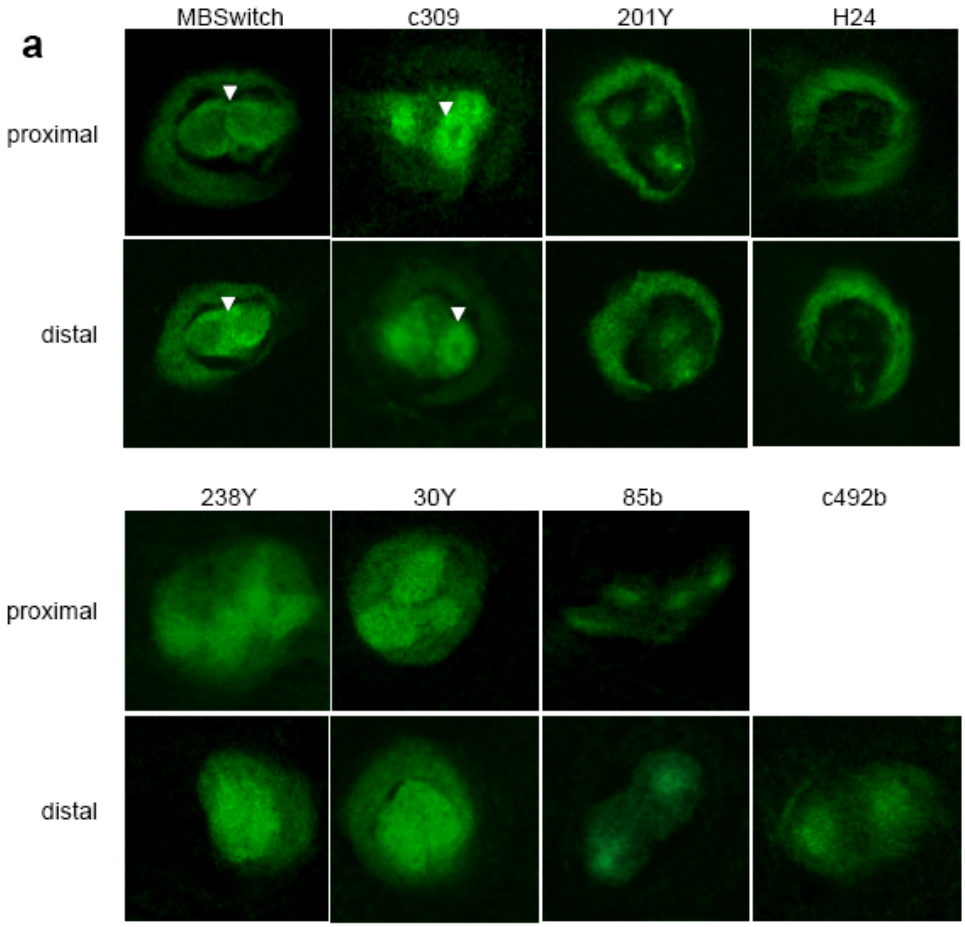
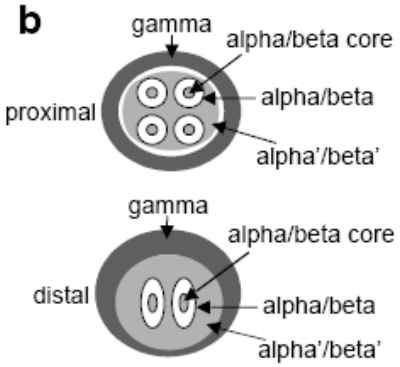


Figure A-2: Localization of GAL4-dependent brain expression. A nuclear targeted GFP (GFPn) was expressed under the control of different GAL4 drivers. a, c309/GFPn; upper arrows refer to a lobes and lower arrows to b lobes of MBs. b, 201Y/GFPn; upper, middle and lower arrows point to a, g and b lobes, respectively, within MBs. c, 104Y/GFPn; the fan-shaped body of the central complex and cells rimming the optic lobes and anterior brain are illuminated. d,

P{MB-Switch}/GFPn with RU486 in the food; labeling is as in b. e, P{MB-Switch}/GFPn without RU486 in the food.

Figure A-3





c

	Expression pattern within the MBs		
Driver	A/B-A'/B'	Core	Gamma
MBSwitch	++	+/-	+
c309	++	-	+
201Y	+/-	++	++
H24	+/-	-	++
238Y	++	++	+
30Y	++	++	+
85b	+	++	+/-
c492b	+	++	+/-

Sehgal Fig S1

Figure A-3: (Figure S1). Expression patterns of eight GAL4 drivers within the MB peduncle. (a) Expression of a nuclear-targeted GFP reporter was driven by each of eight MB GAL4 drivers. Confocal images of brain whole mounts were deconvoluted, and virtual cross-sections were reconstructed through the MB peduncle proximal and distal to Kenyon cell bodies. See (b) for a schematic representation of the structures labeled. Based upon the plane of the section and proximity to the Kenyon cell bodies, all four fiber tracts may not be visible in the proximal sections. Examples of alpha/beta expression are denoted by arrowheads in the MB-Switch and c309 panels. Note that the core region within each tract shows at best weak expression in MB-Switch and appears absent in c309. (b) Schematic of MB peduncle anatomy proximal and distal to Kenyon cell bodies[1]. Axons extend from Kenyon cell bodies through the peduncle, where they form four main tracts. Proximal to the Kenyon cell bodies these can be identified in transverse sections of the peduncle as: four bundles of fibers that extend to the core region of alpha/beta lobes, four groups of fibers surrounding each of the core bundles that also extend to alpha/beta lobes, a diffuse group of fibers surrounding the previous two groups that extend to the alpha'/beta' lobes, and an outer ring of fibers that extends to the gamma lobes. Distal to Kenyon cell bodies the four tracts of core and alpha/beta fibers merge into two groups, and fibers destined for the alpha'/beta' and gamma lobes become more distinguishable in cross-sections of the peduncle[1]. (c) Summary of the expression patterns of MB GAL4 drivers. Expression patterns are denoted with "++" for high levels of fluorescence, "+" for moderate levels of fluorescence, "+/-" for low to

barely detectable levels of fluorescence and "-" for no detectable fluorescence. Since alpha/beta and alpha'/beta' fibers could not always be distinguished, we have grouped them here for simplicity. Expression of 201Y in the alpha/beta core has previously been documented[2]. Lack of expression of c309 in the core region has also previously been described[3]. The pan-neuronal elavGeneSwitch driver (Fig A-1) also expressed weakly in the core region and strongly in alpha/beta-alpha'/beta' fibers (data not shown).

1. Strausfeld, N.J., Sinakevitch, I. & Vilinsky, I. The mushroom bodies of Drosophila melanogaster: an immunocytological and golgi study of Kenyon cell organization in the calyces and lobes. *Microsc Res Tech*. **62**, 151-69 (2003).
2. Wang, J., Zugates, C.T., Liang, I.H., Lee, C.H. & Lee, T. Drosophila Dscam is required for divergent segregation of sister branches and suppresses ectopic bifurcation of axons. *Neuron* **14**, 559-71 (2002).
3. Connolly, J.B., Roberts, I.J., Armstrong, J.D., Kaiser, K., Forte, M., Tully, T. & O'Kane, C.J. Associative learning disrupted by impaired Gs signaling in Drosophila mushroom bodies. *Science* **20**, 2104-7 (1996).

Figure A-4:

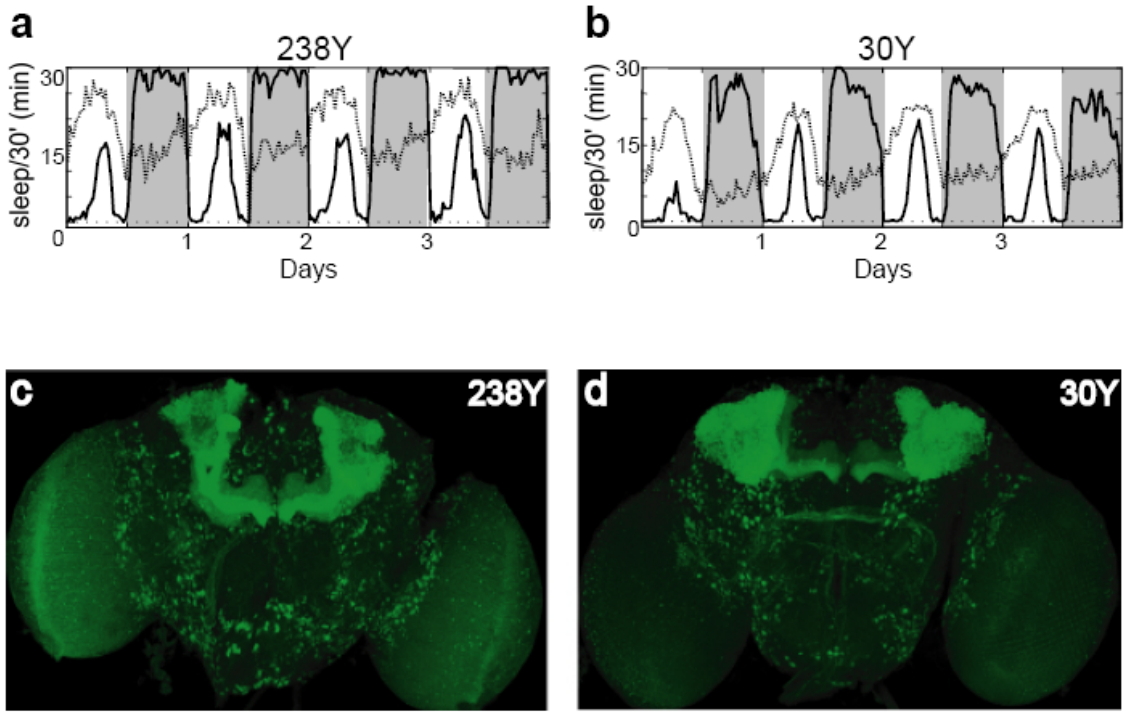


Figure A-4:(Figure S2.) Expression of constitutively active PKA with the 238Y or 30Y GAL4 drivers leads to suppression of sleep at night and enhancement of sleep during the day. (a,b) 238Y/UAS-mc*, 30Y/UAS-mc* and control driver/*y w* females were raised as in Fig A-1. Sleep profiles are means of n=15-16 animals. Data from experimental groups and controls are represented by stippled and solid lines, respectively. (c,d) GAL4 expression profiles from 5-9 day old females carrying both the driver and UAS-GFPn transgene. Brains were prepared as in Fig A-2.

Figure A-5

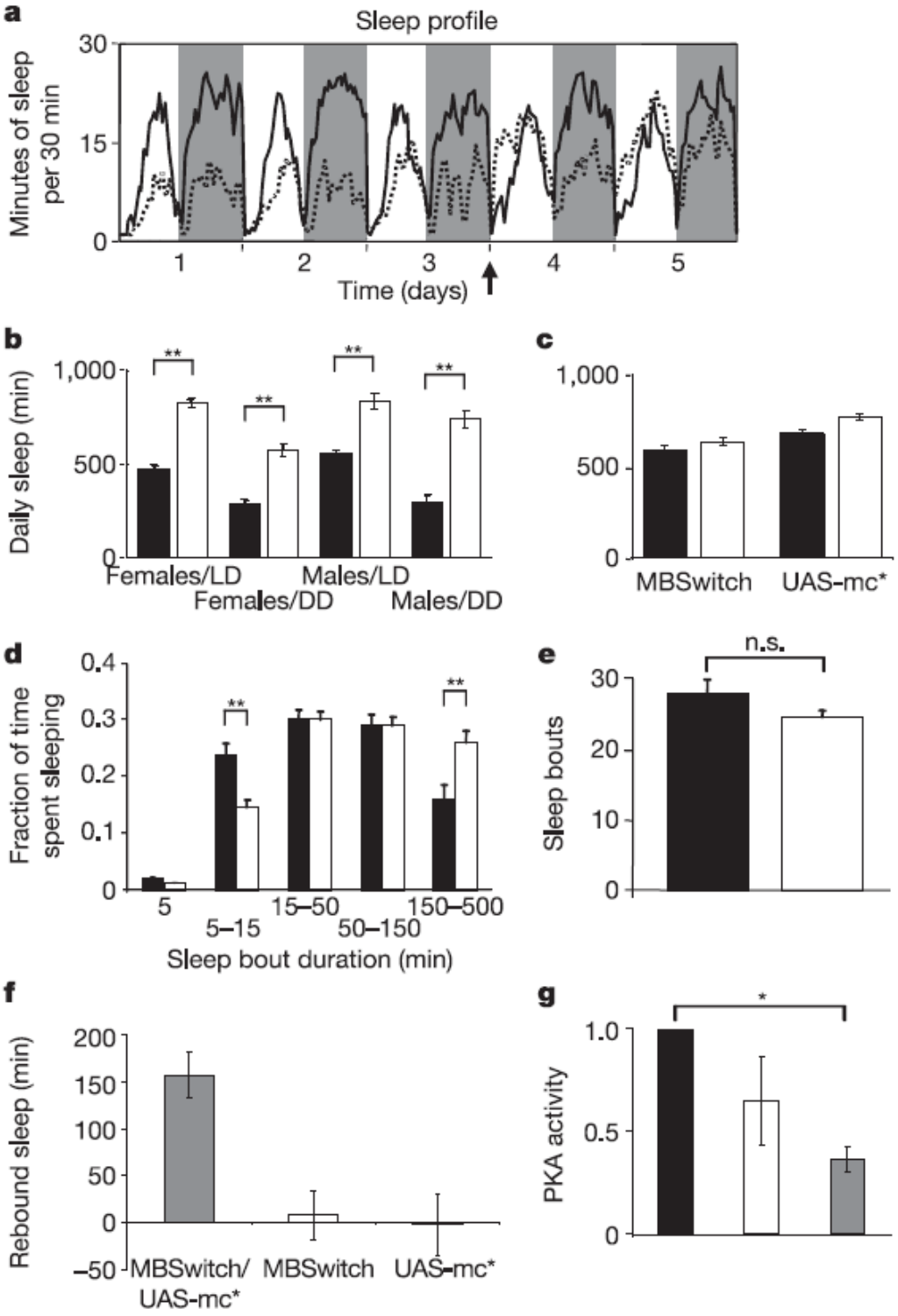

a
Sleep profile
Minutes of sleep per 30 min
30
15
0
1
2
3
4
5
Time (days)
b
Daily sleep (min)
1,000
500
0
**
Females/LD
Females/DD
Males/LD
Males/DD
c
1,000
500
0
MBSwitch
UAS-mc*
d
Fraction of time spent sleeping
0.4
0.3
0.2
0.1
0
5
5–15
15–50
50–150
150–500
Sleep bout duration (min)
e
n.s.
Sleep bouts
30
20
10
0
f
Rebound sleep (min)
200
150
100
50
0
–50
MBSwitch/ UAS-mc*
MBSwitch
UAS-mc*
g
*
PKA activity
1.0
0.5
0

Figure A-5: Inducible expression of PKA in the MBs leads to decreased sleep bout duration and accumulation of sleep debt. a, Sleep was monitored in w1118/y w;UAS-mc*/+;P{MB-Switch}/+ females in the presence (dashed line) or absence (solid line) of 500 mM RU486 (n = 16 for each group). At the end of day 3, animals were transferred to tubes lacking RU486 (arrow). Grey bars represent 12-h dark periods. b, Sleep was measured for females in LD (n = 61 or 62) and in constant darkness (DD, n = 28–30), and for males in LD (n = 45 or 46) and in DD(n = 15 or 16). Black bars represent animals fed RU486; white bars are controls. Plotted values represent averages over three days. c, w1118/yw;;P{MB Switch}/þ or w1118/y w;UAS-mc*/+ female controls were placed in LD for 3 days with (black bars) or without (white bars) RU486 in their food. d, Sleep bouts in LD were binned according to duration for P{MB-Switch}/UAS-mc* females with (black bars) or without (white bars) RU486 (n = 43–45 for each group). e, Sleep bout number in LD is similar for P{MB-Switch}/UAS-mc* females treated with RU486 (black bars) to that in genotypically identical uninduced animals (white bars). n = 43–45 for each group. f, Flies were monitored for four days in the presence or absence of 500 mMRU486 and then transferred to tubes with no drug at diurnal time 0 (ZT0; when the light was turned on). Rebound sleep during the following 12 hours was determined by subtracting sleep in the ‘no drug to no drug’ group from sleep in ‘drug to no drug’ animals. n = 64 for w1118/y w;UAS-mc*/+;P{MB-Switch}/+ experimental animals; n =30 for w1118/yw;;P{MB Switch}/+ and w1118/yw ;UAS-mc*/+ controls. g, elav- GeneSwitch/UAS-mc* females were switched at ZT0 from drug to drug (black bar), from no drug to no

drug (white bar) or from drug to no drug (that is rebound conditions, grey bar). After 2 h, PKA activity was measured in heads from each group. Significance was determined with a one-way analysis of variance. Asterisk, P ≤0.05; two asterisks, p ≤ 0.01; n.s., p . 0.05 by unpaired t-test. Where errors are shown, they are s.e.m.

Figure A-6:

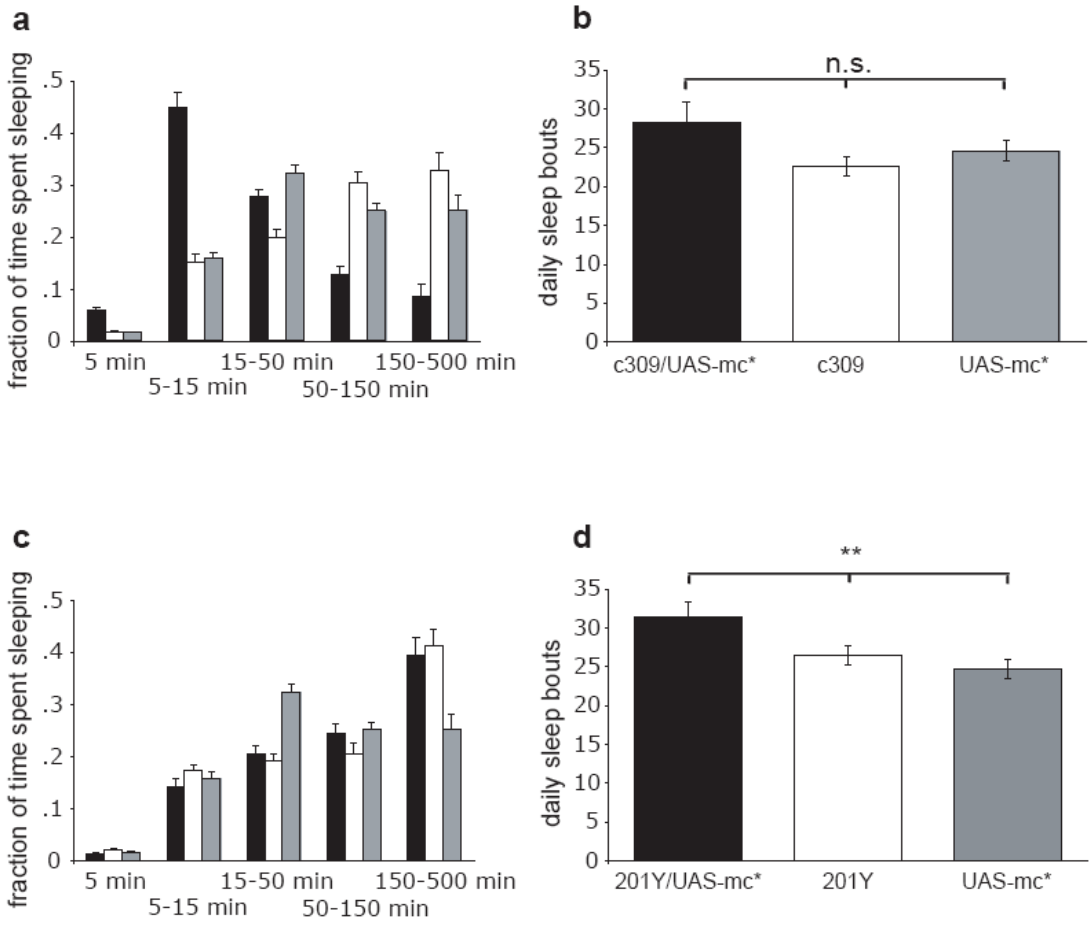


Figure A-6: (Figure S3). Sleep architecture of animals expressing PKA under the control of c309 and 201Y drivers. Animals were prepared as in Fig A-1 and analyzed as in Figs A-5d,e. (a) Sleep bout distribution shifts leftward to reflect overall shorter sleep bouts in c309/UAS-mc* female flies compared to controls. Black bars represent w^{1118}/*y w*;c309/UAS-mc*; white bars represent w^{1118}/*y w*;c309/+; grey bars represent w^{1118}/*y w*;UAS-mc*/+. (b) Average daily number of sleep bouts is similar between w^{1118}/*y w*;c309/UAS-mc* (black bar), w^{1118}/*y w*;c309/+ (white bar) and w^{1118}/*y w*;UAS-mc*/+ (grey bar). (c) Sleep bout

distribution in 201Y/UAS-mc* female flies compared to controls. Black bars represent w^{1118}/*y w*;201Y/UAS-mc*; white bars represent w^{1118}/*y w*;201Y/+; grey bars represent w^{1118}/*y w*;UAS-mc*/+. (d) Average daily number of sleep bouts in w^{1118}/*y w*;201Y/UAS-mc* (black bar) compared to w^{1118}/*y w*;201Y/+ (white bar) and w^{1118}/*y w*;UAS-mc*/+ (grey bar) controls. N = 30-61 female flies in each group. All calculations are plotted as averages $\pm$ SEM. n.s. is not significant and ** p<.01 by 1-way ANOVA. In the case of the latter, posthoc comparison between w;201Y/UAS-mc* and w^{1118}/*y w*; UAS-mc*/+ showed a significant difference while the difference between w;201Y/UAS-mc* and w^{1118}/*y w*;201Y/+ was marginally insignificant.

Figure A-7:

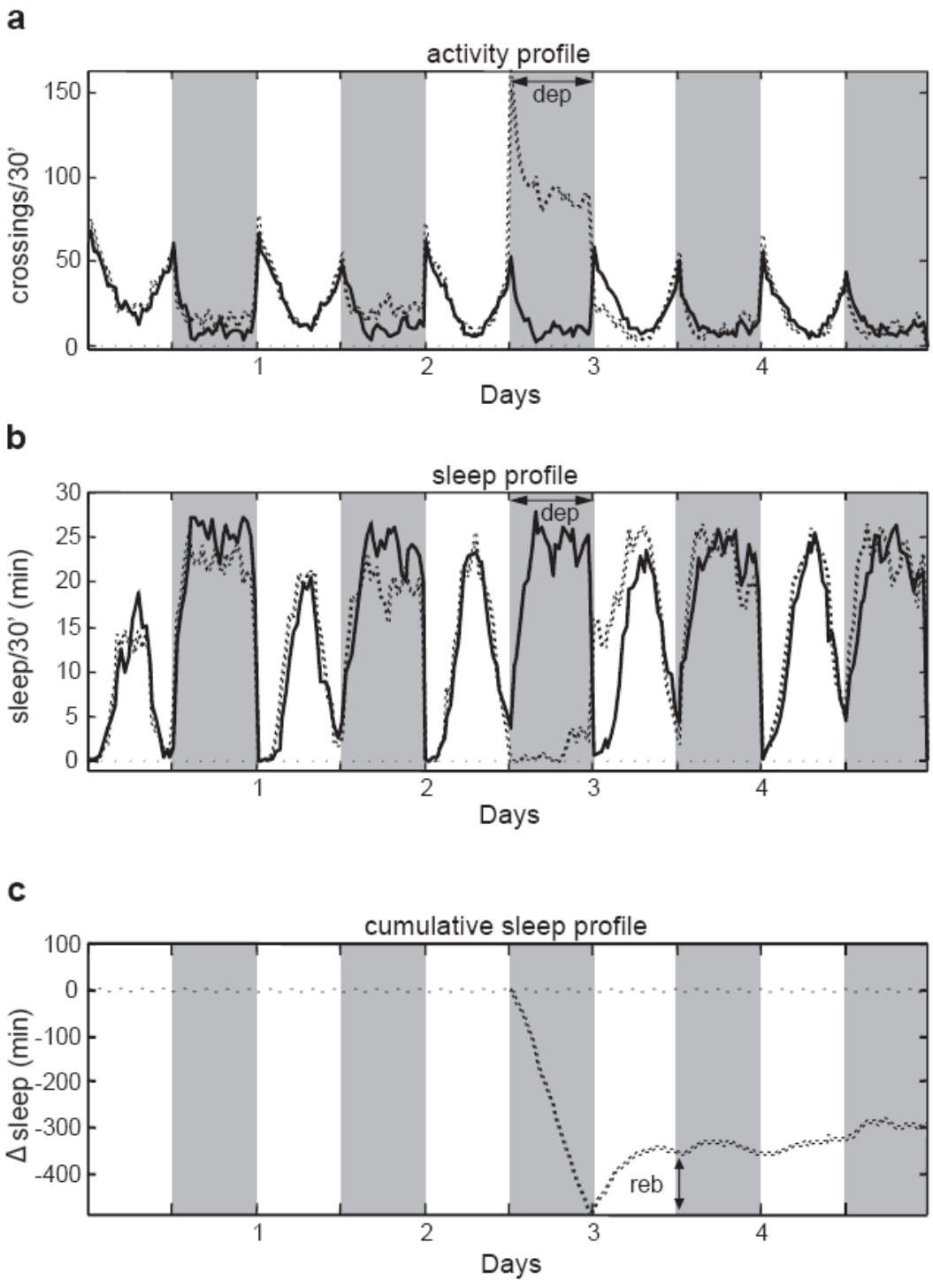

a
activity profile
dep
crossings/30'
150
100
50
0
1
2
3
4
Days
b
sleep profile
dep
sleep/30' (min)
30
25
20
15
10
5
0
1
2
3
4
Days
c
cumulative sleep profile
Δ sleep (min)
100
0
-100
-200
-300
-400
reb
1
2
3
4
Days

Figure A-7: Figure S4. MBSwitch/UAS-mc* female flies show homeostatic rebound sleep following 12 hours of mechanical sleep deprivation that is similar in magnitude to PKA-induced rebound. MBSwitch/UAS-mc* animals were prepared as in Fig A-5. After 1.5 days of acclimation, activity and sleep profiles of uninduced animals were measured for 2.5 days in LD. Beginning at day 2.5 (lights off) and ending at day 3 (lights on), experimental animals were shaken for 2 seconds at randomized intervals with a mean inter-shake interval of 20 seconds. Control animals were undisturbed. (a) During the shaking period, marked in the figure as “dep”, activity was elevated in experimental animals (dotted line), but not in genotypically identical control animals (solid line). (b) Sleep in the experimental group was virtually abolished during the shaking period, while a normal sleep profile was maintained in control animals. For approximately 8 hours after the deprivation period, the experimental group slept substantially more than the control group. (c) Sleep in the control group was subtracted from sleep in the experimental group and integrated over time to keep a running tabulation of cumulative change in sleep. During the deprivation period, experimental animals lost ~500 minutes of sleep compared to controls. Following the deprivation period, the experimental animals recovered 137 $\pm$ 26 min relative to controls before resuming a sleep profile with little net change in sleep relative to controls. Plotted values are derived from means of 21 experimental animals and 25 control animals.

Figure A-8

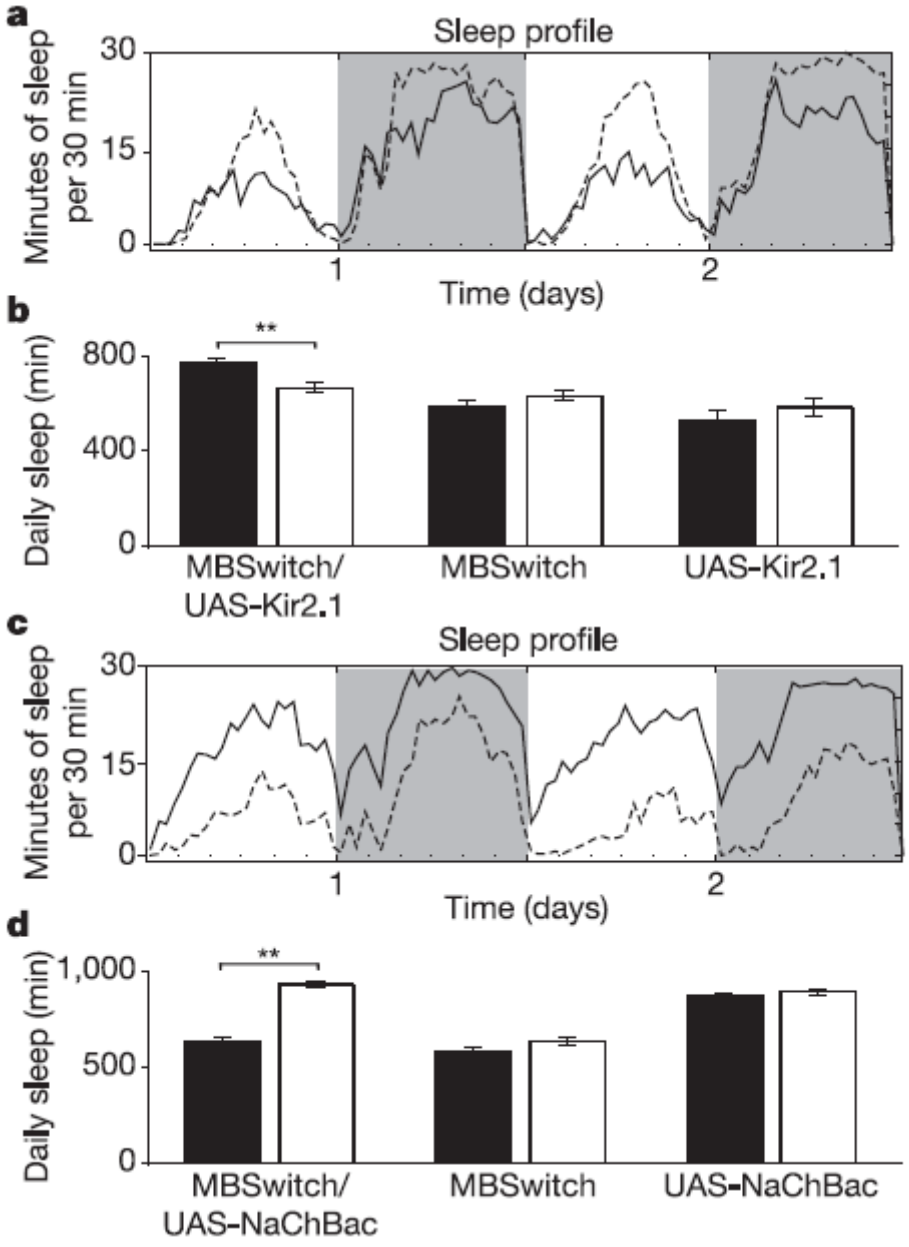


Figure A-8: Decreasing and increasing excitability in a subset of MB neurons have opposite effects on sleep. a, In P{MB-Switch}/UAS-Kir2.1 animals in which K^+ channel expression in the MBs was induced by RU486 (dashed

line), sleep exceeded that of control animals of identical genotype (solid line). b, RU486 significantly increased sleep in MBSwitch/Kir2.1 females (black bars); white bars are uninduced controls. From left to right: for w1118/y w;;MBSwitch/Kir2.1, n = 34–47; for w1118/yw;;MBSwitch/+, n = 30 or 31; for w1118/y w;;UAS-Kir2.1/+, n = 11–26. c, In P{MB-Switch)/ UAS-*NaChBac* flies in which Na^+ channel expression in the MBs was induced by RU486 (dashed line), sleep was significantly suppressed in comparison with control animals of identical genotype (solid line). d, RU486 significantly suppressed sleep in MBswitch/UAS-*NaChBac* females. From left to right: for w1118/y w;UAS-*NaChBac*/+;MBSwitch/+, n = 46 or 47; for w1118/y w;;MBSwitch/+, n = 31; for w1118/y w;UAS-*NaChBac*/+, n = 59 or 60. Two asterisks, $P \leq 0.01$ by unpaired t-test. Where errors are shown, they are s.e.m.

Figure A-9:

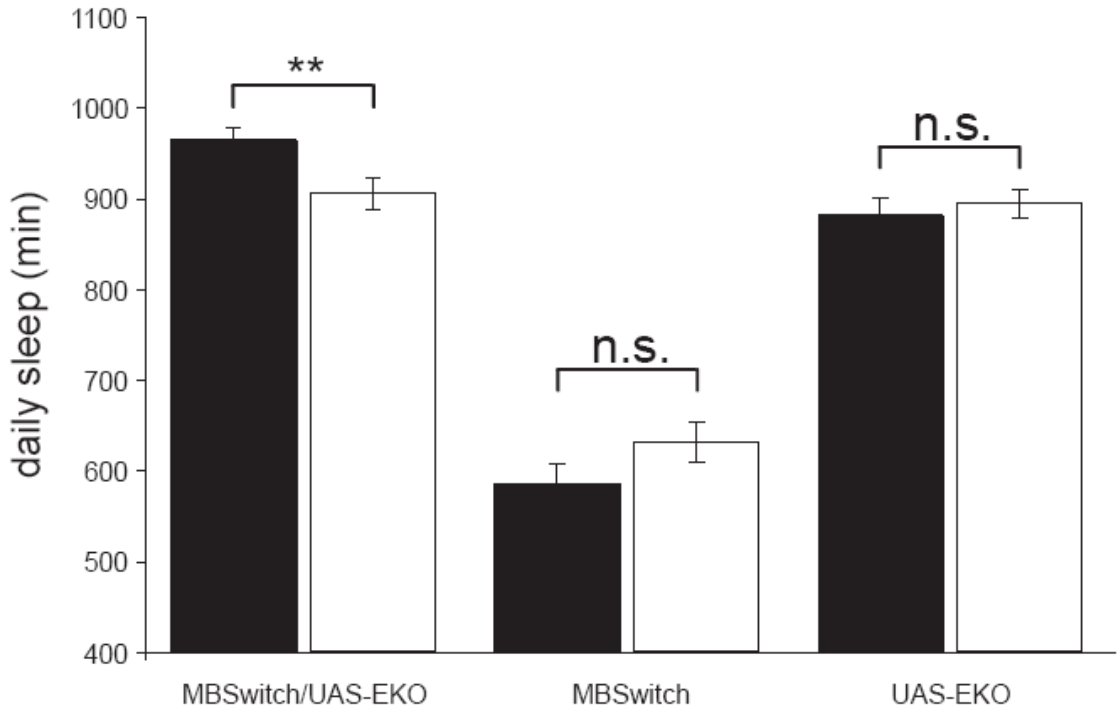


Figure A-9: (Figure S5). Decreasing excitability in a subset of MB neurons using the *EKO* transgene increases sleep. Animals were prepared and assayed for sleep as in Fig 4. RU486 suppressed sleep slightly in female flies bearing either the MBSwitch driver or 3 copies of the transgene for UAS-*EKO* but significantly increased sleep in females carrying the driver/transgene combination. Black bars represent animals that were fed RU486; white bars are controls. From left to right: for w^{1118}/*y w*;2xUAS-*EKO*/+;MBSwitch/1xUAS-*EKO* n = 45; for w^{1118}/*y w*;;MBSwitch/+ n = 30-31; for w^{1118}/*y w*;2xUAS-*EKO*/+;1xUAS-*EKO*/+ n = 29-49. Plotted values represent averages $\pm$ SEM. ** p<.01; n.s. is not significant by unpaired t-test.

Figure A-10:

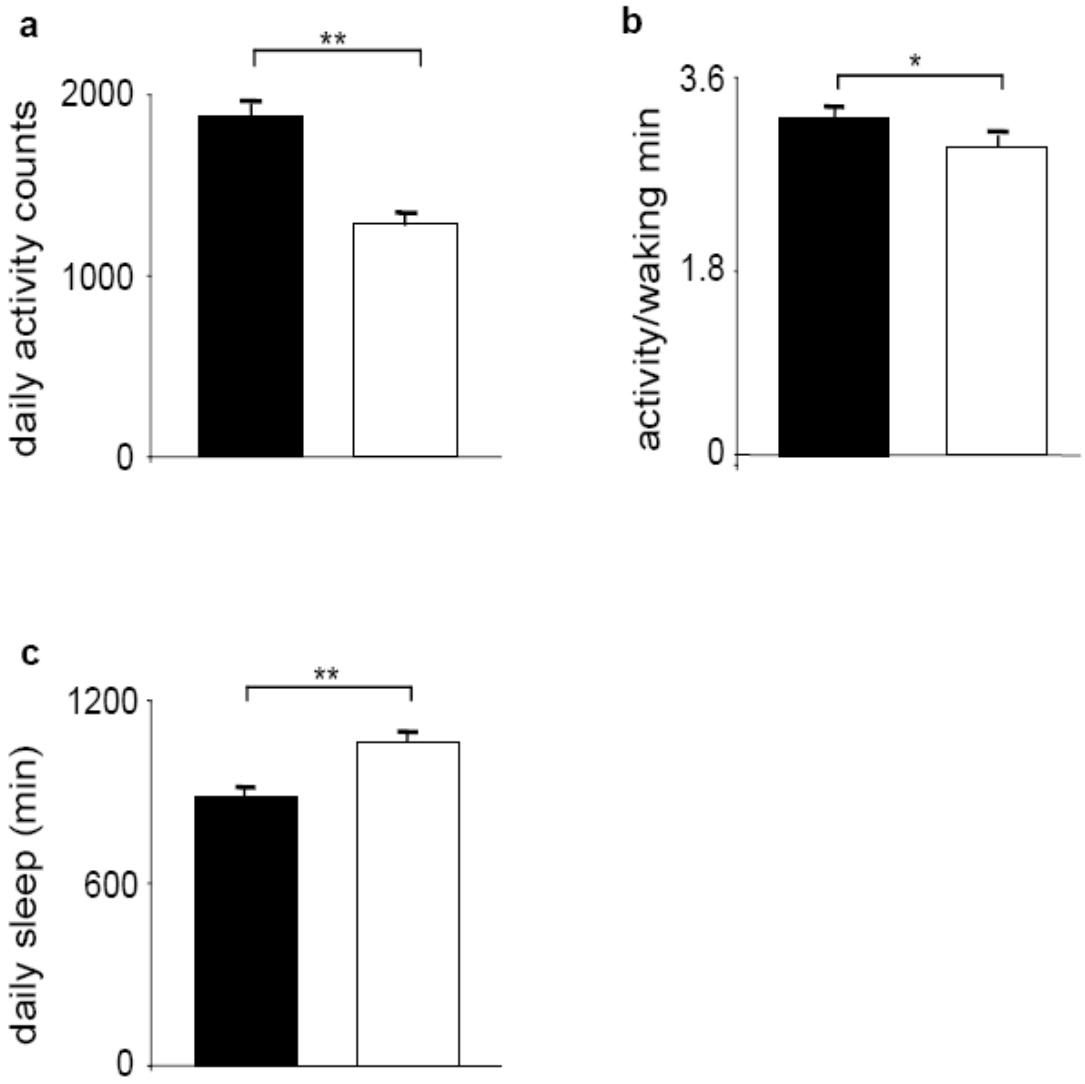


Figure A-10: Figure S6. Ablation of mushroom bodies with hydroxyurea leads to increased activity and decreased rest. (a) Daily locomotor activity of HU-treated (black) vs untreated (white) animals, averaged over 3 days. (b) Average daily locomotor activity divided by waking time for HU-treated (black) vs control (white)

animals. (c) Average daily sleep for HU-treated (black) vs control (white) animals. N=59 and 53 for drug and no drug, respectively. All panels depict averages $\pm$ SEM. ** p<.001; * p<.05 by unpaired t-test.

Figure A-11:

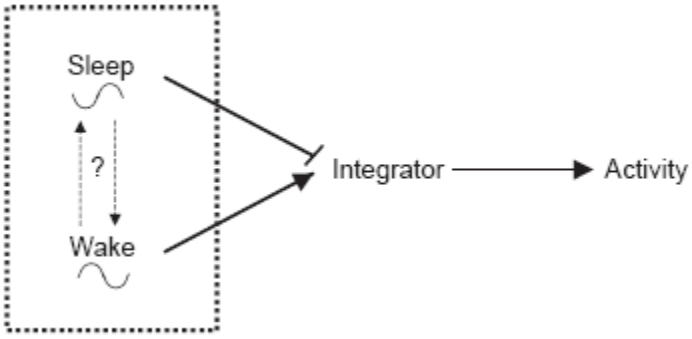


Figure A-11: (Figure S7). Model of sleep-regulating circuitry involving MBs. We propose that within the MBs, sleep-promoting neurons (e.g. 201Y) are normally most active at night, and wake-promoting/sleep-inhibiting neurons (e.g. c309/MBSwitch) are normally most active during the day (diurnal influences are indicated by upward and downward deflections in sinusoids). Antagonistic signals from these two sets of cells are integrated to generate sleep/wake activity cycles. Dashed arrows indicate possible influences of MB neurons on each other. Constitutive expression of PKA in a single set of relevant MB neurons leads to increased sleep or waking at temporally ectopic times of day. When PKA is constitutively expressed in both sets an increase in daytime sleep accompanies a decrease in nighttime sleep with little net change in total daily sleep (Fig A-4). When MBs are ablated the excitatory influence of the integrator increases waking and locomotion.

Table A-1: The GAL4 drivers used are listed alongside their published expression patterns.

GAL4 driver	Major pattern of expression within the brain	References
c309	optic lobe neurons, antennal lobe interneurons, a subset of central complex neurons, mushroom bodies	1,2,3
201Y	mushroom bodies and a small cluster of cells located laterally	1,2,4,5,6,7
85b	antennal lobes, one lateral cell body, mushroom bodies	18
104Y	fan-shaped body of the central complex, optic lobe neurons, small number of neurons diffusely distributed across the brain	3
103Y	mushroom bodies, small number neurons diffusely distributed across the brain	8
GMR	eye	9
c747	antennal lobe interneurons, central complex and mushroom bodies	1,5,3
238Y	large optic lobe neurons and projections, subset of the central complex, mushroom bodies	1,4,7
30Y	subsets of the ellipsoid body and antennal lobes, mushroom bodies, small number of neurons diffusely distributed across the brain	2,4,7
C507	ellipsoid body of the central complex	10
MBSwitch	mushroom bodies	11
elavGeneSwitch	pan-neuronal	12
H24	antennal lobes, ellipsoid body of the central complex, mushroom bodies	7,13
17D	median bundle, mushroom bodies	3,7,13
pdf	lateral neurons	14
per	cells expressing the clock gene *period*	15
129Y	antennal nerve, suboesophageal ganglion	16
1366	various areas of the anterior, medial and posterior brain	17
c739	ellipsoid body and fan-shaped body of the central complex, antennal lobes, mushroom bodies	2,3,6
MJ286	lateral protocerebrum	2
MJ63	lateral protocerebrum	2
c232	ellipsoid body of the central complex	1,2
c492b	antennal lobes, ellipsoid body of the central complex, mushroom bodies	3,19

1. Connolly, J.B, Roberts, I.J., Armstrong, J.D., Kaiser, K., Forte, M., Tully, T. & O'Kane, C.J. Associative learning disrupted by impaired Gs signaling in Drosophila mushroom bodies. *Science* **274**, 2104-7 (1996).
2. Joiner, M.A. & Griffith, L.C. Mapping of the anatomical circuit of CaM kinase-dependent courtship conditioning in *Drosophila. Learn Mem* **6**, 177–192 (1999).

3. Rodan, A.R., Kiger, J.A. & Heberlein, U. Functional Dissection of Neuroanatomical Loci Regulating Ethanol Sensitivity in ***Drosophila.*** *J. Neurosci*. **22**, 9490–9501 (2002).
4. Yang, M.Y., Armstrong, J.D., Vilinsky, I., Strausfeld, N.J. & Kaiser, K. Subdivision of the *Drosophila* mushroom bodies by enhancer-trap expression patterns. *Neuron* **15**, 45-54 (1995).
5. O'Dell, K.M., Armstrong, J.D., Yang, M.Y. & Kaiser, K. Functional dissection of the *Drosophila* mushroom bodies by selective feminization of genetically defined subcompartments. *Neuron* **15**, 55-61 (1995).
6. Armstrong, J.D., de Belle, J.S., Wang, Z. & Kaiser, K. Metamorphosis of the mushroom bodies; large-scale rearrangements of the neural substrates for associative learning and memory in *Drosophila*. *Learn Mem* **5**, 102-114 (1998).
7. Zars, T., Fischer, M., Schulz, R. & Heisenberg, M. Localization of a short-term memory in *Drosophila*. *Science* **288,** 672-675 (2000).
8. Tettamanti, M., Armstrong, J.D., Endo, K., Yang, M.Y., Furukubo-Tokunaga, K., Kaiser, K. & Reichert, H. Early development of the *Drosophila* mushroom bodies, brain centres for associative learning and memory. *Dev Genes Evol* **207**, 242-252 (1997).
9. Moses, K. and Rubin, G.M. Glass encodes a site-specific DNA-binding protein that is regulated in response to positional signals in the developing Drosophila eye. *Genes Dev*. **4**, 583-93 (1991).
10. Renn, S.C., Armstrong, J.D., Yang, M., Wang, Z., An, X., Kaiser, K. & Taghert, P.H. Genetic analysis of the Drosophila ellipsoid body neuropil: organization and development of the central complex. *J. Neuro biol*. **41,** 189–207 (1999).
11. Mao, Z., Roman, G., Zong, L. & Davis, R.L. Pharmacogenetic rescue in time and space of the *rutabaga* memory impairment by using Gene-Switch *PNAS*, **101**, 198-203 (2004).
12. Osterwalder, T., Yoon, K.S., White, B.H. & Keshishian, H. A conditional tissue-specific transgene expression system using inducible GAL4. *Proc. Nat. Acad. Sci. U.S.A*. **98**, 12596-12601 (2001).
13. Martin, J.R., Ernst, R. & Heisenberg, M. Mushroom bodies suppress locomotor activity in *Drosophila melanogaster. Learn Mem* **5**, 179-191 (1998).
14. Kaneko, M., Park, J.H., Cheng, Y., Hardin, P.E. & Hall, J.C. Disruption of synaptic transmission or clock-gene-product oscillations in circadian pacemaker cells of Drosophila cause abnormal behavioral rhythms. *J Neurobiol* **43**, 207-233 (2000).
15. Kaneko, M. & Hall, J.C.: Neuroanatomy of cells expressing clock genes in Drosophila: transgenic manipulation of the period and timeless genes to mark the perikarya of circadian pacemaker neurons and their projections. *J Comp Neurol* **422**, 66-94 (2000).
16. Armstrong, J.D., Kaiser, K., Muller, A., Fischbach, K.-F., Merchant, N. & Strausfeld, N.J. Flybrain, an on-line atlas and database of the Drosophila nervous system. *Neuron*, **15**, 17-20 (1995).
17. Helfrich-Forster, C., Tauber, M., Park, J.H., Muhlig-Versen, M., Schneuwly, S. & Hofbauer, A. Ectopic expression of the neuropeptide pigment- dispersing factor alters behavioral rhythms in Drosophila melanogaster. *J Neurosci* **20**, 3339-3353 (2000).
18. Devaud, J.M., Keane, J. & Ferrus, A. Blocking sensory inputs to identified antennal glomeruli selectively modifies odorant perception in Drosophila. *J Neurobiol*. **56**, 1-12 (2003).
19. Zars, T., Wolf, R., Davis, R. & Heisenberg, M. Tissue-specific expression of a type I adenylyl cyclase rescues the rutabaga mutant memory defect: in search of the engram. *Learn Mem.* **7**, 18-31.(2000)

Part 2: Caffeine changes global levels of cAMP

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Caffeine is one of the most widely consumed stimulants in the world and has been proposed to promote wakefulness by antagonizing function of the adenosine A_{2A} receptor (Huang et al., 2005). Work from our laboratory demonstrates that chronic administration of caffeine reduces and fragments sleep in *Drosophila* and also lengthens circadian period (Wu et al., 2009). However, these effects are not mediated by the caffeine target thought to be relevant for sleep, the adenosine receptor. On the other hand, the effects of caffeine on sleep and circadian rhythms are mimicked by a potent phosphodiesterase inhibitor, IBMX (3-isobutyl-1-methylxanthine). Using *in vivo* fluorescence resonance energy transfer imaging, we find that caffeine induces widespread increase in cAMP levels throughout the brain. We suggest that chronic administration of caffeine promotes wakefulness in *Drosophila*, at least in part, by inhibiting cAMP phosphodiesterase activity.

Methods:

Fluorescent resonance energy transfer imaging of cAMP levels.

Brains from *elav-Gal4/+*; *UAS-Epac1-camps (50A)/+* (Shafer et al., 2008) flies were dissected in ice-cold calcium-free saline containing 46 mM NaCl, 180 mM KCl, and 10 mM Tris, pH 7.2. The brains were then laid at the bottom of a 35 x 10 mm plastic FALCON Petri dish (Becton Dickenson Labware), given a few seconds to adhere and then covered with 1.6 ml of hemolymph-like saline (HL3) containing 70 mM NaCl, 5 mM KCl, 1.5 mM $CaCl_2$, 20 mM $MgCl_2$, 10 mM $NaHCO_3$, 5 mM trehalose, 115 mM sucrose, and 5 mM HEPES, pH 7.1 (Shafer et al., 2008).

Time course fluorescent resonance energy transfer (FRET) imaging of pan-neuronally expressed Epac1-camps was performed on individual brains using a Leica TCS SP5 confocal microscope using a HCX APO L 40x/0.80 dipping objective. 60 µl of 10 mg/ml caffeine was added into the dish for a final concentration of 0.375 mg/ml following 3 min of baseline imaging. In the water control, 60 µl of water was added. To quantify yellow fluorescent protein (YFP) (525 nm)/cyan fluorescent protein (CFP) (475 nm) peak values, spectral analysis was used, taking images from 470 nm to 599 nm in 10 nm increments at 256 x 256 pixels, 700 Hz, and a line average of two every 20 s. Regions of interest (ROIs) on the brains were selected and examined for changes in YFP/CFP peak height value on the spectral analysis.

Results:

Caffeine causes widespread increase in cAMP levels.

If caffeine does not act on sleep by antagonizing *dAdoR* (adenosine receptor) signaling, how else might it act? Caffeine, like other methylated xanthines, inhibits

cAMP PDE in mammalian cells, and indeed cAMP/PKA signaling is implicated in the regulation of sleep in *Drosophila* and mammals (Hendricks et al., 2001; Graves et al., 2003; Joiner et al., 2006). Biochemical data have suggested that the concentration of caffeine required to inhibit phosphodiesterases (PDEs) is higher than would be physiologically relevant in mammals (Fredholm et al., 1999) , but recent data suggest that at least some of the effects of caffeine on human immune function may involve inhibition of cAMP PDE (Horrigan et al., 2006). Thus, we sought to investigate a role for the cAMP-PKA pathway in the effects of caffeine on sleep.

If caffeine acts as a cAMP phosphodiesterase (PDE), one would expect the presence of caffeine to elevate cAMP levels in widespread areas throughout the fly brain. To assess this, we conducted *in vivo* FRET imaging with recently described *UAS-Epac1-camps* flies, which can be used to overexpress Epac1-camps, a FRET-based cAMP sensor (Nikolaev et al., 2004; Shafer et al., 2008). In this system, the presence of cAMP causes a reduction in FRET from donor (CFP) to recipient (YFP) chromophores. In brains where Epac1-camps is expressed pan-neuronally, we find that addition of caffeine leads to an increase in cAMP levels (as measured by a decrease in YFP/CFP signal) in widespread areas throughout the brain, including areas previously implicated in sleep regulation such as mushroom bodies (Joiner et al., 2006; Pitman et al., 2006) and pars intercerebralis (Foltenyi et al., 2007) (Fig. *A-12*).

Figure A-12:

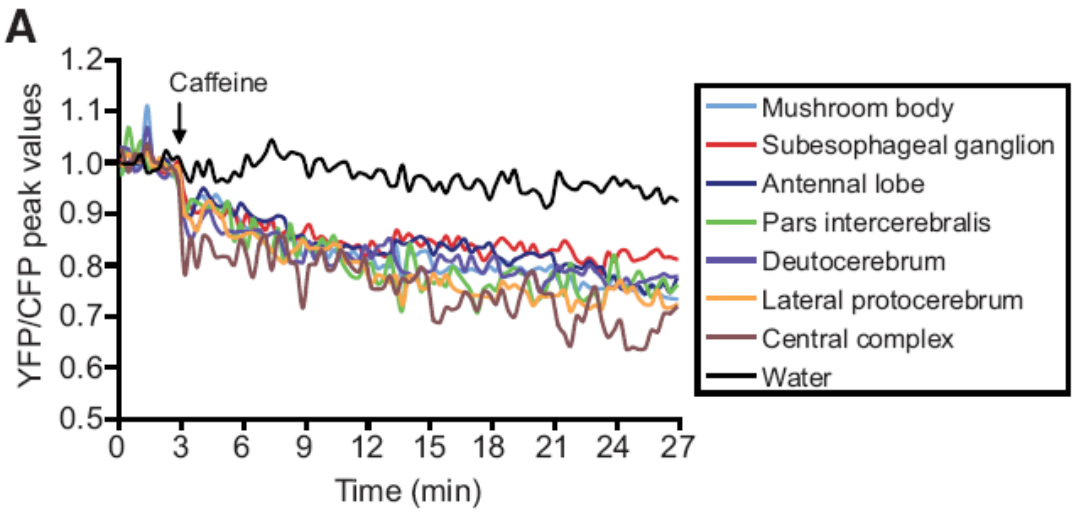

Figure A-12: Caffeine reduces Epac1-camps FRET. Average FRET plots, as measured by YFP/CFP peak signals, for different regions of interest (ROI) following bath application of 0.375 mg/ml caffeine on elav-Gal4/+; UAS-Epac1-camps/+ brains. Decreasing YFP/CFP reflects increasing cAMP levels. Arrow represents the start of caffeine exposure. For each ROI, the following number of brains were analyzed as follows: mushroom body (n =7), subesophageal ganglion (n=8), antennal lobe (n=6), pars intercerebralis (n=3), deutocerebrum (n=6), lateral protocerebrum (n=4), central complex (n=2). The brain ROIs in the five water-treated brains were pooled. YFP/CFP peak signals pooled in 6 min bins were statistically different from water (at least $p<0.05$) in all regions and all bins, except central complex, which was statistically different only for minutes 21–27, and subesophageal ganglion, which was statistically similar for minutes 15–21.

Summary and Perspectives.

An understanding of both the anatomical and cellular signaling pathways underlying a particular behavior is expected to provide insight into the basis of the behavior. For my thesis I set out to understand the mechanisms that underlie sleep in *Drosophila*. I approached this question from many angles, which include analysis of anatomy, investigation of a specific neurotransmitter, octopamine ,and characterization of the intra cellular signaling important for wake behavior in flies.

Octopamine is a very prominent neurotransmitter in invertebrates (Roeder, 2005). Prior to this work octopamine had only been alluded to as an arousal signal, but with the use of *Drosophila* as a model organism to study sleep, we were able to conclusively demonstrate its wake-promoting actions. We show that there is a decrease in sleep in mutants that do not produce octopamine. We also show that flies with octopamine signaling altered through genetic changes in the membrane potential of octopaminergic neurons display significant changes in sleep, such that depolarization causes less sleep and hyperpolarization increases sleep. These effects are all independent of the overall activity of the animals because they either do not have defects in their ability to generate movement or manifest excessive movement.

We then addressed the anatomical pathway important for octopamine regulation of sleep. We demonstrate that a subset of octopamine-producing cells is necessary for generating the wake-promoting actions of octopamine. This signal is then relayed to an area of the brain known as the Pars Intercerebralis (PI), specifically the *Drosophila* insulin (*Dilp2*)-producing neurons within the PI. Effects of octopamine are mediated specifically by the K3 isoform of the OAMB receptor present in *Dilp2* neurons. Activation of the OAMB receptor results in increases in cAMP and closing of the slowpoke K+ channel, thereby causing a prolonged action potential in these neurons. Interestingly the wake-promoting effects of octopamine are independent of *Dilp2* and seem to be mediated by other pathways in *Dilp2* neurons.

Putting these findings in the context of our general understanding of sleep: While there have been many studies addressing why we sleep, we are only just beginning to approach this question in a controlled fashion. Sleep is a fundamental phenomenon with many complexities. Dr. Jerome Siegel postulates that many of the functions we attribute to sleep are due to evolutionary pressure for those activities, such as cellular repair, to occur during sleep but that sleep itself is not necessary for these activities (Siegel, 2005). While this is not a commonly held opinion, it is important to consider when thinking about the function of sleep and the work in this thesis. In this thesis work I have focused on the underlying circuitry and regulation of sleep/wake in *Drosophila*. While I

have not directly addressed function, my work provides correlative evidence for and against different functions of sleep.

The use of *Drosophila* as a model organism for sleep has led to the identification of many anatomical areas and cellular pathways that turn out to be important for other behaviors, thereby creating a resurgence of many old theories for why we sleep (Cirelli, 2009; Crocker and Sehgal, 2010). For instance, we found that specific cells in the pars Intercerebralis (PI), an area important for metabolic regulation, are important for promoting wakefulness. It has long been thought that sleep and metabolism are intimately associated, but until recently it has been difficult to address whether these two processes are mutually dependent. The fly model allows for more straightforward approaches to these questions than does the mammalian model. We have the ability to manipulate sleep and/or metabolic pathways in a time and anatomical specific manner. In doing so, we can address the role of each biological function and examine interdependencies. The difficulty of such approaches in mammals arises, in part, from the fact that major changes in baseline sleep are seldom detected. There are very few if any mouse mutations that abolish sleep to the extent seen in some fly mutants. In addition, the developmental effects of many of these mutations on the sleep pathway and/or the metabolic pathway may prevent them from developing to adults. Tissue and time specific knockouts can now be generated in mammals, but not with the ease possible in flies.

Our recent work in trying to understand the overlap of the metabolic and sleep pathways suggests that each biological function is independent of the

other. Despite being independent, the functions overlap in a key neurotransmitter known as octopamine. We find that octopamine regulates triglyceride levels and promotes wakefulness. One theory is that the wakefulness is what causes the changes in triglyceride levels. But this does not appear to be the case, because many short-sleeping mutants have different effects on triglycerides and short sleep does not always result in high triglycerides. In fact, overexpressing PKA in the mushroom body (an area with no known metabolic effect) results in short sleep and lowered triglycerides. This may be closer to a more pure effect of sleep loss, in flies but since the mushroom body is a main site of olfactory integration we cannot rule out the effects it may have on sensing food. These studies highlight the importance of looking at the whole picture when dealing with sleep and its functions. It is highly likely that in humans there are metabolic effects of sleep deprivation, but they may be a consequence of the pathways activated to elicit the sleep deprivation. This concept was recently shown in stress and insomnia models in mammals where overlap in stress and arousal systems (specifically the locus coeruleus and paraventricular nucleus in the hypothalamus) results in stress induced insomnia (Cano et al., 2008).

The idea that metabolic function affects the homeostatic regulation of sleep is an interesting concept. While this question is not addressed in this thesis ,it could be easily examined by sleep-depriving animals with altered metabolic function. However, It is unclear whether these studies would actually address the function of sleep but they could identify somnogens. If a metabolic

signal, such as insulin or AMP activated kinase (AMPK), is a somnogen then alterations in this signal would only affect the drive to sleep. It would not address sleep function. In order to address this, one would need to show that normal sleep is required for changes in these pathways. This has not been done and may be very hard to do.

Recent work In mammals has tried to address the homeostatic regulation of sleep by cellular metabolic signals. A widely held belief is that sleep has a restorative function (Benington and Heller, 1995). Whether this is actually a sleep function or is postulated based upon the restriction of cellular repair processes to times of rest is unknown, but it is clear that sleep disruption in mammals leads to disrupted metabolism (Spiegel et al., 2009). The idea is that the build-up of adenosine in wake-promoting areas, due to high activity of AMPK, results in these neurons turning off and going off-line to restore normal ATP levels (precursor to ADP and adenosine)(Dworak et al., 2010). A major piece of evidence supporting this model came from data suggesting that caffeine reduces sleep by blocking the adenosine signal (Huang et al., 2005). This presents a congruent story, where reduced sleep further increases adenosine levels; when effects of increased adenosine levels, which would normally lead to sleep, are blocked, the animal extends its wakefulness. Recent work of Dr. Mark Wu in our lab has addressed this question in flies (Wu et al. 2009).

Since sleep is a highly conserved behavior, it is likely that its purpose is similar across species. If this is the case, in flies there should also be a buildup of adenosine telling the animal to sleep. In both flies and mammals, caffeine is an

important wake-promoting signal and in mammals it inhibits the A2a adenosine receptor (Huang et al., 2005). Unfortunately, in both flies and in mammals, the evidence for a role of adenosine receptors in regulating sleep is not strong, so analysis of other caffeine targets is important to understand its effects on sleep and wake. cAMP phosphodiesterase is another well-known target of caffeine and we show here that caffeine treatment leads to overall increases in cAMP across multiple brain regions. In addition, the behavioral data reveal that caffeine promotes global arousal independent of adenosine (Wu et al., 2009), calling into question whether decreased adenosine signaling actually decreases sleep.

Another prevalent theory for sleep function is its role in learning and memory (Walker and Stickgold, 2004). We have shown that the mushroom body, a site of learning and memory in flies, is an important area for sleep regulation. It is reasonable to assume that an area important for integrating sensory signals such as the mushroom body would be wake-promoting. Many sensory systems in mammals when stimulated will produce wakefulness. What is more interesting about this work is the possibility that a subset of this structure is a sleep-promoting area. This effect is likely to be separate from the learning areas of the mushroom body since it does not seem feasible to learn a task while one is asleep. However, the sleep-promoting region may turn out to be important for the consolidation of memory. Evidence for this idea exists in mammals where sleep deprivation impairs consolidation of specific types of memory (Graves et al., 2003a; Walker and Stickgold, 2004).

Despite this work, it is clear we do not have a good understanding of why we sleep. Many current theories are based on correlative data such as what is described in this thesis. As we look closer at some of the main theories for why we sleep we are finding that they may be independent of sleep itself. This brings us back to Dr. Siegel's theory on sleep—that it actually serves no purpose, and that, through evolution and the optimization of both brain and body function, certain biological processes just happen to occur during sleep. Unfortunately, if this is the case it may be impossible to prove, because sleep deprivation would always be associated with the deleterious loss of these processes, thereby making it appear as if they are functions of sleep. But in a society that is perpetually sleep deprived, whether a process is a function of sleep or not is irrelevant; if sleep deprivation causes memory impairment and metabolic changes due to alterations in normal biological functioning, it remains prudent that we avoid loss of sleep.

References:

Adachi, M., Barrot, M., Autry, A.E., Theobald, D., and Monteggia, L.M. (2008). Selective loss of brain-derived neurotrophic factor in the dentate gyrus attenuates antidepressant efficacy. Biol Psychiatry *63*, 642-649.

Adamantidis, A.R., Zhang, F., Aravanis, A.M., Deisseroth, K., and de Lecea, L. (2007). Neural substrates of awakening probed with optogenetic control of hypocretin neurons. Nature *450*, 420-424.

Aeschbach, D., and Borbely, A.A. (1993). All-night dynamics of the human sleep EEG. J Sleep Res *2*, 70-81.

Agosto, J., Choi, J.C., Parisky, K.M., Stilwell, G., Rosbash, M., and Griffith, L.C. (2008). Modulation of GABAA receptor desensitization uncouples sleep onset and maintenance in Drosophila. Nat Neurosci *11*, 354-359.

Allada, R., and Siegel, J.M. (2008). Unearthing the phylogenetic roots of sleep. Curr Biol *18*, R670-R679.

Ambrosius, U., Lietzenmaier, S., Wehrle, R., Wichniak, A., Kalus, S., Winkelmann, J., Bettecken, T., Holsboer, F., Yassouridis, A., and Friess, E. (2008). Heritability of sleep electroencephalogram. Biol Psychiatry *64*, 344-348.

Anderson, M.P., Mochizuki, T., Xie, J., Fischler, W., Manger, J.P., Talley, E.M., Scammell, T.E., and Tonegawa, S. (2005). Thalamic Cav3.1 T-type Ca2+ channel plays a crucial role in stabilizing sleep. Proc Natl Acad Sci U S A *102*, 1743-1748.

Andretic, R., Franken, P., and Tafti, M. (2008). Genetics of sleep. Annu Rev Genet *42*, 361-388.

Andretic, R., van Swinderen, B., and Greenspan, R.J. (2005). Dopaminergic modulation of arousal in Drosophila. Curr Biol *15*, 1165-1175.

Anokhin, A., Steinlein, O., Fischer, C., Mao, Y., Vogt, P., Schalt, E., and Vogel, F. (1992). A genetic study of the human low-voltage electroencephalogram. Hum Genet *90*, 99-112.

Archer, S.N., Robilliard, D.L., Skene, D.J., Smits, M., Williams, A., Arendt, J., and von Schantz, M. (2003). A length polymorphism in the circadian clock gene Per3 is linked to delayed sleep phase syndrome and extreme diurnal preference. Sleep *26*, 413-415.

Armstrong, J.D., de Belle, J.S., Wang, Z., and Kaiser, K. (1998). Metamorphosis of the mushroom bodies; large-scale rearrangements of the neural substrates for associative learning and memory in Drosophila. Learn Mem *5*, 102-114.

Aston-Jones, G., and Bloom, F.E. (1981). Activity of norepinephrine-containing locus coeruleus neurons in behaving rats anticipates fluctuations in the sleep-waking cycle. J Neurosci *1*, 876-886.

Bacon, J.P., Thompson, K.S., and Stern, M. (1995). Identified octopaminergic neurons provide an arousal mechanism in the locust brain. J Neurophysiol *74*, 2739-2743.

Baier, A., Wittek, B., and Brembs, B. (2002). Drosophila as a new model organism for the neurobiology of aggression? J Exp Biol *205*, 1233-1240.

Baines, R.A., Uhler, J.P., Thompson, A., Sweeney, S.T., and Bate, M. (2001). Altered electrical properties in Drosophila neurons developing without synaptic transmission. J Neurosci *21*, 1523-1531.

Barron, A.B., Maleszka, J., Vander Meer, R.K., Robinson, G.E., and Maleszka, R. (2007). Comparing injection, feeding and topical application methods for treatment of honeybees with octopamine. J Insect Physiol *53*, 187-194.

Barron, A.B., Schulz, D.J., and Robinson, G.E. (2002a). Octopamine modulates responsiveness to foraging-related stimuli in honey bees (Apis mellifera). Journal of Comparative Physiology a-Neuroethology Sensory Neural and Behavioral Physiology *188*, 603-610.

Barron, A.B., Schulz, D.J., and Robinson, G.E. (2002b). Octopamine modulates responsiveness to foraging-related stimuli in honey bees (Apis mellifera). J Comp Physiol A Neuroethol Sens Neural Behav Physiol *188*, 603-610.

Benington, J.H., and Heller, H.C. (1995). Restoration of brain energy metabolism as the function of sleep. Prog Neurobiol *45*, 347-360.

Benington, J.H., Kodali, S.K., and Heller, H.C. (1995). Stimulation of A1 adenosine receptors mimics the electroencephalographic effects of sleep deprivation. Brain Res *692*, 79-85.

Berger, M., Gray, J.A., and Roth, B.L. (2009). The expanded biology of serotonin. Annu Rev Med *60*, 355-366.

Berridge, C.W. (2008). Noradrenergic modulation of arousal. Brain Res Rev *58*, 1-17.

Berridge, C.W., and Espana, R.A. (2000). Synergistic sedative effects of noradrenergic alpha(1)- and beta-receptor blockade on forebrain electroencephalographic and behavioral indices. Neuroscience *99*, 495-505.

Beuckmann, C.T., Sinton, C.M., Miyamoto, N., Ino, M., and Yanagisawa, M. (2003). N-type calcium channel alpha1B subunit (Cav2.2) knock-out mice display hyperactivity and vigilance state differences. J Neurosci *23*, 6793-6797.

Bopparaju, S., and Surani, S. (2010). Sleep and diabetes. Int J Endocrinol *2010*, 759509.

Borbely, A.A. (1982). A two process model of sleep regulation. Hum Neurobiol *1*, 195-204.

Brand, A.H., and Perrimon, N. (1993). Targeted gene expression as a means of altering cell fates and generating dominant phenotypes. Development *118*, 401-415.

Brogiolo, W., Stocker, H., Ikeya, T., Rintelen, F., Fernandez, R., and Hafen, E. (2001). An evolutionarily conserved function of the Drosophila insulin receptor and insulin-like peptides in growth control. Curr Biol *11*, 213-221.

Broughton, S.J., Slack, C., Alic, N., Metaxakis, A., Bass, T.M., Driege, Y., and Partridge, L. (2010). DILP-producing median neurosecretory cells in the Drosophila brain mediate the response of lifespan to nutrition. Aging Cell *9*, 336-346.

Busch, S., Selcho, M., Ito, K., and Tanimoto, H. (2009). A map of octopaminergic neurons in the Drosophila brain. J Comp Neurol *513*, 643-667.

Bushey, D., Huber, R., Tononi, G., and Cirelli, C. (2007). Drosophila Hyperkinetic mutants have reduced sleep and impaired memory. J Neurosci *27*, 5384-5393.

Cano, G., Mochizuki, T., and Saper, C.B. (2008). Neural circuitry of stress-induced insomnia in rats. J Neurosci *28*, 10167-10184.

Carpen, J.D., Archer, S.N., Skene, D.J., Smits, M., and von Schantz, M. (2005). A single-nucleotide polymorphism in the 5'-untranslated region of the hPER2 gene is associated with diurnal preference. J Sleep Res *14*, 293-297.

Carpen, J.D., von Schantz, M., Smits, M., Skene, D.J., and Archer, S.N. (2006). A silent polymorphism in the PER1 gene associates with extreme diurnal preference in humans. J Hum Genet *51*, 1122-1125.

Certel, S.J., Savella, M.G., Schlegel, D.C., and Kravitz, E.A. (2007). Modulation of Drosophila male behavioral choice. Proc Natl Acad Sci U S A *104*, 4706-4711.

Charmandari, E., Tsigos, C., and Chrousos, G. (2005). Endocrinology of the stress response. Annu Rev Physiol *67*, 259-284.

Chemelli, R.M., Willie, J.T., Sinton, C.M., Elmquist, J.K., Scammell, T., Lee, C., Richardson, J.A., Williams, S.C., Xiong, Y., Kisanuki, Y., *et al.* (1999). Narcolepsy in orexin knockout mice: molecular genetics of sleep regulation. Cell *98*, 437-451.

Chen, Y.L., Hung, Y.S., and Yang, E.C. (2008). Biogenic amine levels change in the brains of stressed honeybees. Archives of Insect Biochemistry and Physiology *68*, 241-250.

Chentsova, N.A., Gruntenko, N.E., Bogomolova, E.V., Adonyeva, N.V., Karpova, E.K., and Rauschenbach, I.Y. (2002a). Stress response in Drosophila melanogaster strain inactive with decreased tyramine and octopamine contents. J Comp Physiol B *172*, 643-650.

Chentsova, N.A., Gruntenko, N.E., Bogomolova, E.V., Adonyeva, N.V., Karpova, E.K., and Rauschenbach, I.Y. (2002b). Stress response in Drosophila melanogaster strain inactive with decreased tyramine and octopamine contents. Journal of Comparative Physiology B-Biochemical Systemic and Environmental Physiology *172*, 643-650.

Chikahisa, S., Fujiki, N., Kitaoka, K., Shimizu, N., and Sei, H. (2009). Central AMPK contributes to sleep homeostasis in mice. Neuropharmacology *57*, 369-374.

Chung, B.Y., Kilman, V.L., Keath, J.R., Pitman, J.L., and Allada, R. (2009). The GABA(A) receptor RDL acts in peptidergic PDF neurons to promote sleep in Drosophila. Curr Biol *19*, 386-390.

Chung, S.K., Reinhart, P.H., Martin, B.L., Brautigan, D., and Levitan, I.B. (1991). Protein kinase activity closely associated with a reconstituted calcium-activated potassium channel. Science *253*, 560-562.

Church, D.M., Goodstadt, L., Hillier, L.W., Zody, M.C., Goldstein, S., She, X., Bult, C.J., Agarwala, R., Cherry, J.L., DiCuccio, M., *et al.* (2009). Lineage-specific biology revealed by a finished genome assembly of the mouse. PLoS Biol *7*, e1000112.

Cirelli, C. (2009). The genetic and molecular regulation of sleep: from fruit flies to humans. Nat Rev Neurosci *10*, 549-560.

Cirelli, C., Bushey, D., Hill, S., Huber, R., Kreber, R., Ganetzky, B., and Tononi, G. (2005a). Reduced sleep in Drosophila Shaker mutants. Nature *434*, 1087-1092.

Cirelli, C., LaVaute, T.M., and Tononi, G. (2005b). Sleep and wakefulness modulate gene expression in Drosophila. J Neurochem *94*, 1411-1419.

Cirelli, C., Pompeiano, M., and Tononi, G. (1996). Neuronal gene expression in the waking state: a role for the locus coeruleus. Science *274*, 1211-1215.

Cole, S.H., Carney, G.E., McClung, C.A., Willard, S.S., Taylor, B.J., and Hirsh, J. (2005). Two functional but noncomplementing Drosophila tyrosine decarboxylase genes: distinct roles for neural tyramine and octopamine in female fertility. J Biol Chem *280*, 14948-14955.

Cope, D.W., Hughes, S.W., and Crunelli, V. (2005). GABAA receptor-mediated tonic inhibition in thalamic neurons. J Neurosci *25*, 11553-11563.

Corbet, S. (1991a). A fresh look at the arousal syndrome of insects. Adv. Invert. Physiol. *23*.

Corbet, S.A. (1991b). A fresh look at the arousal syndrome of insects. Adv. Invert. Physiol. *23*, 81-116.

Crittenden, J.R., Skoulakis, E.M., Han, K.A., Kalderon, D., and Davis, R.L. (1998). Tripartite mushroom body architecture revealed by antigenic markers. Learn Mem *5*, 38-51.

Crocker, A., and Sehgal, A. (2008). Octopamine regulates sleep in drosophila through protein kinase A-dependent mechanisms. J Neurosci *28*, 9377-9385.

Crocker, A., and Sehgal, A. (2010). Genetic analysis of sleep. Genes Dev *24*, 1220-1235.

Crocker, A., Shahidullah, M., Levitan, I.B., and Sehgal, A. (2010). Identification of a neural circuit that underlies the effects of octopamine on sleep:wake behavior. Neuron *65*, 670-681.

Cueni, L., Canepari, M., Lujan, R., Emmenegger, Y., Watanabe, M., Bond, C.T., Franken, P., Adelman, J.P., and Luthi, A. (2008). T-type Ca2+ channels, SK2 channels and SERCAs gate sleep-related oscillations in thalamic dendrites. Nat Neurosci *11*, 683-692.

Dauvilliers, Y., Maret, S., and Tafti, M. (2005). Genetics of normal and pathological sleep in humans. Sleep Med Rev *9*, 91-100.

Davenport, A.P., and Evans, P.D. (1984). Stress-induced changes in the octopamine levels of insect haemolymph. Insect Biochemistry *14*, 135-143.

David, J.C., and Coulon, J.F. (1985). Octopamine in invertebrates and vertebrates. A review. Prog Neurobiol *24*, 141-185.

de Belle, J.S., and Heisenberg, M. (1994). Associative odor learning in Drosophila abolished by chemical ablation of mushroom bodies. Science *263*, 692-695.

De Gennaro, L., Marzano, C., Fratello, F., Moroni, F., Pellicciari, M.C., Ferlazzo, F., Costa, S., Couyoumdjian, A., Curcio, G., Sforza, E., *et al.* (2008). The electroencephalographic fingerprint of sleep is genetically determined: a twin study. Ann Neurol *64*, 455-460.

DePeyer, J.E., Cachelin, A.B., Levitan, I.B., and Reuter, H. (1982). Ca^{2+}-activated K^{+} conductance in internally perfused snail neurons is enhanced by protein phosphorylation. Proc Natl Acad Sci U S A *79*, 4207-4211.

DiAngelo, J.R., and Birnbaum, M.J. (2009). Regulation of fat cell mass by insulin in Drosophila melanogaster. Mol Cell Biol *29*, 6341-6352.

Dockendorff, T.C., Su, H.S., McBride, S.M., Yang, Z., Choi, C.H., Siwicki, K.K., Sehgal, A., and Jongens, T.A. (2002). Drosophila lacking dfmr1 activity show defects in circadian output and fail to maintain courtship interest. Neuron *34*, 973-984.

Donlea, J.M., Ramanan, N., and Shaw, P.J. (2009). Use-dependent plasticity in clock neurons regulates sleep need in Drosophila. Science *324*, 105-108.

Douglas, C.L., Vyazovskiy, V., Southard, T., Chiu, S.Y., Messing, A., Tononi, G., and Cirelli, C. (2007). Sleep in Kcna2 knockout mice. BMC Biol *5*, 42.

Dubnau, J., Grady, L., Kitamoto, T., and Tully, T. (2001). Disruption of neurotransmission in Drosophila mushroom body blocks retrieval but not acquisition of memory. Nature *411*, 476-480.

Dumermuth, G., Lange, B., Lehmann, D., Meier, C.A., Dinkelmann, R., and Molinari, L. (1983). Spectral analysis of all-night sleep EEG in healthy adults. Eur Neurol *22*, 322-339.

Dworak, M., McCarley, R.W., Kim, T., Kalinchuk, A.V., and Basheer, R. (2010). Sleep and brain energy levels: ATP changes during sleep. J Neurosci *30*, 9007-9016.

Edelson, E. (1992). Sleep (New York: Chelsea House).

Edgecomb, R.S., Harth, C.E., and Schneiderman, A.M. (1994). Regulation of feeding behavior in adult Drosophila melanogaster varies with feeding regime and nutritional state. J Exp Biol *197*, 215-235.

Espinosa, F., Marks, G., Heintz, N., and Joho, R.H. (2004). Increased motor drive and sleep loss in mice lacking Kv3-type potassium channels. Genes Brain Behav *3*, 90-100.

Espinosa, F., Torres-Vega, M.A., Marks, G.A., and Joho, R.H. (2008). Ablation of Kv3.1 and Kv3.3 potassium channels disrupts thalamocortical oscillations in vitro and in vivo. J Neurosci *28*, 5570-5581.

Evans, P.D., and Maqueira, B. (2005). Insect octopamine receptors: a new classification scheme based on studies of cloned Drosophila G-protein coupled receptors. Invert Neurosci *5*, 111-118.

Evans, P.D., and Robb, S. (1993). Octopamine receptor subtypes and their modes of action. Neurochem Res *18*, 869-874.

Ewald, D., Williams, A., and Levitan, I.B. (1985). Modulation of single Ca^{2+}-dependent K^{+} channel activity by protein phosphorylation. Nature *315*, 503-506.

Feil, R., Holter, S.M., Weindl, K., Wurst, W., Langmesser, S., Gerling, A., Feil, S., and Albrecht, U. (2009). cGMP-dependent protein kinase I, the circadian clock, sleep and learning. Commun Integr Biol *2*, 298-301.

Fellin T, Halassa MM, Terunuma M, Succol F, Takano H, Frank M, Moss SJ, Haydon PG. (2009). Endogenous nonneuronal modulators of synaptic transmission control cortical slow oscillations in vivo. Proc Natl Acad Sci. *106*, 15037-42Foltenyi, K., Greenspan, R.J., and Newport, J.W. (2007). Activation of EGFR and ERK by rhomboid signaling regulates the consolidation and maintenance of sleep in Drosophila. Nat Neurosci *10*, 1160-1167.

Ford, G.K., Al-Barazanji, K.A., Wilson, S., Jones, D.N., Harbuz, M.S., and Jessop, D.S. (2005). Orexin expression and function: glucocorticoid manipulation, stress, and feeding studies. Endocrinology *146*, 3724-3731.

Frank, M.G., Issa, N.P., and Stryker, M.P. (2001). Sleep enhances plasticity in the developing visual cortex. Neuron *30*, 275-287.

Fredholm, B.B., Battig, K., Holmen, J., Nehlig, A., and Zvartau, E.E. (1999). Actions of caffeine in the brain with special reference to factors that contribute to its widespread use. Pharmacol Rev *51*, 83-133.

Fuller, P.M., Lu, J., and Saper, C.B. (2008). Differential rescue of light- and food-entrainable circadian rhythms. Science *320*, 1074-1077.

Fussnecker, B.L., Smith, B.H., and Mustard, J.A. (2006). Octopamine and tyramine influence the behavioral profile of locomotor activity in the honey bee (Apis mellifera). J Insect Physiol *52*, 1083-1092.

Gais, S., and Born, J. (2004). Low acetylcholine during slow-wave sleep is critical for declarative memory consolidation. Proc Natl Acad Sci U S A *101*, 2140-2144.

Ganguly-Fitzgerald, I., Donlea, J., and Shaw, P.J. (2006). Waking experience affects sleep need in Drosophila. Science *313*, 1775-1781.

Garofalo, R.S. (2002). Genetic analysis of insulin signaling in Drosophila. Trends Endocrinol Metab *13*, 156-162.

Garofalo, R.S., and Rosen, O.M. (1988). Tissue localization of Drosophila melanogaster insulin receptor transcripts during development. Mol Cell Biol *8*, 1638-1647.

Gilestro, G.F., and Cirelli, C. (2009). pySolo: a complete suite for sleep analysis in Drosophila. Bioinformatics *25*, 1466-1467.

Goddard, A.W., Ball, S.G., Martinez, J., Robinson, M.J., Yang, C.R., Russell, J.M., and Shekhar, A. Current perspectives of the roles of the central norepinephrine system in anxiety and depression. Depress Anxiety *27*, 339-350.

Goddard, A.W., Ball, S.G., Martinez, J., Robinson, M.J., Yang, C.R., Russell, J.M., and Shekhar, A. (2010). Current perspectives of the roles of the central norepinephrine system in anxiety and depression. Depress Anxiety *27*, 339-350.

Gong, Z., Son, W., Chung, Y.D., Kim, J., Shin, D.W., McClung, C.A., Lee, Y., Lee, H.W., Chang, D.J., Kaang, B.K., *et al.* (2004). Two interdependent TRPV channel subunits, inactive and Nanchung, mediate hearing in Drosophila. J Neurosci *24*, 9059-9066.

Goutagny, R., Comte, J.C., Salvert, D., Gomeza, J., Yamada, M., Wess, J., Luppi, P.H., and Fort, P. (2005). Paradoxical sleep in mice lacking M3 and M2/M4 muscarinic receptors. Neuropsychobiology *52*, 140-146.

Graves, L., Dalvi, A., Lucki, I., Blendy, J.A., and Abel, T. (2002). Behavioral analysis of CREB alphadelta mutation on a B6/129 F1 hybrid background. Hippocampus *12*, 18-26.

Graves, L.A., Heller, E.A., Pack, A.I., and Abel, T. (2003a). Sleep deprivation selectively impairs memory consolidation for contextual fear conditioning. Learn Mem *10*, 168-176.

Graves, L.A., Hellman, K., Veasey, S., Blendy, J.A., Pack, A.I., and Abel, T. (2003b). Genetic evidence for a role of CREB in sustained cortical arousal. J Neurophysiol *90*, 1152-1159.

Gruntenko, N., Chentsova, N.A., Bogomolova, E.V., Karpova, E.K., Glazko, G.V., Faddeeva, N.V., Monastirioti, M., and Rauschenbach, I.Y. (2004). The effect of mutations altering biogenic amine metabolism in Drosophila on viability and the response to environmental stresses. Archives of Insect Biochemistry and Physiology *55*, 55-67.

Hallmayer, J., Faraco, J., Lin, L., Hesselson, S., Winkelmann, J., Kawashima, M., Mayer, G., Plazzi, G., Nevsimalova, S., Bourgin, P., *et al.* (2009). Narcolepsy is strongly associated with the T-cell receptor alpha locus. Nat Genet *41*, 708-711.

Hamada, F.N., Rosenzweig, M., Kang, K., Pulver, S.R., Ghezzi, A., Jegla, T.J., and Garrity, P.A. (2008). An internal thermal sensor controlling temperature preference in Drosophila. Nature *454*, 217-220.

Han, K.A., Millar, N.S., and Davis, R.L. (1998). A novel octopamine receptor with preferential expression in Drosophila mushroom bodies. J Neurosci *18*, 3650-3658.

Hardie, S.L., Zhang, J.X., and Hirsh, J. (2007). Trace amines differentially regulate adult locomotor activity, cocaine sensitivity, and female fertility in Drosophila melanogaster. Dev Neurobiol *67*, 1396-1405.

Harik, S.I., Busto, R., and Martinez, E. (1982). Norepinephrine regulation of cerebral glycogen utilization during seizures and ischemia. J Neurosci *2*, 409-414.

Harris, J.W., and Woodring, J. (1992). Effects of Stress, Age, Season, and Source Colony on Levels of Octopamine, Dopamine and Serotonin in the Honey-Bee (Apis-Mellifera L) Brain. Journal of Insect Physiology *38*, 29-35.

He, Y., Jones, C.R., Fujiki, N., Xu, Y., Guo, B., Holder, J.L., Jr., Rossner, M.J., Nishino, S., and Fu, Y.H. (2009). The transcriptional repressor DEC2 regulates sleep length in mammals. Science *325*, 866-870.

Heisenberg, M. (2003). Mushroom body memoir: from maps to models. Nat Rev Neurosci *4*, 266-275.

Helfrich-Forster, C., Wulf, J., and de Belle, J.S. (2002). Mushroom body influence on locomotor activity and circadian rhythms in Drosophila melanogaster. J Neurogenet *16*, 73-109.

Hendricks, J.C., Finn, S.M., Panckeri, K.A., Chavkin, J., Williams, J.A., Sehgal, A., and Pack, A.I. (2000a). Rest in Drosophila is a sleep-like state. Neuron *25*, 129-138.

Hendricks, J.C., Lu, S., Kume, K., Yin, J.C., Yang, Z., and Sehgal, A. (2003). Gender dimorphism in the role of cycle (BMAL1) in rest, rest regulation, and longevity in Drosophila melanogaster. J Biol Rhythms *18*, 12-25.

Hendricks, J.C., and Sehgal, A. (2004). Why a fly? Using Drosophila to understand the genetics of circadian rhythms and sleep. Sleep *27*, 334-342.

Hendricks, J.C., Sehgal, A., and Pack, A.I. (2000b). The need for a simple animal model to understand sleep. Prog Neurobiol *61*, 339-351.

Hendricks, J.C., Williams, J.A., Panckeri, K., Kirk, D., Tello, M., Yin, J.C., and Sehgal, A. (2001). A non-circadian role for cAMP signaling and CREB activity in Drosophila rest homeostasis. Nat Neurosci *4*, 1108-1115.

Hilakivi, I., and Leppavuori, A. (1984). Effects of methoxamine, and alpha-1 adrenoceptor agonist, and prazosin, an alpha-1 antagonist, on the stages of the sleep-waking cycle in the cat. Acta Physiol Scand *120*, 363-372.

Hirashima, A., and Eto, M. (1993). Effect of stress on levels of octopamine, dopamine, and serotonin in the American cockroach. Comparative Biochemistry and Physiology-C Pharmocology Toxicology and Endocrinology *105*, 279-284.

Hodge, J.J. (2009). Ion channels to inactivate neurons in Drosophila. Front Mol Neurosci *2*, 13.

Hoffman, B., and Lefkowitz, R. (1996a). Goodman and Gilman's the Pharmacological Basis of Therapeutics, 9 edn (New York: McGraw-Hill Health Professions Division).

Hoffman, B., and Lefkowitz, R., eds. (1996b). Goodman and Gilman's the pharmocological basis of therapeutics, 9 edn (New York: McGraw-Hill Health Professional Division).

Holets, V.R., Hokfelt, T., Rokaeus, A., Terenius, L., and Goldstein, M. (1988). Locus coeruleus neurons in the rat containing neuropeptide Y, tyrosine hydroxylase or galanin and their efferent projections to the spinal cord, cerebral cortex and hypothalamus. Neuroscience *24*, 893-906.

Honma, S., Kawamoto, T., Takagi, Y., Fujimoto, K., Sato, F., Noshiro, M., Kato, Y., and Honma, K. (2002). Dec1 and Dec2 are regulators of the mammalian molecular clock. Nature *419*, 841-844.

Horrigan, L.A., Kelly, J.P., and Connor, T.J. (2006). Immunomodulatory effects of caffeine: friend or foe? Pharmacol Ther *111*, 877-892.

Hoyer, S.C., Eckart, A., Herrel, A., Zars, T., Fischer, S.A., Hardie, S.L., and Heisenberg, M. (2008). Octopamine in male aggression of Drosophila. Curr Biol *18*, 159-167.

Hu, W.P., Li, J.D., Zhang, C., Boehmer, L., Siegel, J.M., and Zhou, Q.Y. (2007). Altered circadian and homeostatic sleep regulation in prokineticin 2-deficient mice. Sleep *30*, 247-256.

Huang, Z.L., Qu, W.M., Eguchi, N., Chen, J.F., Schwarzschild, M.A., Fredholm, B.B., Urade, Y., and Hayaishi, O. (2005). Adenosine A2A, but not A1, receptors mediate the arousal effect of caffeine. Nat Neurosci *8*, 858-859.

Huber, R., Hill, S.L., Holladay, C., Biesiadecki, M., Tononi, G., and Cirelli, C. (2004). Sleep homeostasis in Drosophila melanogaster. Sleep *27*, 628-639.

Huber, R., Tononi, G., and Cirelli, C. (2007). Exploratory behavior, cortical BDNF expression, and sleep homeostasis. Sleep *30*, 129-139.

Hunsley, M.S., and Palmiter, R.D. (2003). Norepinephrine-deficient mice exhibit normal sleep-wake states but have shorter sleep latency after mild stress and low doses of amphetamine. Sleep *26*, 521-526.

Hwangbo, D.S., Gershman, B., Tu, M.P., Palmer, M., and Tatar, M. (2004). Drosophila dFOXO controls lifespan and regulates insulin signalling in brain and fat body. Nature *429*, 562-566.

Ikeya, T., Galic, M., Belawat, P., Nairz, K., and Hafen, E. (2002). Nutrient-dependent expression of insulin-like peptides from neuroendocrine cells in the CNS contributes to growth regulation in Drosophila. Curr Biol *12*, 1293-1300.

Insel, P.A. (1996). Seminars in medicine of the Beth Israel Hospital, Boston. Adrenergic receptors--evolving concepts and clinical implications. N Engl J Med *334*, 580-585.

Jaramillo, A.M., Zheng, X., Zhou, Y., Amado, D.A., Sheldon, A., Sehgal, A., and Levitan, I.B. (2004). Pattern of distribution and cycling of SLOB, Slowpoke channel binding protein, in Drosophila. BMC Neurosci *5*, 3.

Joiner, M.A., and Griffith, L.C. (1999). Mapping of the anatomical circuit of CaM kinase-dependent courtship conditioning in Drosophila. Learn Mem *6*, 177-192.

Joiner, W.J., Crocker, A., White, B.H., and Sehgal, A. (2006). Sleep in Drosophila is regulated by adult mushroom bodies. Nature *441*, 757-760.

Jouvet, M. (1968). Insomnia and decrease of cerebral 5-hydroxytryptamine after destruction of the raphe system in the cat. Adv Pharmacol *6*, 265-279.

Kanyshkova, T., Pawlowski, M., Meuth, P., Dube, C., Bender, R.A., Brewster, A.L., Baumann, A., Baram, T.Z., Pape, H.C., and Budde, T. (2009). Postnatal expression pattern of HCN channel isoforms in thalamic neurons: relationship to maturation of thalamocortical oscillations. J Neurosci *29*, 8847-8857.

Keckeis, M., Lattova, Z., Maurovich-Horvat, E., Beitinger, P.A., Birkmann, S., Lauer, C.J., Wetter, T.C., Wilde-Frenz, J., and Pollmacher, T. (2010). Impaired glucose tolerance in sleep disorders. PLoS One *5*, e9444.

Kloppenburg, P., and Erber, J. (1995a). The Modulatory Effects of Serotonin and Octopamine in the Visual-System of the Honey-Bee (Apis-Mellifera L) .2. Electrophysiological Analysis of Motion-Sensitive Neurons in the Lobula. Journal of Comparative Physiology a-Sensory Neural and Behavioral Physiology *176*, 119-129.

Kloppenburg, P., and Erber, J. (1995b). The modulatory effects of serotonin and octopamine in the visual system of the honey bee (Apis mellifera L.). II. Electrophysiological analysis of motion-sensitive neurons in the lobula. J. Comp. Physiol. *176A*, 119-129.

Kloss, B., Price, J.L., Saez, L., Blau, J., Rothenfluh, A., Wesley, C.S., and Young, M.W. (1998). The Drosophila clock gene double-time encodes a protein closely related to human casein kinase Iepsilon. Cell *94*, 97-107.

Knutson, K.L., and Van Cauter, E. (2008). Associations between sleep loss and increased risk of obesity and diabetes. Ann N Y Acad Sci *1129*, 287-304.

Koh, K., Joiner, W.J., Wu, M.N., Yue, Z., Smith, C.J., and Sehgal, A. (2008). Identification of SLEEPLESS, a sleep-promoting factor. Science *321*, 372-376.

Kong, J., Shepel, P.N., Holden, C.P., Mackiewicz, M., Pack, A.I., and Geiger, J.D. (2002). Brain glycogen decreases with increased periods of wakefulness: implications for homeostatic drive to sleep. J Neurosci *22*, 5581-5587.

Konopka, R.J., and Benzer, S. (1971). Clock mutants of Drosophila melanogaster. Proc Natl Acad Sci U S A *68*, 2112-2116.

Kryger, M.H., Roth, T., and Dement, W.C. (2005). Principles and practice of sleep medicine, 4th edn (Philadelphia, PA: Elsevier/Saunders).

Kume, K., Kume, S., Park, S.K., Hirsh, J., and Jackson, F.R. (2005). Dopamine is a regulator of arousal in the fruit fly. J Neurosci *25*, 7377-7384.

Langmesser, S., Franken, P., Feil, S., Emmenegger, Y., Albrecht, U., and Feil, R. (2009). cGMP-dependent protein kinase type I is implicated in the regulation of the timing and quality of sleep and wakefulness. PLoS One *4*, e4238.

Laposky, A., Easton, A., Dugovic, C., Walisser, J., Bradfield, C., and Turek, F. (2005). Deletion of the mammalian circadian clock gene BMAL1/Mop3 alters baseline sleep architecture and the response to sleep deprivation. Sleep *28*, 395-409.

Laposky, A.D., Bradley, M.A., Williams, D.L., Bass, J., and Turek, F.W. (2008). Sleep-wake regulation is altered in leptin-resistant (db/db) genetically obese and diabetic mice. Am J Physiol Regul Integr Comp Physiol *295*, R2059-2066.

Laposky, A.D., Shelton, J., Bass, J., Dugovic, C., Perrino, N., and Turek, F.W. (2006). Altered sleep regulation in leptin-deficient mice. Am J Physiol Regul Integr Comp Physiol *290*, R894-903.

Lee, H.G., Rohila, S., and Han, K.A. (2009). The octopamine receptor OAMB mediates ovulation via Ca2+/calmodulin-dependent protein kinase II in the Drosophila oviduct epithelium. PLoS One *4*, e4716.

Lee, H.G., Seong, C.S., Kim, Y.C., Davis, R.L., and Han, K.A. (2003). Octopamine receptor OAMB is required for ovulation in Drosophila melanogaster. Dev Biol *264*, 179-190.

Lee, J., Kim, D., and Shin, H.S. (2004). Lack of delta waves and sleep disturbances during non-rapid eye movement sleep in mice lacking alpha1G-subunit of T-type calcium channels. Proc Natl Acad Sci U S A *101*, 18195-18199.

Lee, T., and Luo, L. (1999). Mosaic analysis with a repressible cell marker for studies of gene function in neuronal morphogenesis. Neuron *22*, 451-461.

Legendre, R., and Peiron, H. (1913). Z. Allgem. Physiol *14*, 235-262.

Leibowitz, S.F. (1991). Brain neuropeptide Y: an integrator of endocrine, metabolic and behavioral processes. Brain Res Bull *27*, 333-337.

Leonard, B.E. (2005). The HPA and immune axes in stress: the involvement of the serotonergic system. Eur Psychiatry *20 Suppl 3*, S302-306.

Lightman, S.L. (2008). The neuroendocrinology of stress: a never ending story. J Neuroendocrinol *20*, 880-884.

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

Lin, L., Faraco, J., Li, R., Kadotani, H., Rogers, W., Lin, X., Qiu, X., de Jong, P.J., Nishino, S., and Mignot, E. (1999). The sleep disorder canine narcolepsy is caused by a mutation in the hypocretin (orexin) receptor 2 gene. Cell *98*, 365-376.

Lisk, D.R. (2009). Kleine-Levin syndrome. Pract Neurol *9*, 42-45.

Liu, L., Wolf, R., Ernst, R., and Heisenberg, M. (1999). Context generalization in Drosophila visual learning requires the mushroom bodies. Nature *400*, 753-756.

Livingstone, M.S., and Tempel, B.L. (1983). Genetic dissection of monoamine neurotransmitter synthesis in Drosophila. Nature *303*, 67-70.

Lowrey, P.L., Shimomura, K., Antoch, M.P., Yamazaki, S., Zemenides, P.D., Ralph, M.R., Menaker, M., and Takahashi, J.S. (2000). Positional syntenic cloning and functional characterization of the mammalian circadian mutation tau. Science *288*, 483-492.

Luan, H., Lemon, W.C., Peabody, N.C., Pohl, J.B., Zelensky, P.K., Wang, D., Nitabach, M.N., Holmes, T.C., and White, B.H. (2006). Functional dissection of a neuronal network required for cuticle tanning and wing expansion in Drosophila. J Neurosci *26*, 573-584.

Mallick, B.N., and Alam, M.N. (1992). Different types of norepinephrinergic receptors are involved in preoptic area mediated independent modulation of sleep-wakefulness and body temperature. Brain Res *591*, 8-19.

Mao, Z., Roman, G., Zong, L., and Davis, R.L. (2004). Pharmacogenetic rescue in time and space of the rutabaga memory impairment by using Gene-Switch. Proc Natl Acad Sci U S A *101*, 198-203.

Maqueira, B., Chatwin, H., and Evans, P.D. (2005). Identification and characterization of a novel family of Drosophila beta-adrenergic-like octopamine G-protein coupled receptors. J Neurochem *94*, 547-560.

Maret, S., Franken, P., Dauvilliers, Y., Ghyselinck, N.B., Chambon, P., and Tafti, M. (2005). Retinoic acid signaling affects cortical synchrony during sleep. Science *310*, 111-113.

Martin, J.R., Ernst, R., and Heisenberg, M. (1998). Mushroom bodies suppress locomotor activity in Drosophila melanogaster. Learn Mem *5*, 179-191.
McBride, S.M., Giuliani, G., Choi, C., Krause, P., Correale, D., Watson, K., Baker, G., and Siwicki, K.K. (1999). Mushroom body ablation impairs short-term memory and long-term memory of courtship conditioning in Drosophila melanogaster. Neuron *24*, 967-977.
McClung, C., and Hirsh, J. (1999). The trace amine tyramine is essential for sensitization to cocaine in Drosophila. Curr Biol *9*, 853-860.
McGuire, S.E., Le, P.T., and Davis, R.L. (2001). The role of Drosophila mushroom body signaling in olfactory memory. Science *293*, 1330-1333.
Miller, M.R., Robinson, K.J., Cleary, M.D., and Doe, C.Q. (2009). TU-tagging: cell type-specific RNA isolation from intact complex tissues. Nat Methods *6*, 439-441.
Monastirioti, M., Linn, C.E., Jr., and White, K. (1996). Characterization of Drosophila tyramine beta-hydroxylase gene and isolation of mutant flies lacking octopamine. J Neurosci *16*, 3900-3911.
Monteggia, L.M., Barrot, M., Powell, C.M., Berton, O., Galanis, V., Gemelli, T., Meuth, S., Nagy, A., Greene, R.W., and Nestler, E.J. (2004). Essential role of brain-derived neurotrophic factor in adult hippocampal function. Proc Natl Acad Sci U S A *101*, 10827-10832.
Monti, J.M., D'Angelo, L., Jantos, H., Barbeito, L., and Abo, V. (1988). Effect of DSP-4, a noradrenergic neurotoxin, on sleep and wakefulness and sensitivity to drugs acting on adrenergic receptors in the rat. Sleep *11*, 370-377.
Naidoo, N., Casiano, V., Cater, J., Zimmerman, J., and Pack, A.I. (2007). A role for the molecular chaperone protein BiP/GRP78 in Drosophila sleep homeostasis. Sleep *30*, 557-565.
Naylor, E., Bergmann, B.M., Krauski, K., Zee, P.C., Takahashi, J.S., Vitaterna, M.H., and Turek, F.W. (2000). The circadian clock mutation alters sleep homeostasis in the mouse. J Neurosci *20*, 8138-8143.
Nikolaev, V.O., Bunemann, M., Hein, L., Hannawacker, A., and Lohse, M.J. (2004). Novel single chain cAMP sensors for receptor-induced signal propagation. J Biol Chem *279*, 37215-37218.
Nitabach, M.N., Sheeba, V., Vera, D.A., Blau, J., and Holmes, T.C. (2005). Membrane electrical excitability is necessary for the free-running larval Drosophila circadian clock. J Neurobiol *62*, 1-13.
Nitabach, M.N., Wu, Y., Sheeba, V., Lemon, W.C., Strumbos, J., Zelensky, P.K., White, B.H., and Holmes, T.C. (2006). Electrical hyperexcitation of lateral ventral pacemaker neurons desynchronizes downstream circadian oscillators in the fly circadian circuit and induces multiple behavioral periods. J Neurosci *26*, 479-489.
Nitz, D.A., van Swinderen, B., Tononi, G., and Greenspan, R.J. (2002). Electrophysiological correlates of rest and activity in Drosophila melanogaster. Curr Biol *12*, 1934-1940.
Nixon, G.M., and Brouillette, R.T. (2002). Sleep and breathing in Prader-Willi syndrome. Pediatr Pulmonol *34*, 209-217.
O'Dell, K.M. (1994). The inactive mutation leads to abnormal experience-dependent courtship modification in male Drosophila melanogaster. Behav Genet *24*, 381-388.

O'Hara, B.F., Ding, J., Bernat, R.L., and Franken, P. (2007). Genomic and proteomic approaches towards an understanding of sleep. CNS Neurol Disord Drug Targets *6*, 71-81.
Oldham, S., and Hafen, E. (2003). Insulin/IGF and target of rapamycin signaling: a TOR de force in growth control. Trends Cell Biol *13*, 79-85.
Orchard, I., Ramirez, J.M., and Lange, B. (1993). A multifunctional role for octopamine in locust flight. Annual Review of Entomology *38*, 227-249.
Osterwalder, T., Yoon, K.S., White, B.H., and Keshishian, H. (2001). A conditional tissue-specific transgene expression system using inducible GAL4. Proc Natl Acad Sci U S A *98*, 12596-12601.
Ousley, A., Zafarullah, K., Chen, Y., Emerson, M., Hickman, L., and Sehgal, A. (1998). Conserved regions of the timeless (tim) clock gene in Drosophila analyzed through phylogenetic and functional studies. Genetics *148*, 815-825.
Ouyang, M., Hellman, K., Abel, T., and Thomas, S.A. (2004). Adrenergic signaling plays a critical role in the maintenance of waking and in the regulation of REM sleep. J Neurophysiol *92*, 2071-2082.
Parisky, K.M., Agosto, J., Pulver, S.R., Shang, Y., Kuklin, E., Hodge, J.J., Kang, K., Liu, X., Garrity, P.A., Rosbash, M., and Griffith, L.C. (2008). PDF cells are a GABA-responsive wake-promoting component of the Drosophila sleep circuit. Neuron *60*, 672-682.
Pascual, A., and Preat, T. (2001). Localization of long-term memory within the Drosophila mushroom body. Science *294*, 1115-1117.
Peterfi, Z., Obal, F., Jr., Taishi, P., Gardi, J., Kacsoh, B., Unterman, T., and Krueger, J.M. (2006). Sleep in spontaneous dwarf rats. Brain Res *1108*, 133-146.
Pitman, J.L., McGill, J.J., Keegan, K.P., and Allada, R. (2006). A dynamic role for the mushroom bodies in promoting sleep in Drosophila. Nature *441*, 753-756.
Pribbenow, B., and Erber, J. (1996). Modulation of antennal scanning in the honeybee by sucrose stimuli, serotonin, and octopamine: behavior and electrophysiology. Neurobiol Learn Mem *66*, 109-120.
Raizen, D.M., Zimmerman, J.E., Maycock, M.H., Ta, U.D., You, Y.J., Sundaram, M.V., and Pack, A.I. (2008). Lethargus is a Caenorhabditis elegans sleep-like state. Nature *451*, 569-572.
Ralph, M.R., and Menaker, M. (1988). A mutation of the circadian system in golden hamsters. Science *241*, 1225-1227.
Ramos, B.P., and Arnsten, A.F. (2007). Adrenergic pharmacology and cognition: focus on the prefrontal cortex. Pharmacol Ther *113*, 523-536.
Rechtschaffen, A. (1998). Current perspectives on the function of sleep. Perspect Biol Med *41*, 359-390.
Robb, S., Cheek, T.R., Hannan, F.L., Hall, L.M., Midgley, J.M., and Evans, P.D. (1994). Agonist-specific coupling of a cloned Drosophila octopamine/tyramine receptor to multiple second messenger systems. EMBO J *13*, 1325-1330.
Rodan, A.R., Kiger, J.A., Jr., and Heberlein, U. (2002). Functional dissection of neuroanatomical loci regulating ethanol sensitivity in Drosophila. J Neurosci *22*, 9490-9501.
Roeder, T. (1992). A new octopamine receptor class in locust nervous tissue, the octopamine 3 (OA3) receptor. Life Sci *50*, 21-28.

Roeder, T. (1999). Octopamine in invertebrates. Prog Neurobiol *59*, 533-561.
Roeder, T. (2005). Tyramine and octopamine: ruling behavior and metabolism. Annu Rev Entomol *50*, 447-477.
Roeder, T., Degen, J., Dyczkowski, C., and Gewecke, M. (1995). Pharmacology and molecular biology of octopamine receptors from different insect species. Prog Brain Res *106*, 249-258.
Roeder, T., Seifert, M., Kahler, C., and Gewecke, M. (2003). Tyramine and octopamine: antagonistic modulators of behavior and metabolism. Arch Insect Biochem Physiol *54*, 1-13.
Rosenberg, L.A., Glusman, J.G., and Libersat, F. (2007). Octopamine partially restores walking in hypokinetic cockroaches stung by the parasitoid wasp Ampulex compressa. J Exp Biol *210*, 4411-4417.
Rossant, J., and McMahon, A. (1999). "Cre"-ating mouse mutants-a meeting review on conditional mouse genetics. Genes Dev *13*, 142-145.
Rulifson, E.J., Kim, S.K., and Nusse, R. (2002). Ablation of insulin-producing neurons in flies: growth and diabetic phenotypes. Science *296*, 1118-1120.
Saper, C.B., Scammell, T.E., and Lu, J. (2005). Hypothalamic regulation of sleep and circadian rhythms. Nature *437*, 1257-1263.
Sara, S.J. (2009). The locus coeruleus and noradrenergic modulation of cognition. Nat Rev Neurosci *10*, 211-223.
Saraswati, S., Fox, L.E., Soll, D.R., and Wu, C.F. (2004). Tyramine and octopamine have opposite effects on the locomotion of Drosophila larvae. J Neurobiol *58*, 425-441.
Sassin, J.F., Parker, D.C., Mace, J.W., Gotlin, R.W., Johnson, L.C., and Rossman, L.G. (1969). Human growth hormone release: relation to slow-wave sleep and sleep-walking cycles. Science *165*, 513-515.
Schank, J.R., Ventura, R., Puglisi-Allegra, S., Alcaro, A., Cole, C.D., Liles, L.C., Seeman, P., and Weinshenker, D. (2006). Dopamine beta-hydroxylase knockout mice have alterations in dopamine signaling and are hypersensitive to cocaine. Neuropsychopharmacology *31*, 2221-2230.
Scheiner, R., Pluckhahn, S., Oney, B., Blenau, W., and Erber, J. (2002). Behavioural pharmacology of octopamine, tyramine and dopamine in honey bees. Behav Brain Res *136*, 545-553.
Scholz, H. (2005). Influence of the biogenic amine tyramine on ethanol-induced behaviors in Drosophila. J Neurobiol *63*, 199-214.
Schulz, D.J., and Robinson, G.E. (1999). Biogenic amines and division of labor in honey bee colonies: behaviorally related changes in the antennal lobes and age-related changes in the mushroom bodies. J Comp Physiol [A] *184*, 481-488.
Schulz, D.J., and Robinson, G.E. (2001a). Octopamine influences division of labor in honey bee colonies. J Comp Physiol [A] *187*, 53-61.
Schulz, D.J., and Robinson, G.E. (2001b). Octopamine influences division of labor in honey bee colonies. Journal of Comparative Physiology a-Sensory Neural and Behavioral Physiology *187*, 53-61.
Schulz, D.J., Sullivan, J.P., and Robinson, G.E. (2002). Juvenile hormone and octopamine in the regulation of division of labor in honey bee colonies. Hormones and Behavior *42*, 222-231.

Schwaerzel, M., Monastirioti, M., Scholz, H., Friggi-Grelin, F., Birman, S., and Heisenberg, M. (2003). Dopamine and octopamine differentiate between aversive and appetitive olfactory memories in Drosophila. J Neurosci *23*, 10495-10502.
Sehgal, A. (2004). Molecular biology of circadian rhythms (Hoboken, N.J.: Wiley-Liss).
Shafer, O.T., Kim, D.J., Dunbar-Yaffe, R., Nikolaev, V.O., Lohse, M.J., and Taghert, P.H. (2008). Widespread receptivity to neuropeptide PDF throughout the neuronal circadian clock network of Drosophila revealed by real-time cyclic AMP imaging. Neuron *58*, 223-237.
Shahidullah, M., Reddy, S., Fei, H., and Levitan, I.B. (2009a). In vivo role of a potassium channel-binding protein in regulating neuronal excitability and behavior. J Neurosci *29*, 13328-13337.
Shao, L.R., Halvorsrud, R., Borg-Graham, L., and Storm, J.F. (1999). The role of BK-type Ca2+-dependent K+ channels in spike broadening during repetitive firing in rat hippocampal pyramidal cells. J Physiol *521 Pt 1*, 135-146.
Shaw, P. (2003). Awakening to the behavioral analysis of sleep in Drosophila. J Biol Rhythms *18*, 4-11.
Shaw, P.J., Cirelli, C., Greenspan, R.J., and Tononi, G. (2000). Correlates of sleep and waking in Drosophila melanogaster. Science *287*, 1834-1837.
Shaw, P.J., Tononi, G., Greenspan, R.J., and Robinson, D.F. (2002). Stress response genes protect against lethal effects of sleep deprivation in Drosophila. Nature *417*, 287-291.
Shen, K.Z., Lagrutta, A., Davies, N.W., Standen, N.B., Adelman, J.P., and North, R.A. (1994). Tetraethylammonium block of Slowpoke calcium-activated potassium channels expressed in Xenopus oocytes: evidence for tetrameric channel formation. Pflugers Arch *426*, 440-445.
Shipley, J.E., Schteingart, D.E., Tandon, R., and Starkman, M.N. (1992). Sleep architecture and sleep apnea in patients with Cushing's disease. Sleep *15*, 514-518.
Siegel, J.M. (2005). Clues to the functions of mammalian sleep. Nature *437*, 1264-1271.
Siegmund, T., and Korge, G. (2001). Innervation of the ring gland of Drosophila melanogaster. J Comp Neurol *431*, 481-491.
Siepka, S.M., Yoo, S.H., Park, J., Lee, C., and Takahashi, J.S. (2007). Genetics and neurobiology of circadian clocks in mammals. Cold Spring Harb Symp Quant Biol *72*, 251-259.
Sinakevitch, I., and Strausfeld, N.J. (2006). Comparison of octopamine-like immunoreactivity in the brains of the fruit fly and blow fly. J Comp Neurol *494*, 460-475.
Sitaraman, D., Zars, M., and Zars, T. (2010). Place memory formation in Drosophila is independent of proper octopamine signaling. J Comp Physiol A Neuroethol Sens Neural Behav Physiol *196*, 299-305.
Sokolowski, M.B. (2001). Drosophila: genetics meets behaviour. Nat Rev Genet *2*, 879-890.
Spiegel, K., Tasali, E., Leproult, R., and Van Cauter, E. (2009). Effects of poor and short sleep on glucose metabolism and obesity risk. Nat Rev Endocrinol *5*, 253-261.

Steinlein, O., Anokhin, A., Yping, M., Schalt, E., and Vogel, F. (1992). Localization of a gene for the human low-voltage EEG on 20q and genetic heterogeneity. Genomics *12*, 69-73.

Szentirmai, E., Kapas, L., Sun, Y., Smith, R.G., and Krueger, J.M. (2007). Spontaneous sleep and homeostatic sleep regulation in ghrelin knockout mice. Am J Physiol Regul Integr Comp Physiol *293*, R510-517.

Tafti, M., Petit, B., Chollet, D., Neidhart, E., de Bilbao, F., Kiss, J.Z., Wood, P.A., and Franken, P. (2003). Deficiency in short-chain fatty acid beta-oxidation affects theta oscillations during sleep. Nat Genet *34*, 320-325.

Takahashi, Y., Kipnis, D.M., and Daughaday, W.H. (1968). Growth hormone secretion during sleep. J Clin Invest *47*, 2079-2090.

Tanaka, N.K., Tanimoto, H., and Ito, K. (2008). Neuronal assemblies of the Drosophila mushroom body. J Comp Neurol *508*, 711-755.

Tang, S., and Guo, A. (2001). Choice behavior of Drosophila facing contradictory visual cues. Science *294*, 1543-1547.

Tobler, I. (2005). Phylogeny of sleep regulation. In Principles and practice of sleep medicine, M.H. Kryger, T. Roth, and W.C. Dement, eds. (Philadelphia: Elsevier/Saunders), pp. 77-90.

Tobler, I., and Borbely, A.A. (1986). Sleep EEG in the rat as a function of prior waking. Electroencephalogr Clin Neurophysiol *64*, 74-76.

Toh, K.L., Jones, C.R., He, Y., Eide, E.J., Hinz, W.A., Virshup, D.M., Ptacek, L.J., and Fu, Y.H. (2001). An hPer2 phosphorylation site mutation in familial advanced sleep phase syndrome. Science *291*, 1040-1043.

Toivonen, J.M., and Partridge, L. (2009). Endocrine regulation of aging and reproduction in Drosophila. Mol Cell Endocrinol *299*, 39-50.

Tononi, G., and Cirelli, C. (2006). Sleep function and synaptic homeostasis. Sleep Med Rev *10*, 49-62.

Trenell, M.I., Marshall, N.S., and Rogers, N.L. (2007). Sleep and metabolic control: waking to a problem? Clin Exp Pharmacol Physiol *34*, 1-9.

van Beijsterveldt, C.E., and Boomsma, D.I. (1994). Genetics of the human electroencephalogram (EEG) and event-related brain potentials (ERPs): a review. Hum Genet *94*, 319-330.

Van Buskirk, C., and Sternberg, P.W. (2007). Epidermal growth factor signaling induces behavioral quiescence in Caenorhabditis elegans. Nat Neurosci *10*, 1300-1307.

Van Cauter, E., Polonsky, K.S., and Scheen, A.J. (1997). Roles of circadian rhythmicity and sleep in human glucose regulation. Endocr Rev *18*, 716-738.

Van Cauter, E., Spiegel, K., Tasali, E., and Leproult, R. (2008). Metabolic consequences of sleep and sleep loss. Sleep Med *9 Suppl 1*, S23-28.

van Swinderen, B., and Greenspan, R.J. (2003). Salience modulates 20-30 Hz brain activity in Drosophila. Nat Neurosci *6*, 579-586.

Vecsey, C.G., Baillie, G.S., Jaganath, D., Havekes, R., Daniels, A., Wimmer, M., Huang, T., Brown, K.M., Li, X.Y., Descalzi, G., *et al.* (2009). Sleep deprivation impairs cAMP signalling in the hippocampus. Nature *461*, 1122-1125.

Verlinden, H., Vleugels, R., Marchal, E., Badisco, L., Pfluger, H.J., Blenau, W., and Broeck, J.V. (2010). The role of octopamine in locusts and other arthropods. J Insect Physiol *56*, 854-867.

Viola, A.U., Archer, S.N., James, L.M., Groeger, J.A., Lo, J.C., Skene, D.J., von Schantz, M., and Dijk, D.J. (2007). PER3 polymorphism predicts sleep structure and waking performance. Curr Biol *17*, 613-618.

Vyazovskiy, V.V., Deboer, T., Rudy, B., Lau, D., Borbely, A.A., and Tobler, I. (2002). Sleep EEG in mice that are deficient in the potassium channel subunit K.v.3.2. Brain Res *947*, 204-211.

Wagener-Hulme, C., Kuehn, J.C., Schulz, D.J., and Robinson, G.E. (1999). Biogenic amines and division of labor in honey bee colonies. J Comp Physiol [A] *184*, 471-479.

Walker, M.P., and Stickgold, R. (2004). Sleep-dependent learning and memory consolidation. Neuron *44*, 121-133.

Webb, W.B., and Agnew, H.W., Jr. (1971). Stage 4 sleep: influence of time course variables. Science *174*, 1354-1356.

White, B.H., Osterwalder, T.P., Yoon, K.S., Joiner, W.J., Whim, M.D., Kaczmarek, L.K., and Keshishian, H. (2001). Targeted attenuation of electrical activity in Drosophila using a genetically modified K(+) channel. Neuron *31*, 699-711.

Williams, J.A., Sathyanarayanan, S., Hendricks, J.C., and Sehgal, A. (2007). Interaction between sleep and the immune response in Drosophila: a role for the NFkappaB relish. Sleep *30*, 389-400.

Winkelmann, J., Schormair, B., Lichtner, P., Ripke, S., Xiong, L., Jalilzadeh, S., Fulda, S., Putz, B., Eckstein, G., Hauk, S., *et al.* (2007). Genome-wide association study of restless legs syndrome identifies common variants in three genomic regions. Nat Genet *39*, 1000-1006.

Winsky-Sommerer, R., Vyazovskiy, V.V., Homanics, G.E., and Tobler, I. (2007). The EEG effects of THIP (Gaboxadol) on sleep and waking are mediated by the GABA(A)delta-subunit-containing receptors. Eur J Neurosci *25*, 1893-1899.

Wisor, J.P., O'Hara, B.F., Terao, A., Selby, C.P., Kilduff, T.S., Sancar, A., Edgar, D.M., and Franken, P. (2002). A role for cryptochromes in sleep regulation. BMC Neurosci *3*, 20.

Wu, J.S., and Luo, L. (2006). A protocol for mosaic analysis with a repressible cell marker (MARCM) in Drosophila. Nat Protoc *1*, 2583-2589.

Wu, M.N., Ho, K., Crocker, A., Yue, Z., Koh, K., and Sehgal, A. (2009). The effects of caffeine on sleep in Drosophila require PKA activity, but not the adenosine receptor. J Neurosci *29*, 11029-11037.

Wu, M.N., Joiner, W.J., Dean, T., Yue, Z., Smith, C.J., Chen, D., Hoshi, T., Sehgal, A., and Koh, K. (2010). SLEEPLESS, a Ly-6/neurotoxin family member, regulates the levels, localization and activity of Shaker. Nat Neurosci *13*, 69-75.

Wu, Y., Bolduc, F.V., Bell, K., Tully, T., Fang, Y., Sehgal, A., and Fischer, J.A. (2008). A Drosophila model for Angelman syndrome. Proc Natl Acad Sci U S A *105*, 12399-12404.

Xu, Y., Padiath, Q.S., Shapiro, R.E., Jones, C.R., Wu, S.C., Saigoh, N., Saigoh, K., Ptacek, L.J., and Fu, Y.H. (2005). Functional consequences of a CKIdelta mutation causing familial advanced sleep phase syndrome. Nature *434*, 640-644.

Yuan, Q., Joiner, W.J., and Sehgal, A. (2006). A sleep-promoting role for the Drosophila serotonin receptor 1A. Curr Biol *16*, 1051-1062.

Zamboni, G., Perez, E., Amici, R., Jones, C.A., and Parmeggiani, P.L. (1999). Control of REM sleep: an aspect of the regulation of physiological homeostasis. Arch Ital Biol *137*, 249-262.

Zars, T., Fischer, M., Schulz, R., and Heisenberg, M. (2000). Localization of a short-term memory in Drosophila. Science *288*, 672-675.

Zhang, F., Wang, L.P., Brauner, M., Liewald, J.F., Kay, K., Watzke, N., Wood, P.G., Bamberg, E., Nagel, G., Gottschalk, A., and Deisseroth, K. (2007). Multimodal fast optical interrogation of neural circuitry. Nature *446*, 633-639.

Zhang, J., Fang, Z., Jud, C., Vansteensel, M.J., Kaasik, K., Lee, C.C., Albrecht, U., Tamanini, F., Meijer, J.H., Oostra, B.A., and Nelson, D.L. (2008). Fragile X-related proteins regulate mammalian circadian behavioral rhythms. Am J Hum Genet *83*, 43-52.

Zheng, X., Yang, Z., Yue, Z., Alvarez, J.D., and Sehgal, A. (2007). FOXO and insulin signaling regulate sensitivity of the circadian clock to oxidative stress. Proc Natl Acad Sci U S A *104*, 15899-15904.

Zhou, C., and Rao, Y. (2008). A subset of octopaminergic neurons are important for Drosophila aggression. Nat Neurosci *11*, 1059-1067.

Zhou, Y., Wang, J., Wen, H., Kucherovsky, O., and Levitan, I.B. (2002). Modulation of Drosophila slowpoke calcium-dependent potassium channel activity by bound protein kinase a catalytic subunit. J Neurosci *22*, 3855-3863.

Zhu, S., Chiang, A.S., and Lee, T. (2003). Development of the Drosophila mushroom bodies: elaboration, remodeling and spatial organization of dendrites in the calyx. Development *130*, 2603-2610.

Zong, H., Espinosa, J.S., Su, H.H., Muzumdar, M.D., and Luo, L. (2005). Mosaic analysis with double markers in mice. Cell *121*, 479-492.